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Enrichment for Submitotic Cell Populations Using Flow Cytometry

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Background: One of the most dramatic events during the course of the mammalian cell cycle is mitosis, when chromosomes condense and segregate, the nuclear envelope breaks down, and the cell divides into two daughter cells. Although cells undergoing mitosis are cytologically distinguishable from nonmitotic cells, few molecular markers are available to specifically identify mitotic cells, especially cells within different stages of mitosis.

Methods: We applied the flow cytometric method of Juan et al. (Cytometry 32:71–77, 1998) to obtain cells with various levels of the molecular markers cyclin B1 and phosphorylated histone H3; fluorescence microscopy was then used to identify sorted cells in different stages of mitosis.

Results: We observed the substantial enrichment of submitotic cell populations.

Conclusions: This method represents an effective approach to obtain an enriched population of submitotic cells without the use of drug treatments or prior synchronization. Cytometry 39:126–130, 2000.

Key terms: phosphorylated histone H3; cyclin B1; mitosis

Traditional approaches to analyze cellular events that take place late in the human cell cycle have usually involved cells specifically blocked in a particular cell cycle phase or synchronized cells. With such techniques, normal cellular biology is often disrupted (1). Flow cytometric approaches are a useful alternative for analyzing cell cycle events because these methods allow examination of asynchronous and otherwise unperturbed cell populations (2,3). Several flow cytometric methods have also been described to detect mitotic cells on the basis of detectable differences in cell scatter (4,5), differential changes in acridine orange fluorescence after acid or heat denaturation (6,7), and detection of mitotic cells based on high levels of the AF-2 protein (8). A flow cytometric approach to distinguish human cells in different phases of the cell cycle by using the levels of cyclin B1 and phosphorylated histone H3 has been recently published (9).

The site-specific phosphorylation of the amino terminus of histone H3 has been described as a mitotic marker (10). This phosphorylation event has been correlated with cell cycle position and chromosomal condensation. In mammalian cells, widespread phosphorylation of this protein at serine 10 has been shown to begin in prophase, peak in metaphase, and fall during anaphase (11,12). Low levels of histone H3 phosphorylation have been initially detected late in the cell cycle in pericentric heterochromatin and found to increase throughout the genome as the cell progresses to metaphase (10). Methods used to initiate premature chromosome condensation have been found to concurrently induce phosphorylation of H3 (13,14), further supporting the association between this posttranslational modification and chromosomal condensation. Conversely, both cell cycle–dependent and drug-induced dephosphorylation of H3 have been associated with chromosome decondensation (11,15). These studies indicate that the state of phosphorylation of histone H3 at serine 10 is a highly specific indicator for mitotic chromosome condensation.

Cyclin B1, the other molecular marker of late cell cycle position, is a member of the class of mitotic cyclins that are involved in regulating cell cycle transitions. Levels of this protein rise abruptly as cells obtain a 4C DNA content, peak during the metaphase/anaphase transition, and fall precipitously as cells complete mitosis (16–18). We previously reported the use of cyclin B1 flow cytometry to isolate cell populations at different times late in the cell cycle (18).

In the present report, we extend the work of Juan et al. (9) and demonstrate that the levels of phosphorylated histone H3 and DNA content can be used to isolate a near-pure population of mitotic cells. In addition, we have

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combined this method with our previously described technique by using cyclin B1 flow cytometry (18) to isolate cell populations enriched for different substages of mitosis. These approaches will be useful for examining the molecular and cellular events that occur as a cell progresses through different phases of mitosis.

MATERIALS AND METHODS

Cell Culture

Lymphoblastoid cell lines were grown as described previously (18). Cell lines used in this study included: FF, derived from a normal male (19), and SK and VK, derived from normal females. Cells were resuspended in fresh media at 5×10^5 cells/ml at least 12 h before use.

Cell Fixation and Staining

Cells were prepared for cytology or flow cytometry by following a modified protocol (18). All centrifugation steps were 7 min at 1,000 rpm in a TJ-6 tabletop centrifuge with buckets (Beckman Instruments, Palo Alto, CA). Cells were centrifuged, the medium was aspirated, and the cell pellet was washed once in phosphate buffered saline (PBS). Cells were resuspended in 1 ml of PBS, forcefully pipetted into 5 ml of ice cold 80% ethanol, and fixed for 2 h at −20°C. After centrifugation, cells were washed once with 5 ml of 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) in PBS, recentrifuged, resuspended in 1 ml of 1% BSA, 0.25% Triton X-100 in PBS, and placed on ice for 5 min. After the addition of 5 ml 1% BSA in PBS, cells were centrifuged and resuspended as described below.

