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Correlation between DNA Replication and Expression of Cyclins A and B1 in Individual MOLT-4 Cells[1]

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Abstract

Cyclins A and B1 activate cyclin-dependent kinases CDK2 and CDC2, which regulate cell progression through S and G_2. Expression of these cyclins is generally measured in populations of synchronized cells, by immunoblotting. Such studies neither provide information regarding intercellular variability in cyclin expression nor yield precise data on a time relationship between initiation and termination of DNA replication in relation to cyclin expression. Furthermore, cell synchronization by DNA polymerase inhibitors or excess of thymidine induces cell growth imbalance and alters expression of cyclins, thereby introducing an experimental bias. Using a novel flow cytometric method of detection of incorporated bromodeoxyuridine (BrdUrd) in the present study, we have been able to correlate expression of immunocytochemically discerned cyclins A and B1 with incorporation of BrdUrd and the cell cycle position of individual MOLT-4 cells. On the basis of differences in amount of incorporated BrdUrd and DNA content, the following cohorts of cells in narrow windows of the cell cycle were identified: (*a*) cells initiating and (*b*) terminating DNA replication during a 1-h pulse of BrdUrd; (*c*) cells replicating DNA throughout the duration of BrdUrd pulse; (*d*) G_1 cells; and (*e*) G_2 cells that remained in G_2 for at least 1 h after exiting S phase. These populations were characterized with respect to expression of cyclins A and B1. Expression of cyclin A was an early event of S phase, and 84% of cells entering S phase during 1 h of exposure to BrdUrd were already cyclin A positive. More than 95% of S-phase cells, as well as the cells exiting S during BrdUrd pulse, were also cyclin A positive. The maximal rate of accumulation of cyclin A was seen during the first hour of progression through S phase. In contrast, the maximal accumulation rate of cyclin B1 showed cells during the first hour of progression through G_2. A strong correlation between expression of cyclin A and the rate of DNA replication, estimated by the degree of BrdUrd incorporation (r = 0.99), was observed.

Introduction

Phosphorylation of different sets of cellular proteins by CDKs[3] takes place during successive phases and checkpoints of the cell cycle (reviewed in Refs. 1–6). Cyclins play a key regulatory role in this process by activating their partner CDKs and targeting them to the respective protein substrates. Several cyclins are expressed discontinuously during the cycle, their synthesis and degradation occurring at sharp and well-defined time points of the cell cycle. Information about the timing of cyclin expression and its relationship to other cell cycle events is of obvious interest for understanding molecular mechanisms of the cell cycle progression machinery.

Timing of expression of particular cyclins in relation to other events of the cell cycle, such as DNA replication or mitosis, is generally studied in experiments that involve cell synchronization in the cycle followed by analysis of the message or protein level by Northern or Western blotting. Unfortunately, the information about individual cells, intercellular variability, detection of cell subpopulations, or direct correlation of the presence of cyclins with other cellular constituents cannot be obtained by such assays. Furthermore, synchronization of tumor-transformed cells either cannot be very precise (*e.g.*, centrifugal elutriation) or requires transient cell arrest in the cycle generally induced by inhibitors of DNA polymerase. In the latter case, expression of many proteins, including cyclins, is perturbed by the arrest itself, which also induces severe growth imbalance and thereby introduces experimental bias (7, 8). Thus, the classical methods do not permit for very accurate analysis of timing of an expression of particular cyclins in relation to other kinetic events of the cell cycle.

