Cyclins are key components of the cell cycle progression machinery. They activate their partner cyclin-dependent kinases (CDKs) and possibly target them to respective substrate proteins within the cell. CDK-mediated phosphorylation of specific sets of proteins drives the cell through particular phases or checkpoints of the cell cycle. During unperturbed growth of normal cells, the timing of expression of several cyclins is discontinuous, occurring at discrete and well-defined periods of the cell cycle. Immunocytochemical detection of cyclins in relation to cell cycle position (DNA content) by multiparameter flow cytometry has provided a new approach to cell cycle studies. This approach, like no other method, can be used to detect the unscheduled expression of cyclins, namely, the presentation of G1 cyclins by cells in G2/M and of G2/M cyclins by G1 cells, without the need for cell synchronization. Such unscheduled expression of cyclins B1 and A was seen when cell cycle progression was halted, e.g., after synchronization at the G1/S boundary by inhibitors of DNA replication. The unscheduled expression of cyclins B1 or E, but not of A, was also observed in some tumor cell lines even when their growth was unperturbed. Likewise, whereas the expression of cyclins D1 or D3 in nontumor cells was restricted to an early section of G1, the presentation of these proteins in many tumor cell lines also was seen during S and G2/M. This suggests that the partner kinase CDK4 (which upon activation by D-type cyclins phosphorylates pRB committing the cell to enter S) is perpetually active throughout the cell cycle in these tumor lines. Expression of cyclin D also may serve to discriminate G0 vs. G1 cells and, as an activation marker, to identify the mitogenically stimulated cells entering the cell cycle. Differences in cyclin expression make it possible to discriminate between cells having the same DNA content but residing at different phases or G1/S and G2/M of a lower DNA ploidy vs. G1 cells of a higher ploidy. The expression of cyclins D, E, A and B1 provides new cell cycle landmarks that can be used to subdivide the cell cycle into several distinct subcompartments. The point of cell cycle arrest by many antitumor agents can be estimated with better accuracy in relation to these compartments compared to the traditional subdivision into four cell cycle phases. The latter applications, however, pertain only to normal cells or to tumor cells whose phenotype is characterized by scheduled expression of cyclins. As sensitive and specific indicators of the cell's proliferative potential, the cyclins, in particular D-type cyclins, are expected to be key prognostic markers in neoplasia.

Key terms: Cell cycle, cell proliferation, cell cycle regulation, cancer

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Fig. 1. Cellular levels of D type cyclins, cyclin E, cyclin A, and cyclin B1 at different phases of the cell cycle. In normal cells and in some tumor cell lines during their exponential growth, the expression of D type cyclins is transient during G1, and the cells entering S phase are cyclin D negative (e.g., see Fig. 2). Also, G0 cells are cyclin D negative (see Fig. 3). In many tumor lines, however, the level of D-type cyclins remains high and invariable throughout the whole cell cycle (see Fig. 6).

Specific and well-defined points of the cycle (Fig. 1, Table 1). Cyclin B1 activates CDK1 (formerly denoted as CDC2) forming Maturation Promoting Factor (MPF), whose kinase activity is essential for cell entrance to M (51,65). Cyclin B1 begins accumulating at the time of cell exit from S, reaches maximal levels as the cell enters mitosis, and breaks down at the transition to anaphase (66). Cyclin A may associate with either CDC2 or CDK2; the kinase activity of the complex drives the cell through S and G2 (21,42,61). Cellular accumulation of cyclin A starts in mid-S, is maximal at the end of G2, and the protein is rapidly degraded in prometaphase. The kinase partner of cyclin E is CDK2 and this holoenzyme is essential for cell entrance to S phase. Cyclin E starts to accumulate in the cell in mid-G1, is maximally expressed at the time of cell entrance to S, followed by its continuous breakdown as the cell progresses through S (18,40,41).

