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Cell cycle synchronization: Reversible induction of G_2 synchrony in cultured rodent and human diploid fibroblasts

(topoisomerase II/Hoechst 33342/VM-26/Chinese hamster ovary cells/flow cytometry)

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ABSTRACT **In accord with a set of prespecified principles of cell synchrony induction, a three-step procedure was developed to arrest cells reversibly in the G_2 phase of the cell cycle. Cultures of Chinese hamster ovary (CHO) cells were presynchronized in early S phase by sequential treatment with isoleucine deficiency and hydroxyurea blockades; then they were switched to medium supplemented with either of two agents that inhibit DNA topoisomerase II activity by different mechanisms, Hoechst 33342 at 7.5 μg/ml for 12 hr or VM-26 at 0.5 μg/ml for 8 hr. Up to 95% of the cells accumulated in G_2 phase under those conditions. After switch of Hoechst 33342-treated cells to drug-free medium, the cells divided as a highly synchronized cohort of cells within 3 hr. Up to 85% of the cells in a culture of human diploid dermal fibroblasts (HSF-55 cells) could be accumulated in G_2 phase by placing cells presynchronized in early-S phase in medium containing Hoechst 33342 at 0.1 μg/ml for 10 hr. Reversal of G_2 arrest in the HSF-55 cultures resulted in cells dividing sychronously over 3.5 hr. By varying the concentration of Hoechst 33342 and the duration of the treatment period, it was possible to alter the position within G_2 phase at which cells accumulated. This synchronization protocol should greatly facilitate study of G_2/M biochemical events in mammalian cells, in particular, those associated with *cdc2* gene regulation of the onset of mitosis.**

Studies of events within the mammalian cell cycle are greatly facilitated by the use of highly synchronized populations in which all or most cells perform the same biochemical operations simultaneously. Among factors to be considered when using or developing a synchronization protocol are the following: (*i*) avoidance of conditions that force cells into a state of "biochemical imbalance" (1) in which the ratios of major macromolecules are grossly perturbed; (*ii*) assurance that the synchronization protocol is completely reversible; and (*iii*) recognition that the synchrony is rapidly lost (2, 3) to the extent that no single synchronization protocol is suitable for study of events throughout the entire cell cycle.

In this manuscript, these principles of synchrony induction have been applied to the development of a protocol to arrest mammalian cells reversibly in G_2 phase. The need for such populations stems from the recent rekindling of interest in studies of the cell cycle, attributable to the development of techniques for elucidating the mechanisms responsible for genetic regulation of cell proliferation. In particular, studies with a variety of biological systems, predominantly involving the lower eukaryotes, have revealed the existence of a series of *cdc2*-like genes shown to play a crucial role in regulating the initiation of mitosis (4–11). These studies are currently focusing on the molecular interactions between the proteins encoded by the *cdc2* gene and cellular constituents that trigger the transition from G_2 phase into mitosis.

Such studies previously were difficult to do with mammalian cells due to the lack of a system in which essentially all cells would progress from G_2 phase into mitosis in highly synchronous fashion. Earlier-derived procedures for accumulating mammalian cells in G_2 phase through use of centrifugal elutriation (9, 12) or temperature-sensitive mutants (13) produced populations in which only 60–70% of the cells resided in G_2 and the cells were slow to recover from the effects of the synchrony-induction method.

Our G_2 synchronization protocol overcomes the shortcomings of these previous procedures. Cells, presynchronized in early S phase, are treated with low doses of either of two different inhibitors of DNA topoisomerase II (Top II) activity, which causes cells to progress slowly through late interphase as a highly synchronized cohort of cells. Up to 95% of the cells can be accumulated in G_2 phase with our procedure and after inhibitor removal, the cells divide in a highly synchronous manner within 3–3.5 hr.

MATERIALS AND METHODS

Cell Growth. Chinese hamster ovary (CHO) cells maintained as either monolayers in 75-cm^2 tissue culture flasks or in suspension in 250-ml spinner flasks, were grown in F-10 medium supplemented with 15% heat-inactivated (56°C for 30 min) bovine calf serum (HyClone) and antibiotics (F10/15C); the $CaCl_2$ component of F-10 was omitted in studies involving suspension cultures. Plating efficiency measurements of colony-forming ability were made in a described manner (14), with cells plated *immediately after reversal* of the final synchrony-inducing blockade. HSF-55 diploid fibroblasts, derived by D. Chen (Los Alamos National Laboratory) from a human neonate foreskin sample, were grown as monolayers in 75-cm^2 tissue culture flasks in α minimum essential medium supplemented with 10% heat-inactivated bovine calf serum and antibiotics (αMEM/10C). Flow-cytometric analysis of DNA content in mithramycin-stained cells was done as described (15). Cell cycle analysis of DNA histograms from highly synchronized populations was carried out with the Multicycle Computer Program (Phoenix Flow Systems, San Diego, CA) developed by P. S. Rabinovitz (University of Washington, Seattle).