Cyclins A and B1 are associated with CDK2 and CDC2, the kinases regulating cell progression through S and G_2 (6, 9, 10). It is of interest, therefore, to relate expression of these cyclins to the time of initiation or termination of DNA replication. We have recently developed a new method of detection of BrdUrd incorporated into cellular DNA, which was applied to analyze DNA replication in individual cells by multiparameter flow cytometry (11, 12). The method is based on DNA SBIP at the sites containing incorporated BrdUrd. The BrdUrd-associated DNA strand breaks are subsequently labeled with fluorochrome-tagged deoxynucleotides in the reaction catalyzed by exogenous terminal deoxynucleotidyl transferase. Unlike the standard assay of BrdUrd detection requiring harsh conditions of acid or heat treatment for DNA denaturation (to have the incorporated BrdUrd accessible to BrdUrd antibody), which destroys many antigenic epitopes, the SBIP is fully compatible with simultaneous immunocytochemical analysis of the cellular phenotype (12). This methodology was presently combined with the detection of cyclin A or cyclin B1 and with multiparameter flow cytometry (reviewed in Ref. 13). This allowed us to reveal a correlation between DNA replication and expression of these cyclins in exponentially growing, unperturbed cultured cells.

Materials and Methods

Cell Labeling with BrdUrd. Human leukemic MOLT-4 cells, obtained from American Type Culture Collection (Rockville, MD), were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics, as described (12). All media, supplements, and antibiotics were obtained from Life Technologies (Grand Island, NY). The cultures were periodically tested for *Mycoplasma* infection. To maintain asynchronous exponential growth, the cultures were passaged by diluting to a concentration of 1×10^5 cells/ml and were repassaged before approaching 5×10^5 cells/ml density. The cells were labeled with BrdUrd by administration of 30 μg/ml of the precursor (Sigma Chemical Co., St. Louis, MO) into the cultures for 1 h. The cells were then centrifuged, approximately 5×10^6 cells were suspended in 1 ml of PBS, and this suspension was transferred to a plastic Petri dish 50 mm in diameter. The Petri dish was then placed on the glass surface of a Fotodyne UV 300 analytic DNA transilluminator containing four 15-W bulbs (Fotodyne Inc., New Berlin, WI), providing maximal illumination at 300 nm wavelength; the dish was then

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[3] The abbreviations used are: CDK, cyclin-dependent kinase; BrdUrd, bromodeoxyuridine; SBIP, strand break induction by photolysis; PE, phycoerythrin; TdT, terminal deoxynucleotidyl transferase; 7-AAD, 7-amino actinomycin D; C, DNA content.

exposed to UV light for 10 min. The average intensity of UV light at the surface on which the cells were exposed, measured by a UVX-25 sensor (UVP, Inc., Upland, CA), was 4.5 mW/cm^2. The cells were then fixed in suspension in ice-cold 80% ethanol and kept at −20°C for 2 h, or overnight, as described (12). Other details concerning culture conditions and labeling with BrdUrd are described in earlier publications (11, 12).

Immunocytochemical Detection of Cyclins A and B1. After fixation, the cells were washed twice with PBS and then suspended in 1 ml of 0.25% Triton X-100 in PBS on ice, for 5 min. After centrifugation, the cell pellet was suspended in 100 μl of PBS containing 0.25 μg of the anticyclin MoAb (PharMingen, San Diego, CA; cyclin A, clone BF683; cyclin B1, clone GNS-1) and 1% BSA and incubated for 1 h at room temperature. The cells were then rinsed with PBS containing 1% BSA and incubated with the PE-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) diluted 1:30 in PBS containing 1% BSA for 30 min at room temperature in the dark. Other details of the immunocytochemical procedure are presented elsewhere (7, 14).

Western Blotting. To reveal specificity of the used cyclin A and B1 antibodies, a standard immunoblotting procedure was used on cellular lysates, as described by us before (7)

Detection of the Incorporated BrdUrd. The photolytically generated DNA strand breaks at the sites of BrdUrd incorporation were labeled with BrdUTP in the reaction catalyzed by exogenous TdT as described before (11, 12, 15). Briefly, the cells were rinsed twice with PBS, and the cell pellet was suspended in 50 μl of TdT reaction buffer containing 10 μl of 5× concentrated buffer solution (1 M potassium cacodylate, 125 mM Tris-HCl (pH 6.6), and 1.26 mg/ml BSA); 5 μl of 25 mM cobalt chloride; 0.5 μl (12.5 units) of TdT (all from Boehringer Mannheim, Indianapolis, IN); 5 μl of stock BrdUTP solution containing 0.25 nmol of this nucleotide (Sigma); and 30 μl of distilled water. The cells were then rinsed with PBS and incubated in 100 μl of a solution containing 0.7 μg of FITC-conjugated anti-BrdUrd MoAb (Becton Dickinson, San Jose, CA), 0.1% Triton X-100, and 1% BSA in PBS for additional 30 min. The cells were then counterstained with 5 μg/ml 7-AAD (Molecular Probes) in PBS. Details of this method are presented elsewhere (12).