Expression of the different members of the D family of cyclins is tissue and cell-type specific, e.g., whereas cyclin D1 is predominant in fibroblasts, cyclins D2 and D3 prevail in cells of lymphocytic lineage, which are cyclin D1 negative (1,3,70,75). D-type cyclins are maximally expressed following mitogenic stimulation of G0 cells or in response to growth factors; their level appears to decrease during exponential cell growth (4,48,49,54,59,68). The only documented role for cyclin D1 is activation of CDK4: the complex phosphorylates the retinoblastoma tumor suppressor gene protein RB (71,73,78). It is likely that cyclins D2 and D3 have a similar role. Phosphorylation of pRB releases E2F factor, which activates transcription of the components of the DNA replication machinery, thereby committing the cell to S phase (73,78). The periodic and timely expression of these cyclins, as with DNA replication and mitosis, represents landmarks of the cell cycle to which timing of particular events, or point of action of antitumor agents, can be related.

It should be stressed that the cellular level of particular cyclins is regulated not only at the transcriptional and translational level but also by the altered rate of their degradation via the ubiquitin pathway (22). Thus, e.g., the relatively long half-life of cyclin B1 during G2 is shortened manyfold following the cell division, when the cell resides in G1, or is forced to overexpress cyclin E (2). At particular phases of the cycle, therefore, the message level may not always correlate with the amount of the respective cyclin protein.

It has recently become possible to detect cyclins immunocytochemically in individual cells and to relate their expression to cell cycle position (estimated by simultaneous measurement of cellular DNA content) by multiparameter flow cytometry (5,23-32,43-47,67,72,77). This approach allows one to assess the intercellular variability in cyclin expression, detect cell subpopulations sharing similar or distinct features, and determine the presence of thresholds in expression of these proteins at particular phases of the cycle (30). The relationship between expression of individual cyclins and their actual cell cycle position can be studied in asynchronous cell populations in a manner that does not perturb cell cycle progression or induce the growth imbalance that almost always accompanies attempts to synchronize cells in the cycle (31,77). Multiparameter analysis of the cyclin proteins also offers entirely new possibilities to study the cell cycle and the cell cycle perturbations caused by intrinsic or exogenous factors. The aim of this article is to demonstrate these possibilities and to provide a comprehensive overview of this explosively growing area of research. This article updates our earlier review on the subject of cyclins (25), focusing primarily on the literature subsequent to this earlier publication. Emphasis is given to the unique applications of the multiparameter analysis of cyclins, namely, to those applications that yield information on the biology of the cell cycle and cancer, or on mechanisms of antitumor drug action and cannot be obtained using the tools of molecular biology.

Critical Aspects of the Methodology

Clevenger et al. (9), and Jacobberger et al. (38) pioneered development of the methodology for immunocytochemical detection of the intracellular antigens by flow cytometry. The critical steps are cell fixation and permeabilization, which often have to be customized to particular antigens for their optimal detection. The fixative is expected to stabilize the antigen in situ and preserve its epitope in a state where it continues to remain reactive with the available antibody. The cell has to be permeable to allow access of the antibody to the epitope. General strategies of cell fixation and permeabilization have been recently described by Bauer and Jacobberger (7).

Most studies on cyclins have employed precipitating fixatives such as 70–80% ethanol (14), absolute methanol (43,72), or a 1:1 mixture of methanol and acetone (5,45–47) cooled to −20–40°C. Brief treatment with 1% formaldehyde followed by 70% cold ethanol appears to be a preferred procedure for fixation of D type cyclins (14), although this cyclin also can be detected following
fixation with cold methanol (24). The choice of fixative thus appears not to be a critical factor for cyclin detection, and although the absolute level of the immunofluorescence may vary, various fixation protocols yield essentially similar cyclin distributions with respect to the cell cycle position. Each fixative has some undesirable effects (e.g., increased cell clumping in the case of ethanolacetone mixture, or cell autofluorescence and poor DNA stainability when formaldehyde is used) and one often has to compromise between these effects and the optimal detection of a particular cyclin.