For staining of phosphorylated histone H3 only, cells were resuspended in 200 μl of PBS containing 1% BSA, 10% normal goat serum (NGS). Antiphosphorylated histone H3 antibody (catalog no. 06-570 rabbit polyclonal IgG; Upstate Biotechnology, Lake Placid, NY) was added at 1 μg per sample. After incubation at 4°C overnight, cells were washed with 5 ml, and resuspended in 200 μl, of PBS containing 1% BSA, 10% NGS (Vector Laboratories Inc., Burlingame, CA). Fluorescein-conjugated goat anti-rabbit IgG (Fab′2 fragment; Jackson Immunoresearch, West Grove, PA) was added at 7 μg per sample and incubated for 1 h at 4°C. After the addition of 5 ml of 1% BSA in PBS, cells were centrifuged and resuspended in 1 ml of propidium iodide staining buffer (9 volumes of 40 mM Tris-HCl [pH 7.4], 0.8% NaCl, 21 mM $MgCl_2$, 0.05% Nonidet P-40, 5 μg propidium iodide plus 1 volume of 10 mg/ml ribonuclease A) per 5×10^6 cells and incubated for 30 min at room temperature.

For simultaneous staining of phosphorylated histone H3 and cyclin B1, the cells were resuspended in 180 μl of PBS containing 1% BSA, 10% NGS. Antiphosphorylated histone H3 antibody (catalog no. 06-570 rabbit polyclonal IgG; Upstate Biotechnology) was added at 1 μg per sample along with 20 μl of anti-cyclin B1 antibody (fluorescein isothiocyanate [FITC]-Conjugated Cyclin B1 Antibody Reagent Set; PharMingen, San Diego, CA). The cells were incubated overnight at 4°C in the dark. Cells were then washed twice with 5 ml of 1% PBS and resuspended in 180 μl of PBS containing 1% BSA, 10% NGS. Rhodamine-conjugated goat anti-rabbit IgG (Fab′2 fragment; Jackson Immunoresearch) was added at 7 μg per sample with 20 μl of anti-cyclin B1 antibody. Cells were incubated for 1 h at 4°C. After the addition of 5 ml of 1% BSA in PBS, cells were centrifuged and resuspended in 1 ml of 4,6-diamidino-2-phenylindole (DAPI) staining buffer (40 mM Tris-HCl [pH7.4], 0.8% NaCl, 21 mM $MgCl_2$, 0.05% Nonidet P-40, and 10 μg/ml DAPI; Accurate Chemical and Scientific Corp., Westbury, NY) and stained at room temperature for 30 min.

Flow Cytometry

Flow cytometry was done on a FACS Vantage flow cytometer (Becton Dickinson, Mountain View, CA). FITC, rhodamine, and propidium iodide were excited with a 488-nm laser. FITC, rhodamine, and propidium iodide data were collected through 530-, 585-, and 630-nm bandpass filters, respectively. A multiline ultraviolet laser was used to excite DAPI. The DAPI data were collected through a 424-nm bandpass filter. Data were acquired and analyzed in CellQuest (Becton Dickinson).

Immunofluorescence Microscopy

For experiments using unsorted cells, a 100-μl aliquot of cells that were stained with an antibody to antiphosphorylated histone H3 were microfuged for 2 min at 5,000 rpm in a 5417C centrifuge (Eppendorf, Hamburg, Germany). The pellet was resuspended in 30 μl Vectashield (Vector Laboratories). Five microliters were applied directly onto microscope slides, covered with a glass coverslip, and sealed with nail polish. Cells were scored for nuclear morphology as detected by propidium iodide fluorescence and levels of phosphorylated histone H3 as detected by fluorescein fluorescence using a Zeiss Axiovert Photomicroscope. Images for Figure 1 were collected on a Bio-Rad MRC600 scanning laser confocal microscope (Bio-Rad, Richmond, CA) mounted on a Nikon Optiphot. Data were collected using a Nikon 40× 0.75 numerical aperture dry objective. Full-frame (786 × 512) 8-bit images were collected for analysis and overlaid in 24-bit RBG using Adobe Photoshop version 4.0.

For experiments analyzing flow cytometry sorted cells, about 1,000 cells having a 4C DNA content, high levels of phosphorylated histone H3, and low, middle, or high cyclin B1 were sorted directly onto microscopic slides. Five microliters of Vectashield were then added before applying a glass coverslip and sealing with nail polish. For each fraction, at least 150 random cells were scored for nuclear morphology as detected by DAPI fluorescence with a Zeiss Axiophot Photomicroscope.

