0196-4763/80/0102-0098$00.00/0
CYTOMETRY
Vol. 1, No. 2, 1980
Printed in U.S.A.

New Cell Cycle Compartments Identified by Multiparameter Flow Cytometry[1, 2]

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Received for publication March 26, 1980, accepted May 30, 1980

Simultaneous measurements of cellular DNA and RNA as well as estimates of the sensitivity of DNA *in situ* to denaturation by acid (which correlates with the degree of chromatin condensation) and cell ability to incorporate 5-bromodeoxyuridine (BUdR), performed on over 40 different cell systems enabled us to subclassify cells into 12 functionally distinct cell cycle compartments. Quiescent cells had low RNA values, DNA very sensitive to denaturation, and they did not incorporate BUdR. Although in most cell systems they had 2C DNA content (G_{1Q}), quiescent cells with higher DNA values (S_Q and G_{2Q}) were also seen. The G_1 phase of exponentially growing cells had two distinct compartments, A and B. G_1 cells entered S phase only from the B compartment. An increase in RNA and a decrease in the sensitivity of DNA to acid denaturation beyond a specific level characterized the transition of cells from the A to B compartments. Since the G_{1A} to G_{1B} transition was not linear but exponential, it may be assumed that a nondeterministic event triggering cell progression into the cycle resides within G_{1A}. Under adverse growth conditions the probability of a G_{1A} to G_{1B} transition decreased. Cells In G_2 had DNA more sensitive to acid denaturation than S or G_1 phase cells. Mitotic cells had the most condensed chromatin and their RNA content was twice that of G_{1A} cells. Differentiated cells were characterized by 2C DNA (G_{1D}) but, depending on the cell type, had varying RNA content and different degrees of chromatin condensation. A hitherto undescribed category of cells undergoing transition from quiescence to the cycle (or vice versa) was distinguished based on their intermediate values of RNA, sensitivity of DNA to acid denaturation and inability to incorporate BUdR at the rate characteristic for S cells. Depending on their DNA content (C) at the time of transition these cells could be classified as G_{1T} (2C), S_T (4>C>2) or G_{2T} (4C).

Key terms: RNA content, chromatin condensation, quiescent and cycling cells.

Three different flow cytometric techniques were developed in our laboratory to analyze the positions of cells in the cell cycle. All three are based on the use of the metachromatic fluorochrome acridine orange (AO) to differentially stain single *versus* double stranded nucleic acids.

In the first technique, after selective denaturation of double stranded RNA, cellular DNA and RNA are stained differentially allowing for discrimination between cells with different RNA content for any given DNA value (15, 22, 25, 51–54). In the second technique, susceptibility of DNA *in situ* to denaturation is measured in cells pretreated with RNase and then exposed to heat or acid (15, 19–21, 23, 26–29, 31, 32). The extent of DNA denaturation reflected as a metachromatic shift after AO staining, varies between cell types depending on chromatin structure and appears to correlate with the degree of chromatin condensation. In the third technique, cells are incubated with the thymidine analog 5-bromodeoxyuridine (BUdR) and then stained with AO; cycling cells are distinguished from noncycling cells by their lowered green fluorescence (30).

Using these techniques, several different parameters related to the cell cycle could be assayed simultaneously. Specifically, we have measured: (*a*) total content of DNA per cell (15, 19, 24, 50, 52); (*b*) content of DNA accessible to AO (22, 24, 25, 52); (*c*) RNA content per cell (22, 25, 51–54); (*d*) sensitivity of DNA *in situ* to acid denaturation (19–23, 26–29, 44); (*e*)

[1] Supported by US PHS Grants 1R01CA23296 and 126CA14134.
[2] Presented at Automated Cytology VII, Asilomar, California, November 25–30, 1979.

incorporation of BUdR (30); and (*f*) cell, or nuclear diameter (pulsewidth) (48). At least forty different cell types including normal and tumor cells *in vivo* and *in vitro*, as well as numerous cell lines at various phases of growth have been investigated. Some findings related to specific cell systems have been previously reported. Final conclusions, however, pertaining to the variability of these parameters during cell growth, their tissue-specificity *versus* general applicability and association with different functional states of the cell could be drawn only after extensive evaluation in a variety of cell types. We present here such an evaluation and describe observations from representative cell systems. Unlike measurements of DNA alone, this multiparameter approach enables one to subclassify cells in a variety of cell cycle compartments previously unrecognized. Since these compartments, easily discernible by flow cytometry, are believed to represent functionally different stages of cell growth, we propose a new classification of cell cycle phases.

Materials and Methods

Cells

We report here summary studies that were performed on at least 40 different cell systems during the last 5 years. Most of these cell types were described in detail in earlier publications. Listed below are only the most extensively studied cell systems; references are given to publications describing these systems.

1. Normal human peripheral blood lyphocytes, quiescent and stimulated by a variety of mitogens (25, 28, 30–32) and in mixed allogeneic reactions (53).
2. Lymph node cells in normal nodes and in nodes draining tumor areas (11).
3. Various types of human leukemic cells from blood or bone marrow. To date over 400 leukemia cases have been analyzed before and during chemotherapy (1, 16, 23, 32, 35).
4. Four human and mouse leukemia cell lines, growing exponentially or suppressed in growth by macrophages (41).
5. Human solid tumors, biopsy and exfoliative samples (12, 13).
6. Granulocytes and other reactive cell types infiltrating solid tumors (11, 12).
7. Human epidermal cells in culture (36).
8. Five different human bladder cell lines (44, 51).
9. Hamster kidney cell lines and their ts mutants (2, 45).
10. Friend erythroleukemia (FL) cells, growing exponentially or differentiating (24, 54).
11. A variety of other cell lines, including SK-L7, L1210, CHO, 3T3 and AF8 cells (2, 22, 26, 27, 34, 45).

Incorporation of BUdR

Studies on BUdR incorporation were performed *in vitro*: cells were incubated with 20 μM BUdR in the presence of 6 μM uridine and 0.4 μM 5-fluorodeoxyuridine (all from Sigma Chemical Co., St. Louis, MO) for approximately one generation time, as described previously (30).

Cell Staining

Simultaneous Staining of RNA and DNA: A detailed description of this technique is given elsewhere (15, 20, 22, 24, 25, 52). Briefly, cells made permeable by Triton X-100 (Sigma) were stained with chromatographically purified acridine orange (Polysciences Inc., Warrington, PA) at a final dye concentration of 13 μM and dye/DNA-P molar ratio >2, in the presence of 1 mM Na-EDTA. Under these conditions interaction of the dye with DNA results in green fluorescence with maximum emission at 530 nm while interaction with RNA gives red metachromasia at 640 nm, the intensity of which is proportional to RNA content (7). The specificity of staining was evaluated by treatment of cells with RNase A (Worthington Biochemical Corp., Freehold, NJ) or with DNase I (Worthington) as described (15, 25, 52).