Synchronization Procedures. To accumulate cultures of CHO cells in G_2 phase, exponentially growing monolayers (initially containing $\approx 10^6$ cells per flask) or suspension cultures (initially containing $\approx 2.2 \times 10^5$ cells per ml of medium) were first maintained for 36 hr in isoleucine-deficient F-10

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Abbreviations: F10/15C, F-10 medium/15% calf serum/antibiotics; αMEM/10C, α minimum essential medium/10% calf serum/antibiotics; Top II, DNA topoisomerase II.
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medium supplemented with 15% dialyzed calf serum to arrest cells in G_1 phase (16), after which the cultures were switched to F10/15C containing 1 mM hydroxyurea for 10 hr to resynchronize the cells in early S phase (16, 17). The cells were then placed in F10/15C containing either Hoechst 33342 at 7.5 μg/ml for 12 hr or VM-26 at 0.5 μg/ml for 8 hr to accumulate cells in G_2 phase. Reversal of the G_2 blockade was accomplished by rinsing the cells twice with drug-free medium followed by resuspension in F10/15C.

Subconfluent cultures of passage 8 HSF-55 cells (initially $\approx 8 \times 10^5$ cells per flask) were maintained for 48 hr in αMEM/0.1% calf serum to accumulate cells in G_0 phase (18), after which the monolayers were exposed to αMEM/10C containing aphidicolin at 5 μg/ml for 24 hr to arrest the cells in very-early-S phase (18). The cultures were then placed in αMEM/10C containing Hoechst 33342 at 0.1 μg/ml for 10 hr to allow the cells to accumulate in G_2 phase. The G_2-rich cultures were released from Hoechst 33342 blockade by rinsing the monolayers twice with phosphate-buffered saline before adding αMEM/10C.

Chemicals used were obtained from the following sources. Hoechst 33342 (bisbenzimide H 33342 fluorochrome) was purchased from Calbiochem–Behring and was dissolved in sterile water. VM-26 (teniposide) was provided by Bristol Laboratories and was dissolved in dimethyl sulfoxide.

RESULTS

Optimization of Accumulation of CHO Cells in G_2 Phase with Hoechst 33342. In view of both the known involvement of DNA Top II in regulation of DNA topological conversions and DNA relaxation and decatenation reactions (19–21) plus the ability of Top II inhibitors to alter the rate of cell progression through G_2 phase (22–24), we explored the feasibility of inducing G_2 synchrony through the use of agents that inhibit the catalytic activity of this essential enzyme. In an initial series of studies with CHO cells, Hoechst 33342 was used as the Top II inhibitor. This fluorochrome and its structural analogs bind on the outside of DNA within the minor groove with preference for A+T-rich regions of the genome (25–27) and they inhibit Top II-mediated DNA relaxation and decatenation reactions (19) by a process other than stabilization of the cleavable complex (20, 25). In preliminary studies with CHO cells employing flow cytometry to monitor DNA distributions, it was determined that: (*i*) treatment of asynchronous populations with Hoechst 33342 produced an enrichment of cells in G_2 phase, but the effects were not readily reversible; (*ii*) treatment of cells presynchronized in early-S phase with Hoechst 33342 during progression through late interphase caused cells to arrest reversibly in G_2; and (*iii*) accumulation of cells in G_2 phase was greatest when Hoechst 33342 was added when the cells were in very-early-S phase. Optimum results were achieved in monolayer cultures of cells presynchronized in early-S phase by sequential incubation in isoleucine-deficient medium and then medium containing hydroxyurea (16, 17), after which the cells were maintained for 12 hr in complete medium containing Hoechst 33342 at 7.5 μg/ml equivalent to 13 μM (Fig. 1). The fraction of cells residing in G_2 phase in the culture shown in Fig. 1 was 94% with 0.2% residing in mitosis and <5% labeling in an autoradiogram after exposure of cells to [^{3}H]-thymidine at 2 μCi/ml (1 Ci = 37 GBq) for 30 min. In repeated preparation of Hoechst 33342-synchronized cultures, the proportions of G_2 cells usually ranged from 90–95%. Hoechst analogs 33258, 33293, or 33378 worked approximately as well as 33342 to induce G_2-rich populations. The ranges of protein and RNA contents were similar in synchronized G_2 cells and in G_2 cells from asynchronous cultures (data not shown), indicating that the cells did not enter a state of gross biochemical imbalance during the process of synchrony induction.