Analysis of the Cellular Fluorescence. Cellular fluorescence was measured using the standard optical filter configuration of the FACScan flow cytometer (Becton Dickinson), as described (14–17). The FITC-associated green fluorescence (FL1 channel) was representative of the BrdUrd incorporation, the PE fluorescence measured in FL2 channel represented immunofluorescence of cyclin antibodies, and the high wavelength red fluorescence of 7-AAD, measured in FL3, represented DNA content. There was less than 10% fluorescence emission spectrum overlap between the channels as measured with control samples that were stained with a single fluorochrome.

The experiment was repeated at several times, yielding essentially identical results. The respective controls consisted of cells that were: (*a*) incubated in the absence of BrdUrd; (*b*) incubated in the presence of BrdUrd, but not illuminated with UV light for SBIP; (*c*) (for labeling DNA strand breaks) incubated with the TdT reaction medium without the TdT enzyme; and (*d*) incubated with isotype IgG instead of anti-cyclin A or B1 antibody, as a control for the detection of the cyclins.

Results and Discussion

MOLT-4 cells were subjected to a 1-h BrdUrd pulse, and the cells incorporating BrdUrd were identified by the SBIP approach (FITC labeling, green fluorescence; Fig. 1). The cyclins were detected immunocytochemically by using either cyclin A- or B1-specific MoAb labeled with a fluorochrome of another color (PE, orange fluorescence). It was shown by us before that the antibodies presently used for cytometry are specific toward the detected cyclins and do not cross-react with other proteins of MOLT-4 cells (13, 16–18). Furthermore, as it is evident in Fig. 1, the G_1 cells that are known to be cyclins A and B1 negative, indeed had the immunofluorescence intensity similar to that of the control cells, incubated with the isotype control IgG (Fig. 1). In addition, immunoblots of the lysates of the cells used in the experiment had a single predominant band at molecular weights corresponding to size of cyclins A and B1, respectively (Fig. 1*A*). DNA was counterstained with a fluorochrome of still another color (7-AAD, long wavelength red fluorescence). Thus, it was possible to measure relative amounts of the incorporated BrdUrd, cyclins A or B1, and DNA simultaneously, in individual cells.

The "paint-a-gate" multiparameter computer analysis as seen in Fig. 1 allowed us to identify five distinct cell subpopulations differing in their DNA content (cell cycle position) and/or incorporation of BrdUrd during the 1-h pulse. Expression of cyclin A and cyclin B1 was studied in each of these subpopulations.

Three cell subpopulations are distinguished among the BrdUrd incorporating cells (Fig. 1*B*). The cells with a variable degree of BrdUrd incorporation and a DNA content between 2.0 and 2.3 C (*R2*) are the cells that during a 1-h pulse of BrdUrd were entering S phase (S_E); *i.e.*, because the rate of DNA replication is relatively constant throughout the S phase (19), the variable level of BrdUrd incorporation indicates that S_E cells were exposed to the precursor for a variable period of time, up to 60 min. Furthermore, S_E cells are characterized by a DNA content between 2.0 and 2.30 C, which indicates that they replicated less than 15% of the genome while exposed to BrdUrd. This fraction of replicated DNA per 1 h is consistent with the approximately 8-h duration of their S phase. However, because incorporation of the BrdUrd can be detected as early as 5 min after administration of the precursor into the cultures (17), one may assume that the time window of the cell cycle through which S_E cells are recognized is actually about 55 min.