Staining specificity varies depending on the cell type. The highest specificity was observed in lymphocytes and cells of lymphocyte origin (leukemias, stimulated lymphocytes, lymphocytic cell lines). In these cells nearly all green fluorescence (F_{530}) was sensitive to DNase and the coefficient variation (cv) of the mean F_{530} of the G_1 population was often below 2%. Over 85% of the red fluorescence ($F_{>600}$) of stimulated lymphocytes was RNase sensitive (25). Most other cell types had cv between 3 and 7% and exhibited good staining specificity for RNA (over 80% of the red fluorescence was sensitive to RNase). However, fibroblasts, some (but not all) cell lines of fibroblast origin (*i.e.*, WI-38), differentiating epidermal cells and squamous epithelial cells had poor staining specificity; some of these cells showed over 20% green fluorescence remaining after DNase treatment and a cv over 10%. Mast cells have high $F_{>600}$ due not to RNA but to the stainability of the glycosaminoglycans.

DNA Denaturation

Cells were fixed in suspension in 70% ethanol or in ethanol:acetone (1:1 v/v) mixture. After fixation cells were resuspended in buffered saline and incubated with 10^3 units/1 ml of RNase A (Worthington) for 30 min at 35°C. Aliquots of 0.1 ml of cell suspensions (0.5–1.0 × 10^5 cells) were then admixed with 0.4 ml of 0.1 M KCl-HCl buffer at pH 1.5 for 30 sec at 24°C. The cells were then stained by addition of 2 ml of AO solution (8 μg/ml) in 0.2 M Na_2HPO_4–0.1 M citric acid buffer at pH 2.6. Other details of the technique are described elsewhere (27–29, 31–32).

In this technique cell pretreatment at low pH followed by staining at pH 2.6 results in partial DNA denaturation, the extent of which is proportional to the degree of chromatin condensation in many cell systems (27–29, 32). The differential staining of double versus single stranded DNA occurs because AO intercalates into double stranded DNA and fluoresces green whereas the dye stacking on denatured sections of DNA results in metachromatic red fluorescence (10, 42). The extent of DNA denaturation is expressed as α_t, which represents the ratio of $F_{>600}$ to $F_{>600} + F_{530}$; $\alpha_t = F_{>600}/(F_{>600} + F_{530})$. After appropriate calibration of photomultiplier sensitivities the α_t value indicates the fraction of DNA that is denatured (20).

Fluorescence Measurements

The fluorescence of individual cells was measured in the FC200 cytofluorograph (Ortho Diagnostic Instruments, Bio/Physics Systems, Westwood, MA.) as described (48). The red fluorescence emission ($F_{>600}$, measured in a band from 600 to 650 nm) and green fluorescence emission (F_{530}, from 515–575 nm) from each cell was separated by optical filters, measured by separate photomultipliers and their integrated values were stored in the computer. The pulsewidth value was also recorded, and was used to distinguish single cells from cell doublets as well as to estimate cell size and nuclear diameter (48). In addition, the pulsewidth value was used to recognize granulocytes among other blood or bone marrow cells, based on their elongated nucleus after differential cell lysis by Triton X-100 (12). All data presented were based on 5 × 10^3 or 10^4 cells per sample.

Results

Cycling Populations

DNA-RNA Stainability: The DNA *versus* RNA staining pattern of logarithmically growing cells is illustrated in Figure 1. This pattern, with minor modification, was observed in all

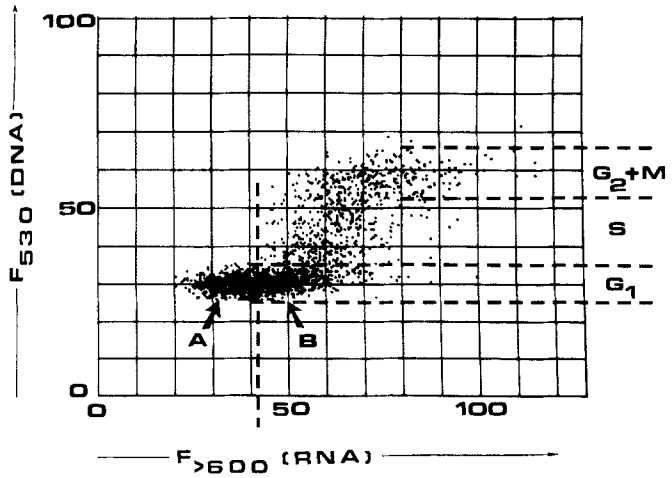

FIGURE 1. Scattergram representing DNA and RNA values of exponentially growing Chinease hamster ovary cells. Based on differences in DNA (F_{530}), cells can be classified in G_1, S and G_2 + M phases. A progressive increase in RNA during the cycle leads to a doubling, on average, of RNA in G_2 + M compared with G_1 cells. It is apparent that only G_1 cells with RNA above the threshold value, indicated by the *broken line* (B compartment) can enter S phase; remaining (G_{1A}) cells have to progress through G_{1B} to enter S. It was shown with synchronized cultures (2, 34) that cells enter the G_{1A} compartment after mitosis; their further progression into G_{1B} is exponential, indicating that the indeterminate cell cycle state is located within G_{1A}.[3] Serum deprivation or cell crowding in cultures results in a dramatic shift of the G_{1A}/G_{1B} ratio toward G_{1A}, with a subsequent decrease in RNA content of G_{1A} cells.

cell types studied. As was shown previously (2, 21, 25, 30, 33, 34) and can be seen from Figure 1, it is possible to discriminate between cells in G_1 *versus* S *versus* G_2 + M phases of the cell cycle based on differences in their green fluorescence. By comparing the G_1, S and G_2 + M populations, it is apparent that RNA content per cell increases during the cycle. Indeed, earlier experiments on synchronized cultures (Chinese hamster ovaries, AF8 cells) have shown that a continuous increase in RNA content takes place during the cell cycle (2, 33, 34).