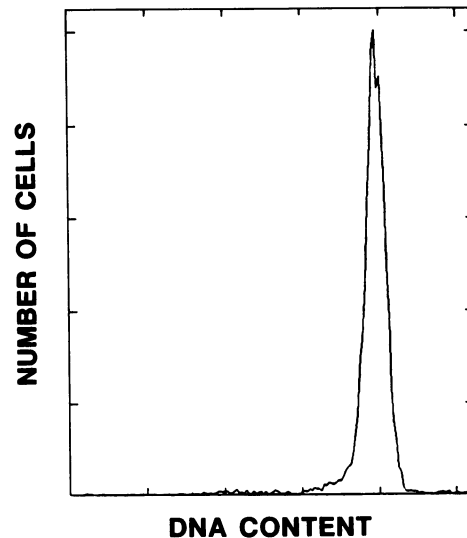

FIG. 1. Population DNA content of a culture of monolayer-grown CHO cells optimally synchronized in G_2 phase through the use of Hoechst 33342. Cells were maintained for 36 hr in isoleucine-deficient F-10 medium after which they were incubated for 10 hr in F10/15C containing 1 mM hydroxyurea, which caused cells to arrest in early-S phase. The cells were then incubated for 12 hr in F10/15C medium containing Hoechst 33342 at 7.5 μg/ml.

Reversibility of Hoechst 33342-Induced G_2-Arrest. To determine the completeness of reversibility of Hoechst 33342 effects, cultures of CHO cells blocked in G_2 phase were washed and returned to drug-free complete medium, and at intervals, aliquots were obtained for determination of cell number and population DNA content (Fig. 2). Examination of the data in Fig. 2 revealed that few cells entered G_1 phase during the first 2 hr after Hoechst 33342 removal; then a highly synchronized cohort of cells divided within the ensuing 3 hr. Because cells did not begin entering G_1 during the first 2 hr after Hoechst 33342 removal and durations of mitosis and G_2 in our CHO cells are 0.5 and 2.0 hr, respectively (28, 29), the leading edge of the G_2-arrested population in Fig. 2, assuming rapid reversibility of Hoechst 33342 effects, was located in early G_2 phase at $\approx$1.5 hr before the G_2/M boundary. We could accumulate cells at a later stage of G_2 either by increasing time of Hoechst 33342 treatment or by reducing the level of Hoechst 33342 used to block cells in G_2 phase. Conversely, use of higher levels of Hoechst 33342 or shorter incubation periods caused cells to accumulate at earlier stages of G_2. To rule out the possibility of *delayed* toxicity associated with our multistep synchronization procedure, the plating efficiency of synchronized cells was determined immediately after wash out of Hoechst 33342 and compared with the plating efficiency of a nonsynchronized control culture. The proportion of cells giving rise to colonies in the synchronized culture was 86 ± 7%, which was comparable to the plating efficiency value of 89.5 ± 5% for the control culture.

Synchronization of HSF-55 Cells in G_2 Phase. We next adapted the Hoechst 33342 procedure to synchronize a totally different, difficult-to-synchronize cell type, the HSF-55 human diploid fibroblast (18). In our CHO studies, we selected conditions that arrested cells in *early* G_2 phase. To demonstrate the flexibility of our procedure, we chose to arrest HSF-55 cells in *late* G_2 phase. The optimized procedure we established involved sequential accumulation of cells initially in G_0 phase in medium with low serum and later in early-S phase by maintenance of cells in complete medium supple-