RESULTS AND DISCUSSION

Detection of Mitotic Cells on the Basis of Histone H3 Phosphorylation

We have confirmed the utility of phophorylated histone H3 as a marker to identify mitotic cells. Treatment of cells with an antibody for the phosphorylated form of histone

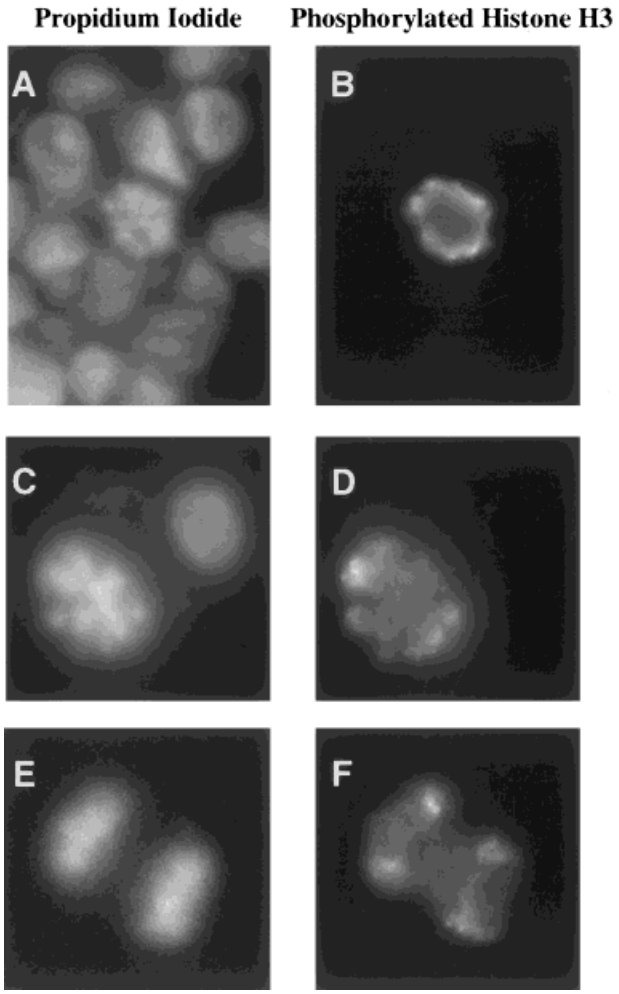

FIG. 1. Immunostaining of phosphorylated histone H3. Cells were stained with an antibody for the phosphorylated form of histone H3 and detected with a flourescein-conjugated secondary antibody. Mitotic cells are easily identified with immunofluorescence microscopy. Interphase cells, uniformly stained in **A** with propidium iodide, show virtually no antibody staining, whereas mitotic cells show intense staining as demonstrated by the prophase cell in **B**. Similarly, the prometaphase cell in **C** and **D** and the anaphase cell in **E** and **F** are stained with the antiphosphorylated histone H3 antibody, whereas the interphase cell is not (C,D).