The characteristic feature of the G_1 population in asynchronous cultures (Fig. 1) is cell heterogeneity with respect to RNA content. One can discriminate two qualitatively different compartments according to RNA content marked A and B, respectively. The threshold dividing the A and B compartments represents the minimal RNA content of S cells, being a continuation of the left outline of the S cluster on the scattergram. It is obvious that cells from the A compartment do not enter S phase; an increase in RNA content beyond the indicated threshold is required for cells to initiate DNA replication. In studies on synchronized cultures it was found that postmitotic cells in early G_1 phase (G_{1A}) are characterized by RNA values lower than those of G_1 cells just before entering S phase (G_{1B}) (2, 34, 45). It should be stressed, however, that in most cell types studied a continuity between G_{1A} and G_{1B} cells is evident since the distribution of G_1 phase cells is unimodal with respect to RNA content. This indicates that the A to B transition does not involve a quantum jump in RNA content.

In reviewing variations in the A–B pattern of various cell types and relating it to cell growth phase, as observed so far, the following generalizations could be made:

1. The proportions of cells in the A *versus* B compartment of the G_1 phase are different in different cell types and are characteristic for each exponentially growing cell line.
2. A dramatic shift in the G_{1A}/G_{1B} ratio (toward G_{1A}) occurs during adverse growth conditions, *i.e.*, as induced by cell crowding or serum deprivation in cultures.
3. The RNA content (per cell) of the G_{1A} population decreases during extended maintenance of cultures under conditions of cell confluency.
4. A short-term Vinblastine block lowers the cell number in the A but not in the B compartment, thus confirming data from experiments on synchronized cells that A cells are younger than B cells.
5. The B compartment is undetectable in cells infected with adenovirus 2. Such cells enter S phase from G_1 without any significant increase in RNA (45a).

DNA Denaturation *In Situ*: The typical pattern of cell stainability with AO, after extraction of cellular RNA and partial DNA denaturation, is shown in Figure 2. Based on differences in both green and red fluorescence it is possible to subclassify exponentially growing cells into G_1 *versus* S *versus* G_2 *versus* M populations, respectively, as described in detail in earlier publications (23, 26–29, 31, 32). We would like to stress here, however, a previously unanalysed phenomenon, namely, the heterogeneity of the G_1 population. Thus, as in the case of cells stained for DNA and RNA, the G_1 population may be subdivided into two qualitatively different compartments. Cells in the A compartment (Fig. 2), characterized by the lowest F_{530} values do not enter S phase directly. In contrast, G_1 cells with higher F_{530} and lower $F_{>600}$ (B compartment) are in continuity with S phase cells. It is obvious from the scattergrams that progression of cells from the G_{1A} compartment into S phase involves their transit through G_{1B}. This is characterized by an increase in cellular F_{530} and decrease in $F_{>600}$ which is manifested as a decrease in α_t. Here again, experiments on synchronized cultures have confirmed that the early G_1, postmitotic cells do belong to the A population (more easily denatured) while the cells in late G_1 are within the B cluster (more resistant to DNA denaturation). As in the case with RNA staining (Fig. 1) there is also a continuity (nonquantum transition) between the A and B compartments recognized by DNA denaturation. This is reflected by the unimodal distribution of all G_1 cells along the α_t axis.

In summary, both RNA content and chromatin structure may be used independently to distinguish qualitatively different compartments within the G_1 phase. The first compartment the postmitotic, G_{1A} cells, increase their RNA and decrease α_t up to a certain threshold. This threshold represents the minimum RNA content per cell and maximum α_t, below which cells cannot enter the S phase. After the threshold is passed the cells still remain in G_1 phase (G_{1B} compartment) but now can enter the S phase without additional changes in RNA content or chromatin stainability.

Noncycling Populations

DNA-RNA Stainability, BUdR Incorporation: Figure 3 illustrates the staining pattern of lymphocytes stimulated