Another subpopulation of cells with a variable level of BrdUrd incorporation represents cells exiting S phase (entering G_2; G_{2E}) during the pulse (*R6*; Fig. 1*B*). Because at the time of cell harvesting they were already in G_2, their DNA content is 4 C. The third subpopulation of cells that incorporate BrdUrd represent cells with a variable DNA content between 2.3 and 4.0 C and a relatively constant level of BrdUrd fluorescence (*R5*). These are S-phase cells that were exposed to the precursor for the whole duration of the pulse. The two subpopulations of cells that did not incorporate BrdUrd have DNA content 2.0 or 4.0 C. These are G_1 or G_2 cells that did not replicate DNA during the pulse; *i.e.*, they had not yet entered S phase (*R3*), or they entered G_2 (*R1*) just before the addition of the precursor.

There were 44.3 ± 2.2, 16.1 ± 2.9, 27.9 ± 0.7, 7.0 ± 1.5, and 4.9 ± 0.5% cells in regions G_1, S_E, S, G_{2E}, and G_2-M, respectively, as estimated in seven separate measurements of different cultures. The lower percentage of cells entering G_2 compared with the cells entering S is expected due to the exponential distribution of the cells' age in these exponentially growing cultures. The observed difference, however, (16.1 *versus* 7.0%) is somewhat higher than expected, perhaps due to an underestimate in G_{2E} cells.

Using the multiparameter "paint-a-gate" computer analysis of individual cells, each of the subpopulations can be analyzed with respect to either cyclin A or cyclin B1 expression (Fig. 1, *C–E*; Table 1). The most attractive feature of this multiparameter approach is the possibility of analysis of cyclin expression in a very narrow time (1 h) window located at the entrance to and exit from S phase, and correlation of these cyclins with DNA replication and cell cycle position. Cells in G_1 were essentially cyclin A negative, with fewer than 10% presenting measurable expression of this protein (Table 1). However, 84% of cells entering S phase during BrdUrd pulse (S_E), *i.e.*, the cells that were replicating up to 15% of their genome (DNA content 2.0–2.3 C), were cyclin A positive. Thus, the onset of expression of cyclin A occurs very early during S phase, almost coinciding with initiation of DNA replication. Nearly all of the cells (98%) replicating the remaining 85% of their genome (cells with DNA content 2.3–4.0 C), as well as 97% of the cells that entered G_2 during the pulse (G_{2E} cells), were cyclin A positive. On the other hand, only 74% cells with 4 C DNA content but BrdUrd unlabeled were cyclin A positive. This