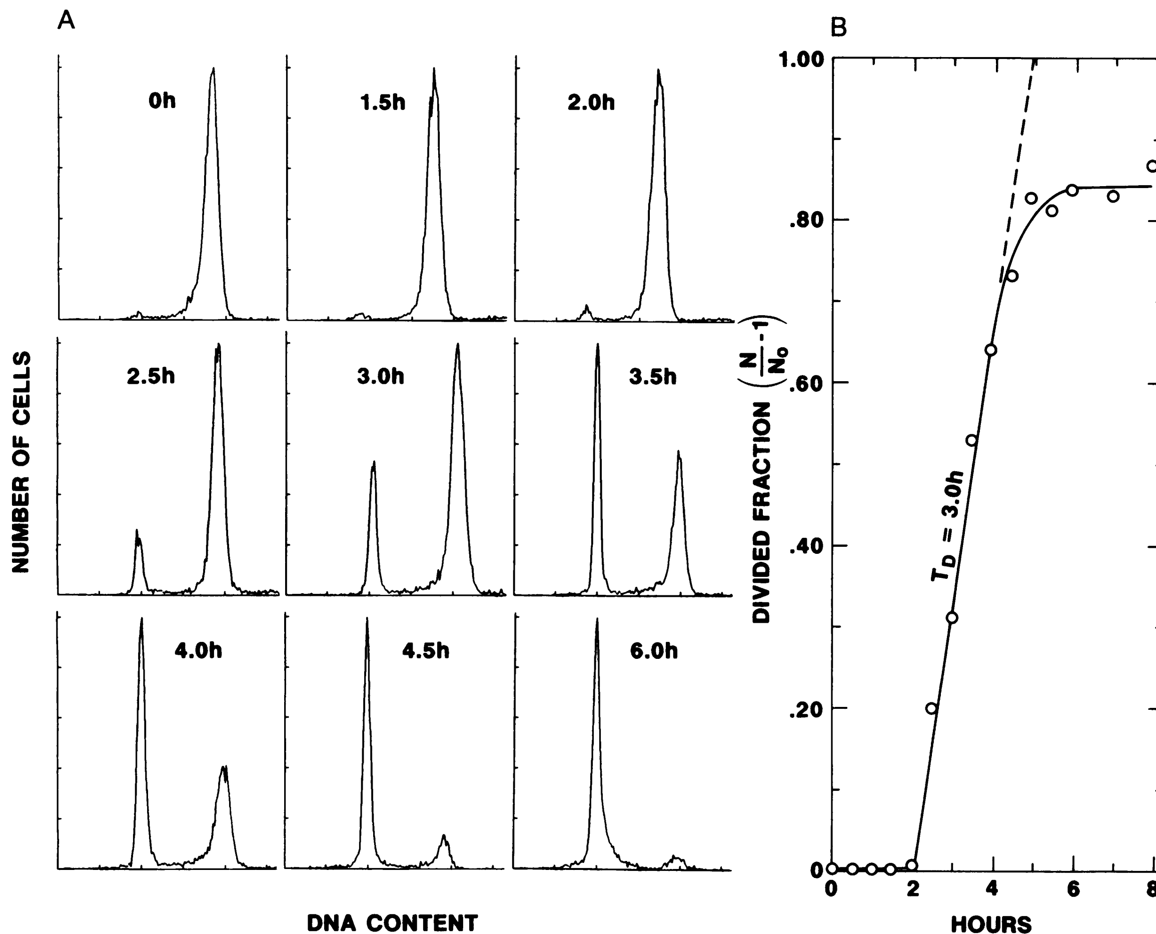

FIG. 2. Reversibility of Hoechst 33342-mediated arrest of CHO cells in G_2 phase. Cultures of monolayer-grown CHO cells were synchronized as in Fig. 1, and at $t = 0$ hr, the cultures were washed and placed in complete, Hoechst 33342-free F-10 medium. At intervals thereafter, cultures were trypsinized, and aliquots were removed to obtain population DNA distributions via flow cytometric analysis of mithramycin-stained cells (*A*) and cell counts (*B*). In this instance, T_D is arbitrarily defined as the time required for all cells in the population to divide if they all divide at the rate characteristic of the highly synchronized, majority cohort of cells.

mented with aphidicolin (18). After additional exposure to medium containing 0.17 μM Hoechst 33342 at 0.1 μg/ml for 10 hr, populations containing 80–85% G_2 cells were obtained (0-hr sample in Fig. 3). Cells began entering G_1 phase within 1 hr after Hoechst 33342 removal (indicating that the cells were located in the late-G_2 phase, as desired), and they divided as a highly synchronized cohort over a 3.5-hr period.