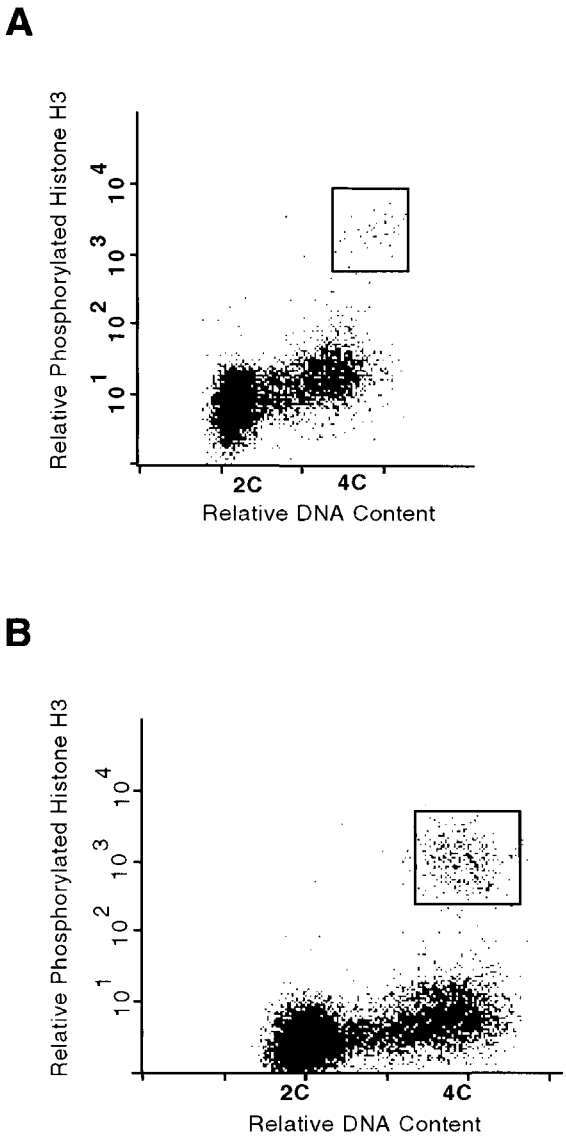

FIG. 2. Multivariate flow cytometric analysis of DNA and phosphorylated histone H3. Cells were simultaneously stained with propidium iodide (X axis) and an antiphosphorylated histone H3 antibody, detected with a fluorescein-conjugated secondary antibody (Y axis). **A:** Cells identified cytologically as being mitotic have a 4C DNA content and high levels of phosphorylated histone H3 (boxed area). **B:** The size of the mitotic population is increased in cells treated for 1 h with colcemid (boxed area).

H3, detected with a fluorescein-conjugated secondary antibody, allows for easy recognition of mitotic cells with immunofluorescence microscopy (10). Cells in prophase, prometaphase, metaphase, and anaphase show intense staining for H3P, whereas interphase cells and telophase cells do not show high levels of staining (Fig. 1A–F and data not shown). Some cells show low levels of staining that appear punctate within the nucleus. Hendzel et al. (10) observed similar staining patterns and found that these punctate localizations represent chromosome regions of pericentric heterochromatin in which histone H3 has become phosphorylated late in the G2 period or early in prophase. Therefore, it seems likely that the cells we observed with low levels of punctate staining are similarly late in the G2 period or early in prophase.

Cytologic studies indicated that the level of the phosphorylated form of histone H3 can be used as a sensitive method for identifying mitotic cells using flow cytometry, and this was confirmed by Juan et al. (9) (Fig. 1). Bivariate flow cytometric analysis of DNA content and the level of phosphorylated histone H3 shows that cells positive for high levels of phosphorylated H3 have a 4C DNA content (Fig. 2A). Mitotic arrest with colcemid enlarges the population of cells with high levels of phosphorylated histone H3 (Fig. 2B). The maximal level of antibody staining to phosphorylated histone H3 is approximately 20–50-fold