Much more critical for the detection of cyclins is the choice of a proper antibody. Very often the antibody, although applicable to immunoblotting, fails in immunocytochemical applications, and vice versa. This may be due to differences in accessibility of the epitope or differences in the degree of denaturation of the antigen on the immunobLOTS compared to its in situ location. Some epitopes may not be accessible in situ at all. Since there is strong homology between different cyclin types, cross-reactivity also may be a problem. Because commercially available MoAbs may differ in specificity, degree of cross-reactivity, etc., it is essential for the authors to provide information (the vendor and hybridoma clone number) of the reagent used in their study. It frequently has been noticed by us that concentrations of MoAbs generally lower than those advised by the vendors provide better cyclin staining due to decreased background fluorescence.

The relative cellular content of a particular cyclin plays a role in its detection. We have observed that the signal to noise ratio (ratio of fluorescence intensity of the cyclin positive cells to the control cells, stained with the isotype immunoglobulin) is much higher in the case of cyclin B1 than in the case of cyclins E or A. The level of expression of D type cyclins varies markedly dependent on the cell type and the phase of cell growth. High sensitivity of the instrument and low level of cell autofluorescence, therefore, are of greater importance for the detection of cyclins E or A than of cyclin B1 or D type cyclins.

**Scheduled Expression of Cyclins B1, A, E, and D**

The scheduled timing of expression of cyclins B1, A, E, and D1 in relation to the major phases of the cell cycle, as mentioned at the beginning of this report, is reflected by a very characteristic pattern of the bivariate cyclin vs. cellular DNA content distributions as shown in Figure 2 for normal human proliferating lymphocytes (cyclins B1, A, and E) and fibroblasts (cyclin D1). As it is evident from the cytograms (Fig. 2), the expression of cyclin B1 is essentially limited to late S phase cells and the cells with a G2/M content of DNA, although early- and mid-S phase cells show a very low level of this protein. Expression of cyclin A is progressively increasing during S phase and is maximal in cells having a G2/M DNA content; most G1 cells are either cyclin A negative or show minimal level of this protein. Expression of cyclin E can be summarized as follows: (1) the maximal level of this protein is detected in the cells undergoing transition from G1 to S, (2) its level continuously decreases during cell progression through S, with the result that G1/M cells are essentially cyclin E negative, and (3) a distinct threshold in cyclin E expression is apparent at the G2/S transition. As it is evident from the continuity of the cell clusters on scatterplots (Fig. 2) or contours on the bivariate contour map (see Figs. 4 and 5) the cells have to accumulate cyclin E above the threshold level to enter S phase. Similar patterns of expression of cyclins B1, A, and E to that presented by proliferating lymphocytes (Fig. 2) were observed in normal fibroblasts and in several tumor cell lines.

The presence of cyclin D1 in exponentially growing normal fibroblasts is limited to cells in G0/G1 (Fig. 2). Most cells in S and G2/M are cyclin D1 negative, with the exception of a very few cells with a G2/M DNA content. The latter may be G1 cell doublets, since not all doublets can be identified by analysis of the shape (pulse width) of the electronic signal (70). Expression of cyclin D2 in U2-OS sarcoma and C82 hybridoma cell lines measured by Lukas et al. (46) shows patterns similar to that of cyclin D1 in fibroblasts. Likewise, expression of cyclin D3 by mitogenically stimulated human lymphocytes during exponential growth is also similar, being limited primarily to a subset of G0/G1 cells with most of the S and G2/M cells being negative (24) (Fig. 3).

The expression of cyclin D1, as shown in Figure 2, is suggestive that only early G1 may be cyclin D1 positive. Because G2/M cells are cyclin D negative, it is most likely that the immediately postmitotic cells do not inherit this protein, but it is transiently synthesized early in G1, and degraded prior to entrance to S phase. Kinetic studies (e.g., stathmokinesis in M) are needed to estimate the length of time post mitosis when the cells are cyclin D negative, and the length of time of expression of this cyclin in G1.

The choice of fixative...
expression of cyclin D1 in human normal fibroblasts and cyclins E, A, and B1 in PHA-stimulated human lymphocytes. The trapezoid windows represent the level of fluorescence of the respective control cells stained with the isotype IgG rather than the respective cyclin MoAb, prior to fluoresceinated secondary antibody. The G0 and G2/M populations gated based on DNA content are marked by broken lines.