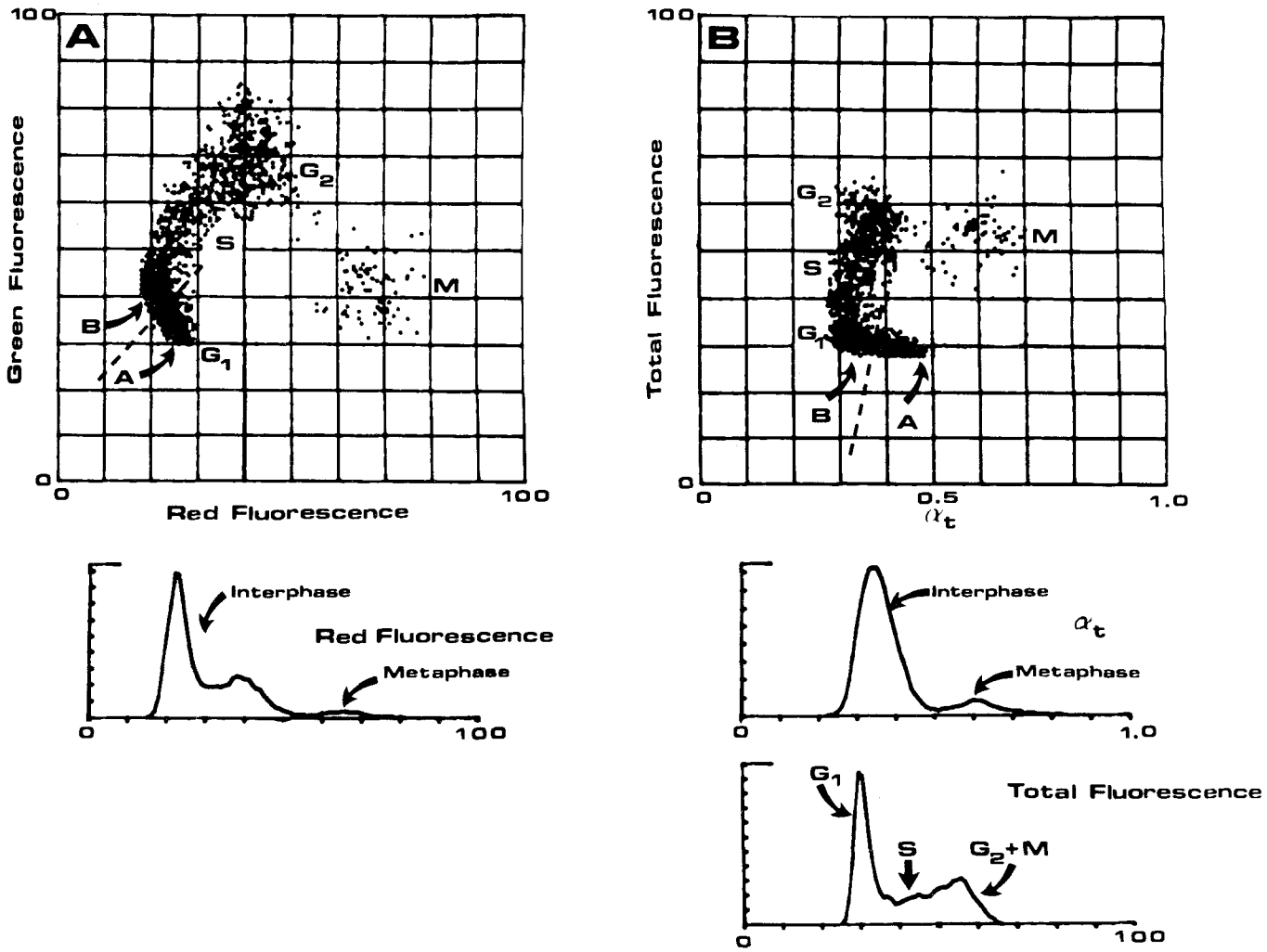

FIG. 2. Characteristic pattern of stainability with AO of cycling cell populations after partial DNA denaturation *in situ*. Exponentially growing FL cells were fixed, incubated with RNase, then with acid (pH 1.5) and finally stained with AO at pH 2.6. Under these conditions native (double helical) DNA fluoresces green, denatured (single stranded) DNA fluoresces red, RNA has been removed. The relative intensities of red and green fluorescence for each cell correlate with the extent of DNA denaturation by acid, which in turn reflects the degree of chromatin condensation (31, 32). Total cell fluorescence is proportional to content of DNA per cell. *A*, distribution of cells with respect to green and red fluorescence. *B*, total fluorescence (red + green) and α_t values of individual cells in the population (α_t = red fluorescence/total fluorescence). The lower histograms are single parameter frequency histograms of red fluorescence, α_t, and total fluorescence of these cells, respectively. G_1 cluster may be subdivided into A and B compartments as shown by the *broken line*. It is evident that G_{1A} cells do not enter S phase directly. A change in chromatin structure accompanies the transition from G_{1A} to G_{1B} and is manifested by an increase in green and a small decrease in red fluorescence. No additional change in chromatin is seen when G_{1B} cells enter S phase. However, their further progression through S does involve a gradual alteration in chromatin (increase in α_t) which is evidenced by the nonvertical distribution of total fluorescence values for S-phase cells when plotted against α_t (Fig. 2*B*). The range of α_t values for G_{1A}, G_{1B}, S, G_2 and M cells is 0.35–0.5, 0.27–0.41, 0.3–0.45 and 0.5–0.7, respectively.

by mitogens. Such cultures always consist of cycling (mitogen responsive cells) and quiescent (nonresponsive) populations. Details of cell stainability related to DNA and RNA staining specificity, time sequence after stimulation, experiments on synchronized cultures, etc. are all presented in earlier publications (25, 33, 34). The point we want to stress here is the distinction between noncycling quiescent lymphocytes (G_{1Q} cells) and the cycling population, with special reference to the heterogeneity of the G_1 population.

The population of mitogen-stimulated cells, which as in the case of other cycling cells contains G_{1A} and G_{1B} subpopulations, is fully separated from quiescent cells based on cellular RNA content (Fig. 3). All quiescent lymphocytes (G_{1Q} cells) irrespective of whether they represent a mitogen-nonresponsive subpopulation in stimulated cultures, or are analysed before stimulation, or after being maintained in cultures in the absence of mitogens, always have a 2C DNA content and very low RNA content, distinctly lower than G_{1A} lymphocytes at the time of maximal stimulation. During the first 48 hr of stimulation by phytohemagglutinin (i.e., when cells slowly and asynchronously enter the cycle) as well as after 4 days in phytohemagglutinin cultures (*i.e.*, when cycling cells begin to

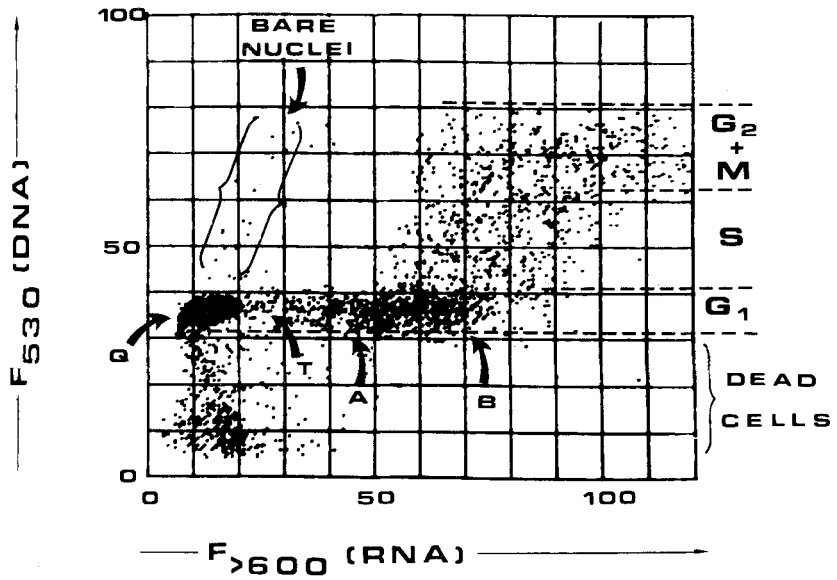

FIG. 3. DNA and RNA values of mitogen-stimulated lymphocytes at the time of maximal proliferation in cultures (25, 31, 53). Subpopulations, as indicated, were identified in studies of synchronized cultures (25). Nonstimulated lymphocytes (G_{1Q}) have very low RNA (2–20 units). Mitogen-responsive cells, depending on their DNA, can be classified as G_1, S and G_2 + M. Among G_1 cells, G_{1A} and G_{1B} can be recognized as in Fig. 1. Few cells in transition (G_{1T}) with RNA values between 20–30 units are seen in these cultures. G_{1T} cells are most numerous at the early and late stages of stimulation (see text). Dead cells, with pyknotic nuclei have low F_{530} (25). It should be emphasized that broken cells, and bare nuclei have low RNA values, and thus may be mistakenly taken as quiescent cells. In contrast to living cells, however, broken cells disappear from suspension after incubation with trypsin and DNase.