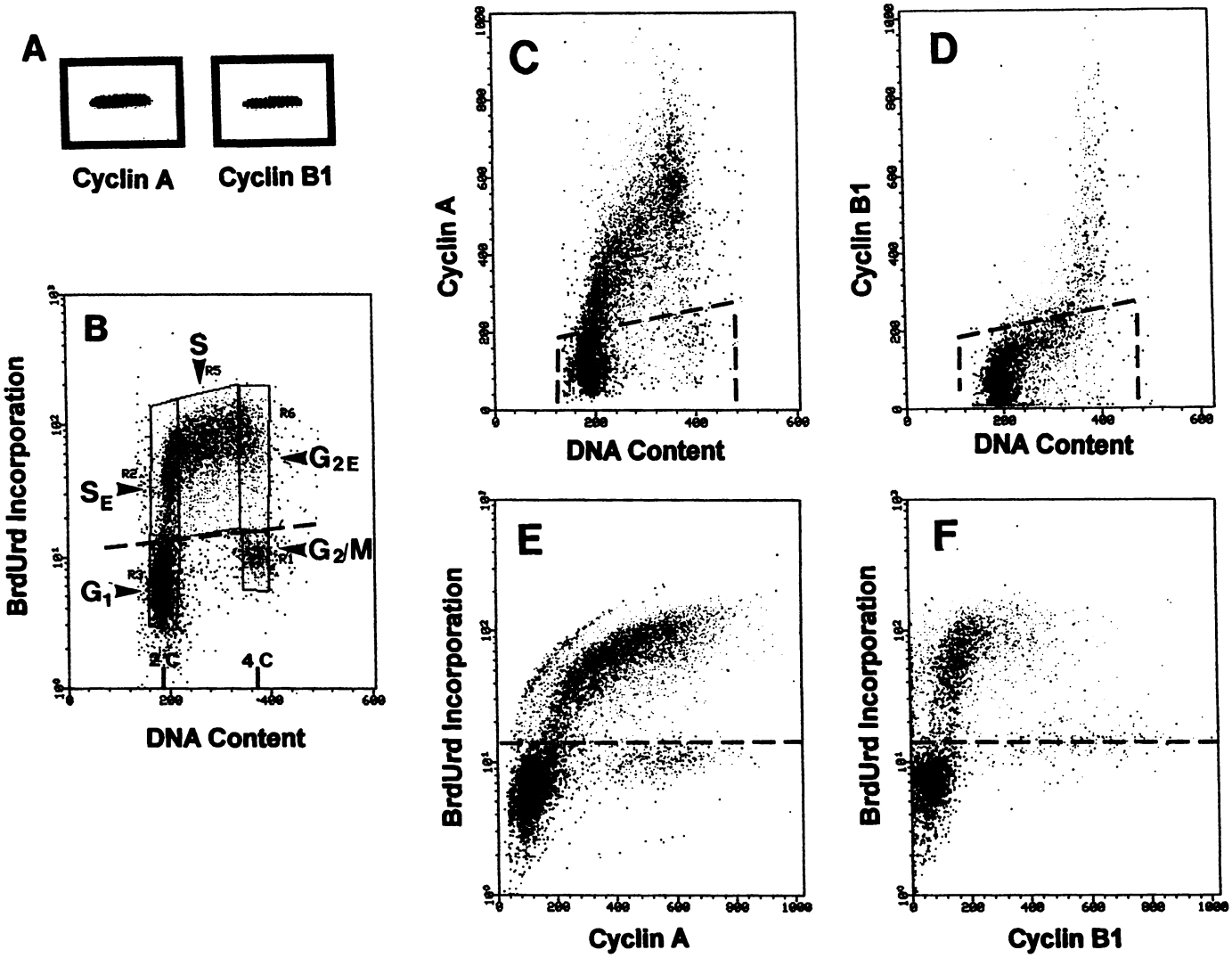

Fig. 1. Analysis of expression of cyclin A and cyclin B1 in relation to the cell position in the cell cycle and incorporation of BrdUrd. *A*, Western blots of the lysates from MOLT-4 cells obtained with MoAbs used for the immunocytochemical detection of cyclin A and cyclin B1 by flow cytometry. On the entire lanes of these immunoblots, the most prominent single bands corresponded to proteins of approximately M_r 58,000 and 61,000, respectively. *B–F*, five different cell subpopulations can be distinguished on the scattergram representing cellular DNA content and BrdUrd incorporation (*B*), as described in the text. During a "paint-a-gate" analysis, each subpopulation is tagged with a different color. Reprocessing the data allows one to reveal correlations between expression of cyclin A or cyclin B1 *versus* DNA content (*C* and *D*) or *versus* BrdUrd incorporation (*E* and *F*). These correlations can be then analyzed separately for each of the tagged subpopulations, which are distinguishable in *C–F* by their color. *Dashed lines* represent the extent of the background fluorescence of the control cells not incubated with BrdUrd (*B*, *E*, and *F*) or incubated with isotypic IgG instead of anticyclin MoAb (*C* and *D*).

Table 1 *Expression of cyclins A and B1 in relation to initiation and termination of DNA replication phase in MOLT-4 cells*

The data present frequency (percentage, left columns) of cells expressing cyclin A or cyclin B1 at different time windows of the cell cycle (G_1, S_E, S, G_{2E}, and G_2-M) defined as shown in Fig. 1. The mean values of intensity of cyclin A- or B1-associated immunofluorescence (right columns) were obtained for each of these populations from gating analysis. The means were normalized for each cyclin (the highest value = 100).