FIG. 3. Expression of cyclin D3 during lymphocytes stimulation by PHA. Nonstimulated cells were cyclin D3 negative. Their stimulation led to a rapid induction of cyclin D3, whose expression declined especially on the third day of stimulation. Relatively few cyclin D3 positive cells in G1 were observed on the third day of stimulation, which are not shown on these contour maps. The broken line represents the level of the isotypic control. For details, see (24).

Expression of Cyclin D Discriminates G0 vs. G1 Cells

Normal quiescent cells (G0 phase) such as nonstimulated lymphocytes from peripheral blood are cyclin B1, A, E, D2, and D3 negative, at least to the extent that these cyclins cannot be detected by flow cytometry (24). Their stimulation initially triggers expression of cyclins D2 and D3, then cyclins E, A, and B1 (1,3,24). The increased level of cyclin D3 is evident as early as 4 h after administration of the mitogen (Fig. 3). Thus cyclins D2 and D3 in the case of lymphocytes (or cyclins D2 and D1 in fibroblasts) may be considered an early activation antigen, a marker of the G0 to G1 transition (24). Expression of D type cyclins, by virtue of their well-defined role in the cell cycle, is more advantageous as a marker of cell stimulation, compared with less specific markers such as increased cellular RNA content (16) or changes in gross chromatin structure reflected by altered in situ DNA denaturability (15).

It should be stressed, however, that because expression
of D type cyclins is transient in G₁ and cells entering S are normally cyclin D1 negative (Fig. 2), the absence of this protein in the cell per se cannot be considered evidence of the cell's G₀ status. Its presence, however, is the positive evidence that the cell has exited G₀.

Unscheduled Expression of Cyclins B₁, A, and E as a Result of Growth Perturbation

Perturbation of cell cycle progression such as cell synchronization by agents that interfere with DNA replication results in significant changes in expression of cyclin proteins (31, 77). The changes in cyclins B₁, A, E, and D3 in leukemic MOLT-4 cells synchronized at the G₁/S boundary by a double thymidine block are illustrated in Figure 4. Unperturbed, exponentially growing MOLT-4 cells exhibit perfectly scheduled expression of cyclins B₁, A and E (Fig. 4). A dramatic change in expression of these three cyclins, however, is apparent in cells synchronized at the G₁/S boundary. Thus cyclin E is grossly overexpressed (5-fold increase, ref. 30) in comparison with unperturbed G₁/S cells. Overexpression of cyclin E may be expected considering that the cells were being held arrested at G₁S, i.e., in a phase when cyclin E is normally synthesized and accumulates in the cell, for extended period of time. More surprising, however, is the pattern of expression of cyclins B₁ and A in the synchronized cells. In exponentially growing cultures, the cells at the G₁/S boundary are cyclin B₁ and A negative, whereas the G₁/S cells from the synchronized cultures are distinctly cyclin B₁ and A positive; actually the level of these cyclins in the cells entering S approaches that of G₂ cells in exponentially growing cultures (Fig. 4). The synchronization by double thymidine block thus induces untimely (unscheduled) expression of these cyclins. By "unscheduled" we mean the presentation of G₁ cyclins (i.e., cyclin E) by the cells in G₂/M, or alternatively, G₂/M cyclins (i.e., cyclins A and/or B₁) by G₁ cells. Virtually identical patterns of unscheduled expression of these cyclins were observed when MOLT-4, CHO or HeLa S3 cells were treated with other inhibitors of DNA replication such as mimosine or aphidicolin (31, 77).

The mechanism of induction of unscheduled expression of cyclins B₁ and A in G₁ may be associated with the stabilization of these proteins (whose message is present during G₁) by cyclin E (2). The latter is overexpressed in cells arrested by inhibitors of DNA replication (see Fig. 4). Alternatively, cell arrest in S, or at the entrance to S, by inhibitors may not prevent the induction of expression of cyclins A and B₁, which ordinarily occurs during S. It is unknown at present to what extent other factors that affect the rate of cell cycle progression (growth factors, agents that interfere with signal transduction, etc.) can modulate the cellular level of cyclins B₁, A, and E, and whether they may induce their unscheduled expression.