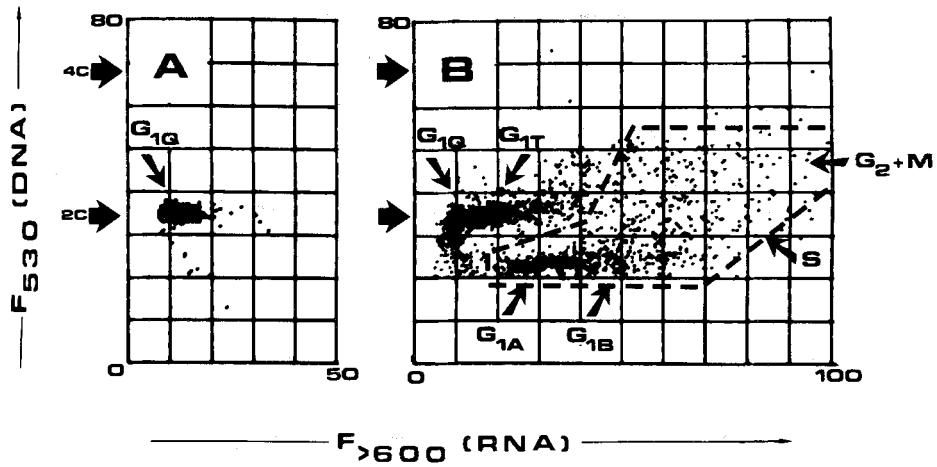

FIG. 4. Incorporation of BUdR by cycling cells in cultures of phytohemagglutinin-stimulated lymphocytes. *A*, nonstimulated lymphocytes incubated with BUdR for 24 hr. The main cluster represents quiescent cells with low RNA and 2C DNA content (G_{1Q}). The position of this cluster is identical with the locus of nonstimulated lymphocytes grown in the absence of BUdR. *B*, lymphocytes stimulated to proliferation by phytohemagglutinin in 3-day-old cultures; BUdR was present during the final 24 hr, *i.e.*, for the approximate duration of the cell cycle of these cells (30). Cycling lymphocytes in G_1 phase (G_{1A} and G_{1B}) have lowered F_{530} in comparison with G_{1Q} cells (see Fig. 2). Cells in transition (T) have F_{530} similar to and $F_{>600}$ higher than G_{1Q} cells.

return to quiescence) there are numerous cells with RNA values intermediate between those of quiescent, G_{1Q} cells and G_{1A} lymphocytes. These cells in transition (G_{1T}) have a 2C DNA content.

It was shown before that incubation with BUdR for one generation time suppresses the DNA stainability with AO (F_{530}) of cycling cells by about 40% thus allowing one to discriminate between cycling and noncycling cells (30). Fig. 4*B* illustrates the pattern of cell stainability in phytohemagglutinin-stimulated cultures incubated in the presence of BUdR during the final 24 hr, *i.e.*, for the approximate duration of the generation cycle of stimulated lymphocytes (34). By comparison with Figure 3 and Figure 4A it is apparent that cycling cells have diminished green fluorescence. The char-

acteristic DNA *versus* RNA pattern of the cycling population reveals the presence of G_{1A} and G_{1B} subpopulations as well as of cells in S and G_2 + M phase. Cells in transition from G_{1Q} to G_{1A} (G_{1T} cells) are also present although in limited numbers. These cells originate from the G_{1Q} cluster, have a 2C DNA content, higher RNA content than G_{1Q} cells and do not incorporate BUdR. G_{1T} cells, however, are most numerous during the first 48 hr of stimulation.

Cultured cell lines suppressed in growth by deficiency of essential medium components (*i.e.*, serum deprivation) display a somewhat different DNA-RNA stainability pattern than lymphocytes. Thus, AF8 (2, 45), 3T3 or WI-38 cells maintained with <0.5% serum for 48–72 hr cease proliferation and become blocked with a 2C DNA content. The RNA content of these cells, however, is only slightly (about 20%) lower than that of G_{1A} cells from exponentially growing cultures (2, 45). Therefore, unlike the G_{1Q} population in lymphocyte cell systems, these blocked cells cannot be distinguished from their respective counterparts in the G_{1A} compartment based on RNA content. However, while G_{1A} cells rapidly increase their RNA then move to G_{1B} and enter S phase, the cells blocked by serum deprivation require a long period of time to initiate DNA synthesis following stimulation.

In contrast to serum-deprived cells, we observed that 3T3 cells upon reaching confluency in cultures with normal serum content, and then being maintained at high cell density for an additional 4–5 days have an RNA content much lower than that of G_{1A} cells.[3] Under these conditions they remain viable, resemble G_{1Q} lymphocytes and may be stimulated by replating at lower cell densities. It appears, therefore, that in the case of 3T3 cells, two steps of quiescence may be discerned. The first one seen in 48–72 hr serum-deprived cultures is not reflected by gross RNA changes and resembles the G_{1T} state of the lymphocyte system. The second step, deep quiescence, observed under conditions of extended confluency (3), involves a reduction of RNA to the relative level seen in G_{1Q} lymphocytes.

While in most of the cell systems studied quiescent cells were characterized by 2C DNA content, exceptions were also seen. One such exception is shown in Figure 5. Exponentially growing Friend leukemia (FL) cells have a typical DNA *versus* RNA distribution pattern. These cells cease growth and most of them differentiate into erythroblasts when incubated in the presence of dimethylsulfoxide (DMSO). The population of noncycling FL cells is characterized by low RNA content (Fig. 5, see also Reference 54) such that there is no overlap in RNA values between these cells and cells growing exponentially. In this cell system, although noncyling cells are preferentially blocked with a 2C DNA content (G_{1Q}), there are also noncycling cells that are arrested with the DNA content of S and G_2 + M cells. Extensive and repeated studies of FL cells induced to quiescence under a variety of experimental conditions and characterized by varying degree of erythroid differentiation always revealed the presence of cells with S and G_2 + M DNA content which were viable and yet did not progress through the cycle (54). In addition to FL cells, such quiescent subpopulations were observed in some leukemia cell lines whose

[3] Darzynkiewicz Z, Traganos F, Melamed MR: In preparation.

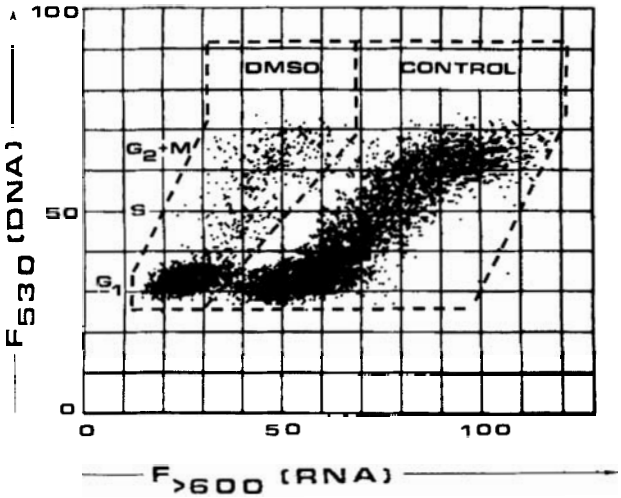

FIG. 5. DNA and RNA values of FL cells growing exponentially (control) or during quiescence, as seen in cultures treated with DMSO for 4 days (DMSO). To show all cells on the same scattergram equal numbers of cells from quiescent and cycling cultures were mixed, stained and measured. To identify populations, as shown, control measurements were also done on the original cell suspensions from exponential and quiescent cultures. Differences in RNA between cycling and quiescent cells are of such magnitude that the respective populations do not overlap in their RNA values. Although there is no cell proliferation in DMSO cultures (no thymidine incorporation, no increase in cell number, no cells in mitosis; see Reference 54) there are still cells with S and G_2 DNA content in such cultures. These S_Q and G_{2Q} cells with low RNA content and with condensed chromatin are alive, excluding trypan blue and withstanding trypsin and DNase treatments.