A study similar in design to that shown in Fig. 3 was also done with mithramycin, an agent that, like Hoechst 33342, binds in the minor groove of DNA, but unlike Hoechst 33342, exhibits affinity for G+C-rich regions of DNA (30). Exposure of presynchronized HSF-55 cells to mithramycin at 0.5 μg/ml for 10 hr enriched the proportion of G_2 cells (≈80%), but the effects of this agent were totally irreversible.

Reversible Synchrony Induction in G_2 Phase with VM-26. To confirm our supposition that Hoechst 33342-induced accumulation of cells in G_2 phase resulted primarily from its effects on Top II activity, we next attempted to synchronize CHO cells with VM-26, a compound that, unlike Hoechst 33342, does not bind to DNA and inhibits Top II-mediated DNA relaxation and decatenation reactions by entrapment of Top II in the cleavable complex (25, 31, 32). Greater than 95% of the cells accumulated in G_2 phase after treatment of early-S-phase-presynchronized cells with VM-26 at 0.5 μg/ml for 8 hr (0-hr sample in Fig. 4). After removal of the VM-26, the cells began dividing after an initial delay of 4 hr, indicating that VM-26 can also induce a reversible state of G_2 arrest.

DISCUSSION

The success obtained with two very different cell types confirms the validity of use of multiple cell-progression-interrupting procedures to achieve highly synchronized populations in G_2 phase. Interference with Top II activity appears an important component of our protocol because highly enriched G_2 populations were obtained with two inhibitors of Top II, differing in their mechanism of interference with this DNA conformation-altering enzyme. Because the effects of Hoechst 33342 were found to be more readily reversible (Fig. 2) than those associated with exposure to VM-26 (Fig. 4), we recommend Hoechst 33342 as the synchrony-induction agent of choice. Because the optimum concentration of Hoechst 33342 was 75-fold greater in CHO cells (Fig. 1) than in HSF-55 cells (Fig. 3), optimum conditions will need to be established for each new cell type to be studied; however, we suspect that most cells will require very low levels of Hoechst 33342 (0.1–0.2 μg/ml) added in very-early-S phase for optimal induction of G_2 synchrony. We also suspect that agents such as distamycin, DAPI [(4′,6-diamidino-2-phenylindole)-dihydrochloride], netropsin, and berenil, which interact with DNA similarly to that of Hoechst 33342 (19, 26), should be useful agents for inducing G_2 synchrony.

Agents such as Hoechst 33342 analogs, distamycin, and DAPI have been shown to inhibit, in *in vitro* reactions, Top II-catalyzed relaxation of supercoiled DNA and the decatenation of highly catenated DNA (19). In addition, distamycin has been reported to inhibit transcription, presumably

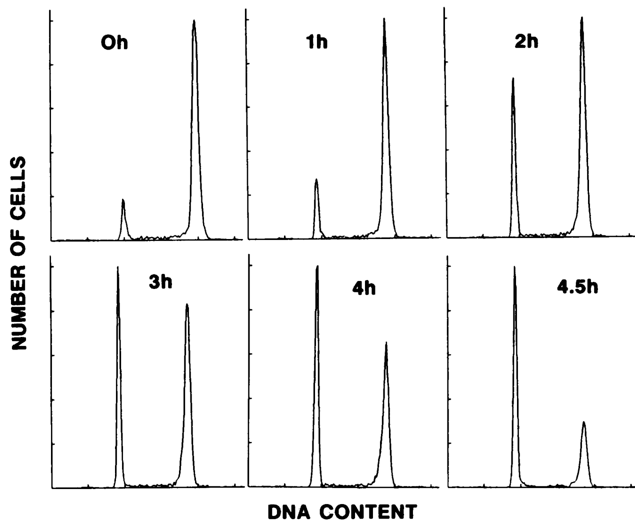

FIG. 3. Reversibility of Hoechst 33342-mediated G_2-arrest in cultures of HSF-55 human diploid fibroblasts. Monolayer cultures were maintained for 48 hr in low-serum medium before being switched to complete medium containing aphidicolin at 5 μg/ml for 24 hr. Cells were then placed in medium supplemented with Hoechst 33342 at 0.1 μg/ml for 10 hr to accumulate cells in G_2 phase. At t = 0 hr, the G_2 arrest was reversed by switching washed cells to Hoechst 33342-free medium. At times indicated, cultures were trypsinized, and aliquots were obtained for later staining with mithramycin and determination of population DNA content by flow cytometry.

through both an alteration in the extent of relaxation in the region of the genome to be transcribed and through interference with the interaction between RNA polymerase and the DNA (33). Partial inhibition of *relaxation* of DNA should result in a decreased rate of DNA replication that would slow down the rate of progression through S phase, whereas partial inhibition of *transcription* would be expected to cause cells to progress slowly through both S and G_2 phases because transcriptive activity appears to be required throughout both these phases of the cell cycle (34). In contrast, prevention of Top II-mediated *decatenation* reactions should result in a reduced ability to disentangle sister chromatids (20, 21), which would prevent cells from initiating mitosis.