Unscheduled Expression of Cyclins B₁ and E in Tumor Cell Lines

The majority of the tumor cell lines exhibit patterns of expression of cyclins B₁, A, and E similar to the "scheduled" expression seen for normal fibroblasts or lymphocytes. For example, the bivariate distributions (cyclin vs. cellular DNA content) of the T-lymphocytic leukemia MOLT-4 cells are identical to those of normal PHA stimulated T-lymphocytes for cyclins B₁, A, and E (24). Some tumor cell lines, however, have distinctly "unscheduled" expression of these cyclins even when observed under conditions of exponential, unperturbed growth. Figure 5 presents such examples.

The cyclin most easily recognized when expressed in an unscheduled fashion is cyclin B₁. Thus unlike in cultures of normal cells where expression of cyclin B₁ is limited to G₂/M cells, this protein also is detected in G₁ and in early S phase in some tumor lines. This is the case for human promyeloctytic leukemia HL-60, breast carcinoma Hs578T and T-47D cells (Fig. 5).

Another example is unscheduled expression of cyclin E. In some cell lines (e.g., Hs 578, Colo 320DM), this protein is expressed not only in late G₁/early S but also in G₂/M (Fig. 5). However, before this particular pattern can be accepted as evidence of genuine unscheduled expression of cyclin E, two sources of a possible error should be excluded. First, it may be possible that doublets of G₁ cells (which are expected to be cyclin E positive) are mistak-
Fig. 5. Examples of the scheduled and unscheduled expression of cyclins E and B1 in different cell lines. PHA stimulated (3 days) normal peripheral blood lymphocytes (PBL) and lymphocytic leukemic MOLT-4 cells provide an example of the scheduled expression of these cyclins. Another leukemic line, Jurkat, expresses cyclin E at minimal levels and only included in the G2/M population. This possibility can be ruled out if a high proportion of cells with a G2/M DNA content are cyclin E positive, their electronic pulse is characteristic of single cells and there is low level of cell clumping. A second instance in which cyclin E expression could mistakenly be thought to exist is when cells grow at multiple DNA ploidy levels. In such instances, the cyclin E positive tetraploid G1 cells can be mistakenly identified as diploid G2/M. Such an eventuality can be excluded based on the absence of DNA tetraploid cells with an S and G2/M (octaploid) DNA content (27,76).

Jurkat cells demonstrate yet another example of unscheduled expression of cyclin E (Fig. 5). These cells lack the characteristic threshold of cyclin E in G1; the cells enter S phase with minimal expression of this protein. It should be stressed that these patterns of unscheduled expression of cyclin B1 (e.g., by HL-60 cells), or cyclin E (Jurkat cells) are highly reproducible and characteristic to these cell lines and provide a fingerprint (distinct phenotype) that allows one to identify these cell lines.

The frequency of unscheduled expression of cyclin B1 or E in various solid or hematopoietic tumor cell lines is relatively low (23,30). However, while screening over 20 cell lines of different lineage we have not been able to detect a single case of unscheduled expression of cyclin A (unpublished).

It is likely that unscheduled expression of cyclins B1 or E observed in certain tumor lines is associated with deregulation of the cell cycle drive machinery. Namely, the presence of G1 cyclins during G2/M, and vice versa, suggests that their partner CDKs may remain persistently active throughout the cell cycle. This may result in a loss of the regulatory control mechanisms at particular checkpoints of the cycle. It is possible, therefore, that the evidence of unscheduled expression of cyclins may be of prognostic value in oncology.

Expression of D Type Cyclins in Tumor Cell Lines

As mentioned, expression of D type cyclins in normal cells is very sensitive to any change in growth conditions. This is evidenced by the fact that patterns of cyclin D expression for a specific cell type are markedly different in subconfluent cultures, or after addition of nutrients or growth factors, etc., compared to the same cells main-
FIG. 6. Expression of cyclin D1 in normal fibroblasts, fibrosarcoma, and breast carcinoma (MCF-7, T47-D, and Hs 578T) cell lines. In contrast to normal fibroblasts that express cyclin D1 transiently during G1 and show absence of this protein during S, the cells of tumor lines show high levels of cyclin D1 expression throughout the entire cell cycle. A similar pattern of expression of cyclin D3 was seen in some leukemic cell lines, e.g., MOLT-4 (31).