	G_1		S_E		S		G_{2E}		G_2-M	
	%	Mean	%	Mean	%	Mean	%	Mean	%	Mean
Cyclin A	9	9	84	36	98	90	97	100	74	61
Cyclin B1	0	0	7	12	11	32	61	65	91	100

drop in the percentage of cells expressing cyclin A in this cell subpopulation is due to the presence of a significant number of mitotic cells; degradation of cyclin A occurs at prometaphase (6), and therefore post-prometaphase cells are cyclin A negative (18).

The mean intensity of cyclin A immunofluorescence was highest for the subpopulation of cells that entered G_2 during the pulse of BrdUrd (G_{2E}; Table 1). This value was 3-fold higher compared with cyclin A immunofluorescence of the cells that were entering S phase during the pulse (S_E). Thus, the cells progressing through S phase between the points at which 15 and 100% of their DNA was replicated (from 2.3 to 4.0 C on the DNA content scale) showed, on average, a 3-fold increase in expression of cyclin A. The duration of S phase of our MOLT-4 cells is approximately 8 h. Hence, the average increase in cyclin A expression during the cell progression through S between these points (separated by 7 h) is approximately 45% per hour, or 35% per 10% of replicated DNA. Judging from the slopes illustrating the rate of accumulation of cyclin A during cell progression through S (Fig. 1*C*), it is evident that this rate was much faster during the first hour of S phase (S_E cells) than in the period afterward (S cells).

G_1 cells were cyclin B1 negative. In contrast to cyclin A expression, however, relatively few cells entering (7%) and progressing through (11%) S phase expressed cyclin B1. The onset of this protein expression was evident in a subpopulation of cells entering G_2 during the pulse of BrdUrd. There were 61% cyclin B1-positive cells in this subpopulation. The maximal expression of cyclin B1, however, was seen in the subpopulation of G_2-M BrdUrd unlabeled cells, *i.e.*, the late G_2 and mitotic cells. These cells were more than 90% cyclin B1