growth was suppressed by macrophages (41) as well as in populations of blast cells from patients with chronic myeloid leukemias during blastic crisis (16, 32). These quiescent cells with an S and G_2 + M DNA content always had very low RNA content, distinctly lower than their cycling counterparts.

DNA Denaturation In Situ: In a variety of cell systems the noncycling cells were shown to have a different structure of nuclear chromatin as compared with cells in the cycle (see References 5 and 6). Cytometric techniques for their identification based on difference in stability of DNA *in situ* to heat- or acid-induced denaturation were described in detail in earlier publications (20, 21, 28, 31, 32). Since then these methods were applied to other cell types and although details of the staining pattern (i.e., green *versus* red fluorescence scattergrams) of cycling and noncycling cells varied somewhat from one cell system to another, the general finding was confirmed in all systems. Namely, the extent of DNA denaturation (α_t value) always correlated with degree of chromatin condensation that in turn was related to cell quiescence. In the case of normal lymphocytes quiescent cells had very condensed chromatin (high α_t) and 2C DNA content (G_{1Q} cells). Their transition into the cycle was paralleled by a substantial decrease in α_t occurring prior to S phase (28, 31, 32). Due to a large difference in α_t, G_{1Q} cells were easily distinguished from G_1 cells. The distinction of cells in transition (G_{1T}) as well as the discrimination between cycling lymphocytes of G_{1A} and G_{1B} compartments was also possible (32). Quiescent 3T3 cells in

cultures maintained at confluency were also characterized by high α_t values and a 2C DNA content. Populations of quiescent cells with high α_t values in stationary cultures of FL (54) and chronic myeloid leukemia (16, 32) had cells not only with 2C (G_{1Q}) but also with higher DNA content (S_Q, G_{2Q}). Our observations on a variety of cell systems in which cycling and quiescent cells were analysed, are summarized in Table 1.

Discussion

Subdivision of the cell cycle into traditional phases based only on differences in DNA content per cell suffers shortcomings. Numerous factors essential for the evaluation of cell growth cannot be estimated from the analysis of this single parameter. In particular, the presence of noncycling cells, cells undergoing transition from quiescence into the cycle (or vice versa), their rate of transition, the heterogeneity of cycling cell populations with regard to the rate of cycle progression and the distinction between deterministic and indeterminate compartments of the cycle all escape detection in a routine analysis of the cell cycle based on DNA content alone.

Multiparameter analysis based on simultaneous measurements of DNA and RNA, sensitivity of DNA *in situ* to acid

Table 1
Main features of the respective phases of the cell cycle that may be distinguished by multiparameter, flow cytometric cell analysis[a]

Phase	DNA (C)	RNA	Chromatin Condensation; α_t	Next Phase Transition	Separation From Other Phases	Examples
G_{1Q}	2	Very low	Highly condensed (α_t = 0.5)	G_{1A}; slow; exponential	No overlap	Peripheral blood lymphocytes; confluent 3T3 cultures
G_{1A}	2	Low	α_t = 0.4	G_{1B}; fast; exponential	Continuity with G_{1B}	All cycling populations; A/B ratio changes with change of growth rate
G_{1B}	2	High	Diffuse; (α_t = 0.34)	S; linear (?)	Continuity with G_{1A} and S	
G_{1D}	2	Varies depending on cell type		Cell death	Often full separation	Granulocytes, differentiated FL cells, epidermal, plasma or mast cells
S	2<C<4	High, often increasing	Diffuse; (α_t = 0.36)	G_2	Continuity with G_{1B} and G_2	All cycling populations
S_Q	2<C<4	Low	Condensed (α_t = 0.45)	S	No overlap with S	Some leukemias (*i.e.*, chronic myloid leukemia-blastic crisis) and solid tumors
G_2	4	Nearly twice G_{1A}	Undergoing condensation (α_t = 0.39)	M	Continuity with S	All cycling populations
G_{2Q}	4	Low	Condensed; (α_t = 0.48)	G_2	No overlap with G_2	Some leukemias and solid tumors
M	4	Twice G_{1A}	Highly condensed (α_t = 0.6–0.7)	G_{1A}	No overlap with G_2	All cycling populations
			Cells in transition			
G_{1T}	2			Between G_{1Q} and G_{1A}		Phytohemagglutinin-lymphocytes; serum deprived 3T3 cultures
S_T	2<C<4			Between S_Q and S		Chronic myeloid leukemia-blastic crisis cells in short-term culture
G_{2T}	4			Between G_{2Q} and G_2		

[a] RNA estimates relate to the same cell type in other phases of the cycle. Sensitivity of DNA *in situ* to acid denaturation is expressed in α_t units; calibration of red and green photomultiplier sensitivities for α_t measurements was described (20). In most cell systems α_t correlated with the degree of chromatin condensation. In our preent studies, nonstimulated, peripheral blood lymphocytes were used as a standard for α_t = 0.5, *i.e.*, the photomultipliers were set so that the value of $F_{>600}$ was equal to that of F_{530}.

denaturation and BUdR incorporation allows one to subclassify cells into a variety of easily discernible compartments. These compartments represent different functional states, or phases of the cell cycle that we believe are relevant for the evaluation of cell growth potential. The general characteristics of these compartments, illustrated in Figure 6 and summarized in Table 1, are discussed below.