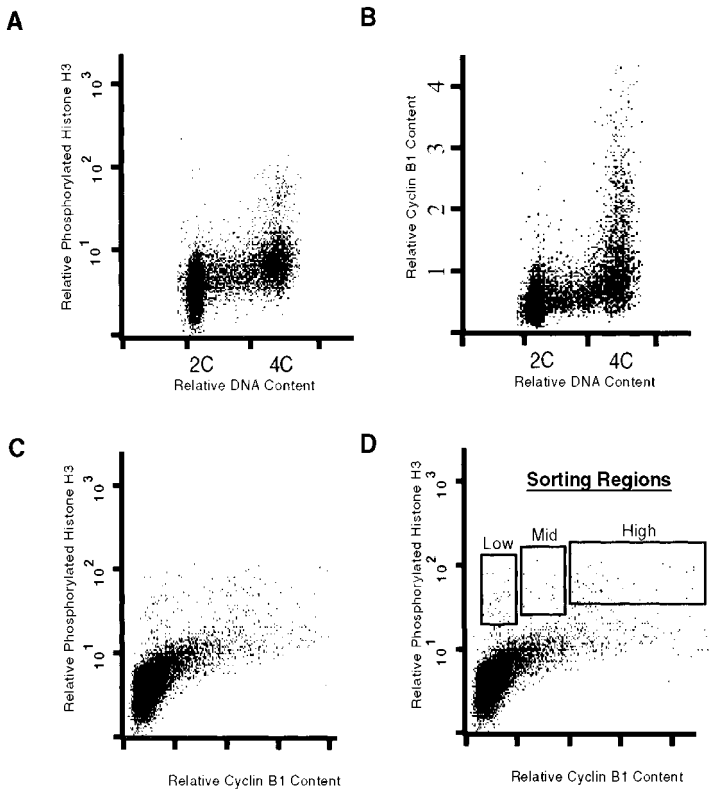

FIG. 3. Trivariate flow cytometric analysis of DNA, phosphorylated histone H3, and cyclin B1. Cells were simultaneously stained with 4,6-diamidino-2-phenylindole, an antiphosphorylated histone H3 antibody, detected with a rhodamine-conjugated secondary antibody, and a fluorescein isothiocyanate (FITC)-conjugated anti–cyclin B1 antibody. **A:** Mitotic cells are detected as having a 4C DNA content and high levels of phosphorylated histone H3. The separation of 4C cells with low and high levels of phosphorylated histone H3 was not as distinct in these trivariate flow cytometry experiments, perhaps due to higher background staining from the FITC-conjugated cyclin B1 antibody. **B:** Cells with a 4C DNA content have heterogeneous levels of cyclin B1. **C:** Cells with a 4C DNA content were gated and separated on the basis of the levels of cyclin B1 and phosphorylated histone H3. **D:** The 4C cells with high levels of phosphorylated histone H3 were separated into the fractions having low, middle, or high cyclin B1 and were used to collect the data presented in Table 1.

Table 1
*Separation of Cells Within Different Stages of Mitosis Using the Levels of Phosphorylated Histone H3 and Cyclin B1**

	Phase				
Cyclin B1 fraction	Interphase	Prophase	Prometaphase	Metaphase	Anaphase
Low	0	28	23	3	46
Middle	1	44	40	12	3
High	0	31	33	28	8

* Cells with a 4C DNA content and high levels of phosphorylated histone H3 were collected from three fractions with low, middle, or high cyclin B1 levels (see Fig. 3D) and scored on the basis of nuclear morphology. Data from each sorting fraction are presented as the percentage of sorted cells found within a particular stage of the cell cycle. Cells in either prophase or prometaphase were found to be most enriched in the middle cyclin B1, high phosphorylated histone H3 fraction; metaphase cells were most enriched in the high cyclin B1, high phosphorylated histone H3 fraction; and anaphase cells were enriched in the low cyclin B1, high phosphorylated histone H3 fraction.

higher for mitotic cells compared than for G1 cells or for cells stained with fluorescein-conjugated secondary antibody alone (Fig. 2A,B and data not shown).

Cells with high levels of phosphorylated histone H3 (box, Fig. 2A) were flow sorted onto slides and scored for nuclear morphology to identify their cell cycle position. More than 99% of the sorted cells were mitotic, including 30% prophase, 31% prometaphase, 23% metaphase, and 15% anaphase. No telophase cells were found, in agreement with previous reports that by telophase the levels of phosphorylated histone H3 fall to interphase levels (11). The anaphase population, however, included cells near the anaphase/telophase transition, as identified by the presence of chromosomes that are clearly segregated, similar to the cell illustrated in Figure 1E,F. This observation suggests that rapid dephosphorylation of histone H3 occurs during the anaphase/telophase transition, consistent with previous results (11).

Multivariate Flow Cytometric Analysis Using Cyclin B1 and Histone H3P

To separate cells in different stages of mitosis, we used trivariate flow cytometric analysis to determine the levels of cyclin B1, phosphorylated histone H3, and DNA content. Cells with a 4C DNA content have heterogeneous levels of phosphorylated histone H3 (Fig. 3A) and cyclin B1 (Fig. 3B). These 4C cells were separated by the levels of phosphorylated histone H3 and cyclin B1 (Fig. 3C),

sorted from each of three fractions (Fig. 3D), and scored for nuclear morphology. There was substantial enrichment for cells within different periods of mitosis among each of the sorting fractions (Table 1). Cells with either a prophase or prometaphase nuclear morphology were found to be most enriched (84%) in the mid-cyclin B1, high phosphorylated histone H3 fractions; metaphase cells were most enriched (28%) in the high cyclin B1, high phosphorylated histone H3 fraction. These findings are in agreement with data from previous studies using only cyclin B1 flow cytometry (18). Anaphase cells were markedly enriched in the low cyclin B1, high phosphorylated histone H3 fraction (46%). We previously reported that mitotic cells with low levels of cyclin B1 are found to be primarily in telophase (18). In the present study, we did not observe any telophase cells isolated in the fraction of cells with both high levels of phosphorylated histone H3 and low levels of cyclin B1. This observation is consistent with previous reports showing that histone H3 becomes dephosphorylated as cells transit to telophase (11). Thus, staining for DNA content, the levels of cyclin B1, and the phosphorylated form of histone H3 can be used to isolate cell populations enriched for specific stages within mitosis. To our knowledge, this represents the first technique that can be used to enrich for submitotic populations without the use of drug treatments or prior synchronization. This methodology will provide a valuable technique for separation of cells at several periods late in the cell cycle, which are then suitable for molecular and biochemical analysis.

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