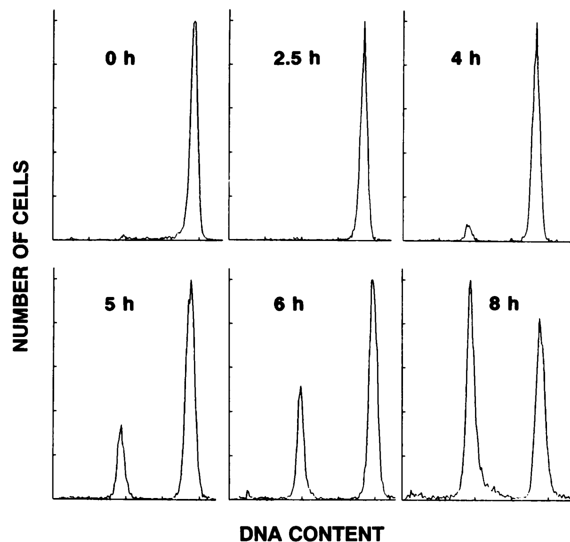

FIG. 4. Reversibility of VM-26-mediated arrest of CHO cells in G_2 phase. Suspension cultures of CHO cells were presynchronized in early-S phase by sequential incubation in isoleucine-deficient medium and hydroxyurea-supplemented complete medium as in Fig. 1. Cells were then incubated in F10/15C medium containing VM-26 at 0.5 μg/ml for 8 hr before being washed and resuspended in drug-free medium at t = 0 hr. At intervals thereafter, aliquots were obtained for later staining with mithramycin and determination of population DNA content by flow cytometry.

We observed a general slowdown in rate of progression of cells through both S and G_2 phases in Hoechst 33342-treated cultures [12 hr required for optimal accumulation of CHO cells in G_2 in Hoechst 33342-treated cultures (Figs. 1 and 2) versus 6 hr to achieve the maximum fraction of G_2 cells in drug-free cultures (data not shown)]. These results may indicate that the inhibitory effects of Hoechst 33342 were directed primarily against DNA relaxation and transcriptive events. In addition to its effects on Top II activity, binding of Hoechst 33342 to DNA might also induce a steric impediment to packaging of DNA, which would be reflected as an alteration in the chromatin-condensation cycle as cells progress through the division cycle (35–40).

In contrast to Hoechst 33342 effects, CHO cells exposed to VM-26 traversed S and G_2 phases at a nearly normal rate of progression [requiring only 8 hr to attain a maximum fraction of G_2 cells (Fig. 4)], but they were delayed from entering mitosis for a period of 4 hr after VM-26 removal. These results may indicate that the primary effect of VM-26 under our culture conditions was interference with Top II-catalyzed decatenation reactions. In agreement with that suggestion was an observed accumulation of preprophase-like cells with partially condensed chromosomes during the 4-hr period between VM-26 removal and resumption of cell division.

Regardless of the nature of the underlying mechanisms, our protocols should provide an important tool for examining, in mammalian cells, the biochemical events associated with passage of cells through G_2 phase and into mitosis. An important property of our protocol is the capability to preselect both the precise location of the arrest point in G_2 phase and the rate of progression of cells across the G_2/M boundary. This capability, in turn, should permit the most detailed studies to date of the *sequence* of events associated with activation of the *cdc2*-encoded protein kinase at G_2/M and subsequent phosphorylation of cellular proteins required for initiation of mitosis. The production of populations initiating mitosis with a high degree of synchrony available with our protocol may also find application in the high-resolution cytogenetics' studies of Yunis *et al.* (41, 42), in which thousands of G-bands can be characterized in haploid sets of chromosomes from cells synchronized in mid-prophase. Finally, application of these principles of synchronization to phases of the cell cycle other than G_2 may provide an improved quality of synchrony for them as well.

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