The G_{1Q} cell population represents cells with a 2C DNA content in "deep" quiescence. Their characteristic feature is minimal RNA content and highly condensed chromatin. G_{1Q} cells are distinctly different from their cycling counterparts since there is no overlap in RNA and α_t values between cells in these two states. Nonstimulated peripheral blood lymphocytes are the most typical examples of G_{1Q} cells (25, 28, 30–33). 3T3 cells or normal cells in primary cultures maintained at confluency for an extended period of time also approach the G_{1Q} state. Similarly, low RNA, nonclonogenic EMTG cells

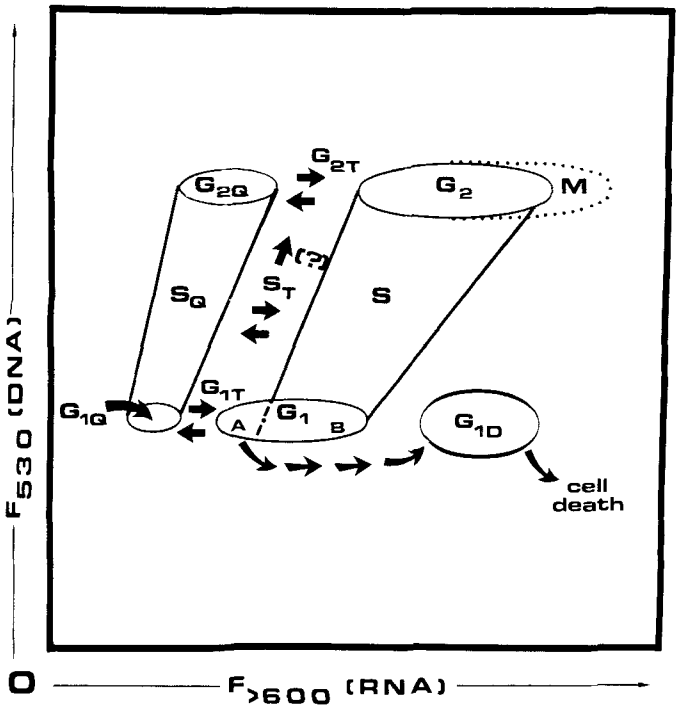

FIG. 6. Schematic representation of all cell cycle phases as they may be distinguished after simultaneous staining of RNA and DNA. The respective position of cell clusters on DNA-RNA scattergrams are outlined and the arrows indicate the possible transition points. Quiescent cells are characterized by minimal RNA. Although in most cases only 2C-DNA quiescent cells could be seen (G_{1Q}), in some cell types noncycling cells have also S- or G_2-DNA content (S_Q or G_{2Q} cells, see text). The transition of quiescent cells into the cycle is paralleled by an increase in RNA and it may occur at any DNA level (G_{1T}, S_T, G_{2T}). It is unknown at which RNA level the S_T cells start DNA replication; perhaps having intermediate RNA values they already slowly traverse the S phase. The typical pattern of cycling cells as seen at the *right* was described in the legend to Figure 1. Differentiated cells (G_{1D}), are outlined here as having high RNA, *e.g.*, as in the case of plasma cells. In other cases, however, G_{1D} cells have distinctly lower RNA than their cycling progenitors, *i.e.*, granulocytes, nucleated erythrocytes. It is likely that G_{1A} rather than G_{1B} cells of the stem cell population undergo differentiation (as shown by the *arrows*) since the latter are already committed to enter the S phase.

described by Watson and Chambers (55) fulfill the criteria of G_{1Q} cells. However, in quiescent diploid cell cultures there are always some cells present (about 5%) with RNA and α_t values that overlap those of the cycling populations. When G_{1Q} cells are stimulated to proliferate (*e.g.*, lymphocytes by mitogens) the inductive phase preceeding DNA replication is long, varying between 24–48 hr (25, 32, 33).

Despite repeated attempts, we have not been able to observe any significant difference between G_{1Q} and cycling cells in the accessibility of DNA in intact chromatin *(in situ)* to small intercalating probes like ethidium, propidium or AO (15, 25, 28). The earlier reports of such differences (40) were subsequently discounted by the same (9) and other authors (8, 43, 47) and were shown to be artifacts unrelated to cell proliferation (8, 14, 43). However, binding of intercalating probes of high molecular weight with bulky side chains protruding into DNA grooves (*e.g.*, actinomycin D), was shown to be substantially lowered in G_{1Q} (17, 18) and mitotic (46) cells.

G_{1A} cells are present in all cycling cell populations. These cells are characterized by lower RNA content and by higher α_t compared with the remaining G_1 cells, *i.e.*, cells about to enter S phse (G_{1B}). In studies on synchronized cultures, the G_{1A} cells were shown to be an early, postmitotic G_1 cell population (2, 34). The proportion of cells in G_{1A} increases markedly during suppression of cell growth, *i.e.*, as induced by cell crowding or paucity of serum in cultures. Extended suppression of cell growth results in a decrease of RNA content of G_{1A} cells and a further condensation of their chromatin, such that these cells approach the locus of G_{1Q} cells on DNA-RNA scattergrams.

It should be emphasized, however, that cells arrested in the G_1 phase by hydroxyurea, or 5-fluorodeoxyuridine display an increased RNA content (33, 34). Thus, in contrast to the effect of serum deprivation or cell crowding, which block cells in G_{1A} (subsequently moving them into the G_{1Q} compartment), hydroxyurea or 5-fluorodeoxyuridine arrest cells specifically in G_{1B} phase, or more precisely at the border between G_{1B} and S (33, 34).

A mitotic block (by Colcemid or Vinblastine) of exponentially growing cultures results in a depletion of cells from the G_{1A} compartment, before any changes in cell number in G_{1B} or S. Our studies on three different cell types, which will be published elsewhere[4] indicate that depletion of the G_{1A} compartment during this block is represented by an exponential decay curve in contrast to cell progression through the G_{1B} or S phase, which appears to be more linear. Thus, the main indeterminate state of the cell cycle, as postulated by Smith and Martin (49) is located within the G_{1A} compartment.

G_{1B} cells are cells in transition from G_{1A} to S phase. They are characterized by the lowest α_t among G_1 cells and RNA values similar to those of cells in very early S phase. In the cell types studied so far (L1210, Chinese hamster ovaries, 3T3) the G_{1B} compartment appears to present the deterministic subphase of G_1, through which cells progress linearly.

G_{1D} cells are terminally differentiated cells that left the cell

[4] Darzynkiewicz Z, Sharpless T, Staiano-Coico L, Melamed MR: Submitted for publication.

cycle and under normal conditions never cycle again. Their content of RNA and degree of chromatin condensation vary extensively depending on cell type. At one end of the spectrum are granulocytes or nucleated erythrocytes with minimal RNA and maximally condensed chromatin ($\alpha_t = 0.6$). Macrophages have a somewhat higher RNA content and their chromatin is less condensed. On the other end of the spectrum are plasma cells which are characterized by extremely high RNA content and moderately condensed chromatin ($\alpha_t = 0.4$).