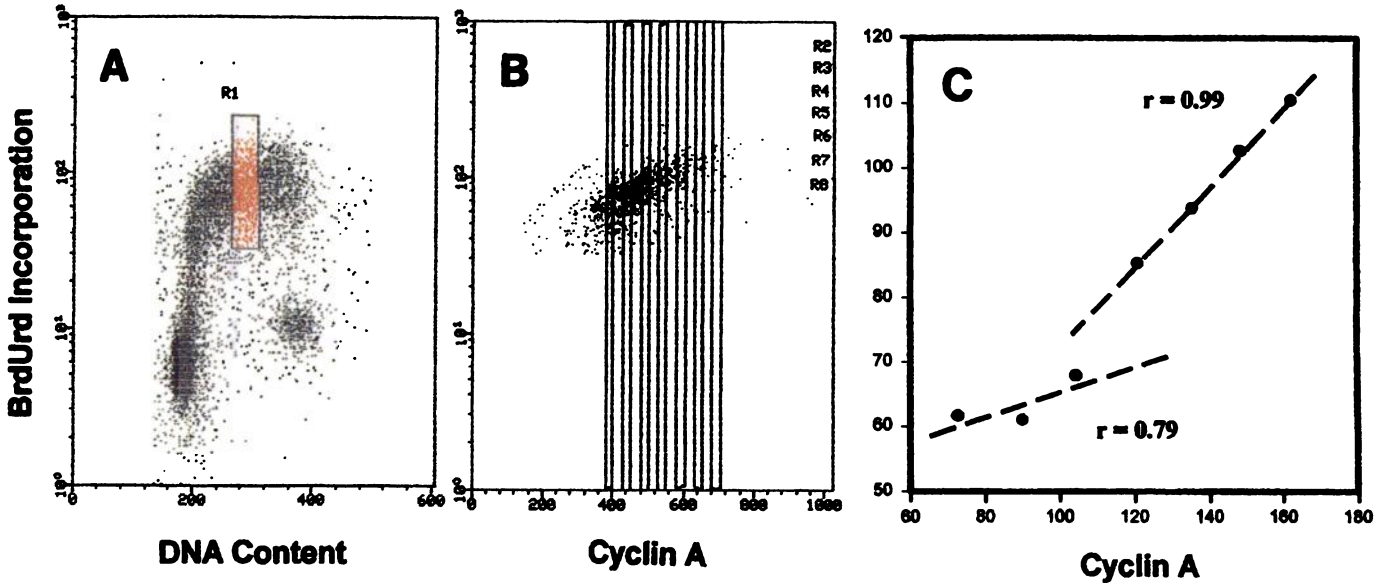

Fig. 2. Analysis of a correlation between expression of cyclin A and BrdUrd incorporation in individual MOLT-4 cells progressing through mid-S phase. The mid-S-phase-gated cell population (*A*) was analyzed by the subsequent gating to reveal cell subpopulations differing in expression of cyclin A (*B*). The mean values of the cyclin A *versus* BrdUrd-associated immunofluorescence (background fluorescence subtracted) is then plotted for each of the narrowly gated subpopulations (*C*). A strong correlation is apparent for the cells characterized by a high level of cyclin A ($r = 0.99$), and a weaker correlation is seen for the cells with low cyclin A expression ($r = 0.79$). Note that the BrdUrd coordinate scale in *A* and *B* is logarithmic, whereas in *C* it is linear.

positive, and the level of this protein presentation in individual cells was 54% higher compared with the cells entering G_2 during the pulse (G_E).

Bivariate analysis of cyclin A or cyclin B1 expression *versus* incorporation of BrdUrd is illustrated in Fig. 1, *E* and *F*. Each of the five cell subpopulations representative of different time windows of the cell cycle can be discriminated on these plots based on their characteristic color tagging during the "paint-a-gate" analysis. Thus, each subpopulation can be analyzed separately with respect to a direct correlation between incorporation of BrdUrd and expression of cyclins. Note that because the overall intensity of the cells' fluorescence, as well as their intercellular variability, was significantly greater in the case of BrdUrd- compared with cyclin-labeling, the coordinate representing BrdUrd incorporation is given in the logarithmic scale (Fig. 1). It is evident from these scattergrams, which represent raw data, that the incorporation of BrdUrd and the expression of cyclin A are correlated in all three cell subpopulations. The correlation between the expression of cyclin B1 and BrdUrd incorporation is less apparent. This may be due to the fact that a relatively small fraction of DNA-replicating cells expressed this protein (Table 1).

To obtain more definite evidence of a correlation between expression of cyclin A and BrdUrd incorporation, the mid-S-phase cells were gated, and the correlation was analyzed only for the selected subpopulation (Fig. 2). This was done by the subsequent gating analysis, by "slicing" this subpopulation onto many narrow gates as shown in Fig. 2*B*. The mean values of the BrdUrd-associated cell fluorescence of cells in each of the narrow gates were then plotted against the mean values of the respective cyclin-associated fluorescence of the same cells (Fig. 2*C*). Two distinct regression slopes, each indicating a correlation between these variables, are evident. The regression plot representing the cells with low expression of cyclin A shows lesser correlation ($r = 0.79$) compared with the slope representing the cells with greater level of cyclin A expression ($r = 0.99$).

The observed correlation between expression of cyclin A and the incorporation of BrdUrd most likely indicates that the rate of DNA replication in individual mid-S-phase cells is correlated with the content of cyclin A in these cells. This finding is consistent with the data in the literature (20, 21), which indicate that overexpression of this cyclin triggers the cells to enter S phase and accelerates the rate of DNA replication. The present results suggest that the intercellular variability in the cell rate of traverse through S phase is a reflection of a variability in the expression of cyclin A.

The present approach, which combines multiparameter analysis of cyclin expression with cell cycle position (DNA content) and DNA replication (BrdUrd incorporation), reveals a direct relationship between these variables within the same cell population, provides information about individual cells and intercellular variability, and does not require cell synchronization in the cycle. Thus, this approach complements traditional methods of analysis of such relationships based on cell transfection with the vector carrying the cyclin gene, the transcription of which can be enforced, and/or on the use of synchronized cell cultures.

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