Two types of differentiated cells (G_{1D}) have been found that contain AO-binding cytoplasmic products insensitive to RNase and DNase. The first type, mast cells, have intense red fluorescence of granules due to the stainability of heparin. The second type, differentiated epidermal cells have moderate green cytoplasmic fluorescence related to keratohyalin granules. These two examples emphasize the necessity of control incubation with nucleases prior to the interpretation of scattergram data in every new cell system studied. The DNA content and chromatin sensitivity to acid denaturation of these cell types were evaluated by measurements of their isolated nuclei.

Altered accessibility of DNA *in situ* to small intercalating probes such as ethidium or AO is a characteristic feature of some G_{1D} cells. Thus, normal erythropoiesis (39) or erythroid differentiation of FL cells (24, 54) is paralleled by a decrease in binding of such probes. During spermatogenesis there is also a marked decrease in DNA accessibility (38). Mature macrophages, however, are characterized by a somewhat increased binding of AO in comparison with other diploid cells (53). Terminal cell differentiation, thus unlike the reversible cell transitions from cycle to quiescence, or transient chromatin condensation during the cycle (at mitosis) involves changes in chromatin that are reflected by altered binding of not only large size ligands such as actinomycin D (17, 18) but also of small intercalators.

Cells in S, G_2 and M are easily recognized based on differences in their DNA content and chromatin structure (27–29, 31, 32). A correlation between the rate of cell progression through S phase and RNA content was observed in Chinese hamster ovary cells (34) and in stimulated lymphocytes synchronized by hydroxyurea (33). If this phenomenon is confirmed in other cell systems, the position of S cells on DNA/RNA scattergrams with respect to their RNA content could then be considered as generally reflecting the rate of progression of these cells through S. The higher the RNA value of the individual cell, the faster this cell advances through S phase. Similarly, the width (in RNA values) of the S cluster could then be considered as an indication of cell heterogeneity with regard to DNA replication rates in a given cell population. The rate of cell progression through G_{1B}, like that observed through S (33) may also be correlated with cellular RNA. Consequently, the indeterminate state (A) of the cell cycle may not necessarily be the sole, or even the main factor responsible for large intercellular variation in generation times.

A progressive accumulation of RNA per cell during the S phase is reflected by an inclination of the S-cell cluster with respect to the DNA/RNA axes (Fig. 1), thus indicating a correlation between an increase in DNA and RNA. With a minimal RNA increase during S phase the S cluster is nearly vertical. This contrasts with its diagonal position when an increase in RNA is substantial. Although no special studies were done to compare RNA accumulation during S phase, we observed a large variation in the angle of inclination of the S cluster on the DNA/RNA scatterplots, depending on the cell type investigated. This suggests that an increase in RNA per cell during the S phase may vary in various cell types. However, irregardless of whether the main increase in RNA occurs during G_1 phase or during S phase, G_2 + M cells have nearly twice the RNA content of G_{1A} cells (2, 34). It should be emphasized that an accurate estimate of RNA increases during S phase should involve parallel measurements of RNase-treated cells in order to subtract the nonspecific red fluorescence component, which also increases during S phase (7, 34).

Sensitivity of DNA *in situ* to acid denaturation increases during cell transition from S to G_2 and from G_2 to mitosis, as reflected by an increase in the α_t value up to 0.6 (Table 1). In no case was an overlap in α_t values observed between G_2 and metaphase cells.

S_Q and G_{2Q} phase cells are cells with S and G_2 DNA content that are noncycling. They are characterized by very low RNA content and by condensed chromatin which distinctly separates them from their cycling counterparts (Figs. 5 and 6, see also Reference 32). Although the presence of G_{2Q} cells was documented in some cell systems (see Reference 37) the presence of S_Q cells (4) is still regarded as controversial. We have observed the presence of S_Q cells in three different cell systems. In the first case, S_Q cells were seen in cultures of leukemia cell lines whose growth was suppressed by macrophages (41). These cells did not incorporate tritiated thymidine; their block in S_Q was fully reversible. Induction of quiescence, paralleled by cell differentiation in FL cell system (Fig. 5, see Reference 54) resulted in a preferential block of cells in G_{1Q} (and then G_{1D}). However, some cells were also blocked in the S_Q compartment. Finally S_Q cells were observed among populations of blast cells in several cases of chronic myeloid leukemia during blastic crisis (16). These quiescent cells, in contrast to S cells, were refractory to treatment with cell cycle specific drugs (16).

A characteristic feature of S_Q cells is the rapidity with which they initiate DNA synthesis upon stimulation. We observed, for instance, that S_Q cells from a chronic myeloid leukemia patient (blastic crisis) when transferred to tissue culture doubled their RNA content within 2 hr. At that time they became indistinguishable from cycling S cells. In this respect, S_Q cells behave as described by Gelfant "G_1-blocked cells" (37) which may initiate DNA replication shortly after their stimulation, without the inductive phase which characterizes G_{1Q} cells.

During stimulation of quiescent cells, or when cycling cells return to quiescence, they pass through a transition phase often remaining in this phase for a considerable period of time. Although this is a functionally distinct phase of cell growth, unique in the multiplicity of biochemical events required in preparation for cell proliferation and associated with "genome activation" (see reference 5), this phase is neither recognized during routine cell cycle analysis nor is it specifically defined. In the multiparameter flow cytometry cell cycle analysis, however, cells in transit between the quiescent and prolifera-

tive compartments can be distinguished as a special category based on intermediate values of RNA and chromatin condensation. Depending on their DNA content at the time of transition they may be classified as either G_{1T} (2C), S_T (4<C<2) or G_{2T} (2C). A population of G_{1T} cells was recently described by Richman (46a).

It should be stressed here that diploid cell lines subjected to serum deprivation do arrest with a mean RNA content typical of G_{1T} rather than G_{1Q} cell populations, and with a substantial overlap in RNA values with G_{1A} cells (2, 45). Based on this observation and taking into account that in confluent cultures these cells ultimately do reach the G_{1Q} state (3) it is tempting to classify cells in a serum deprived culture as in the G_{1T} state. Alternatively, serum starved cells may represent still another quiescent state, different from G_{1Q}, G_{1T} or G_{1A} ("blocked G_{1A}?"). Further studies are obviously needed to clarify the observed differences between quiescent cells in confluent, as opposed to serum-depleted cultures.

The cell classification system presently proposed, based on multiparameter flow cytometry offers new details on the functional state of cell populations at various phases of the cycle. Additional parameters (*e.g.*, cell volume, protein content, activity of specific enzymes) may be of great value in further characterization of these populations or perhaps in even more detailed distinction of functionally different states of cell growth.

ACKNOWLEDGMENT

We thank Miss Robin Nager for her assistance in the preparation of the manuscript.

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