Examples of the differences in expression of D-type cyclins between normal cells (e.g., as exemplified by normal fibroblasts, see Fig. 2) and many tumor cell lines, are presented in Figure 6. In contrast to normal fibroblasts, which at the time of cell entrance to and progression through S phase become cyclin D3 positive) and the absolute level of this protein (increase) in the cell (24). Due to this extreme sensitivity to environmental factors, it is critical that expression of D-type cyclins in different cell types be compared under identical conditions of cell growth, e.g., for exponentially growing cells during asynchronous, unperturbed growth, and with full access to growth factors.

As with cyclins E and B1 (Fig. 5), the patterns shown in Figure 6 are highly reproducible.

As mentioned, cyclin D1 forms a complex with, and activates CDK4. This complex phosphorylates pRB leading to a release of the E2F transcription factor, which in turn induces transcription of the DNA replication machinery committing cells to enter S (71,73,78). Cyclins D2 and D3 also associate with CDK4. The pattern of expression of cyclin D1 in normal fibroblasts (Fig. 2), or cyclins D2 and D3 in normal lymphocytes (23), indicates that CDK4 activity ceases prior to entrance to S and that this kinase becomes reactivated after mitosis. This pattern is consistent with the status of phosphorylation of pRB in normal cells; namely, pRB is underphosphorylated early in G1, prior to cell commitment to S. It should be mentioned, however, that pRB also can be phosphorylated by CDK2 and CDK1 during S and G2, respectively, and it becomes underphosphorylated during mitosis and in postmitotic cells. In contrast to normal cells, however, expression of D type cyclins in most tumor cell lines is not restricted to early G1 cells (ref. 30, Fig. 6). This would suggest that phosphorylation of pRB by CDK4 is not restricted to an early portion of G1 phase in these cells, but continues throughout most of the cell cycle, including mitosis and immediately postmitotic phase.

D-type cyclins play a major role in development and progression of many tumor types (review, 63). Thus there is considerable evidence that their inappropriate expression due to chromosomal translocation, DNA amplification, retroviral integration, or gene mutation contributes to the development of specific cancers (5,6,35,39,45,50,52,55,69). It was recently reported that overexpression of cyclin D1, similar to mutation of p53, is associated with instability of the genome (79). Furthermore, the cyclin D genes may undergo untimely activation due to persistent upstream mitogenic signaling, e.g., as a result of the oncogenically altered components of the signal transduction pathway or altered receptors to growth factors and mitogens (e.g., 12,18). Recent experiments with cyclin D1 gene-deficient mice demonstrated that this protein is of special importance for the proliferation of breast tissue, retina, and brain cells (74). Considering this evidence of cyclin D involvement in tumor development and progression, one may expect that its unscheduled expression, or overexpression, may have a strong prognostic value in oncology, in particular in breast cancer. The unique possibility that cytometry pro-
Cytometry of Cyclin Proteins

Distinction of Cells Having the Same DNA Content But Residing in Different Phases of the Cell Cycle

Often a need exists to discriminate between cells that have the same DNA content but reside in different phases of the cell cycle. This is the case, e.g., during the cell cycle analysis when cells grow at two different DNA ploidies, e.g., after administration of a drug that impairs cytokinesis resulting in an accumulation of G₂ diploid and G₁ tetraploid cells. A similar situation exists when there is a mixture of DNA diploid normal and tetra- or near tetraploid tumor cells in tumor samples. Discrimination of G₂/M diploid from G₁ tetraploid cells in such cases can be accomplished only when an additional marker of G₁ or G₂/M is available. Cyclins E or B₁ are such markers for G₁ and G₂/M, respectively, and the bivariate analysis of DNA content and cyclin E or B makes it possible to discriminate between these cells (Fig. 7).

The possibility of discrimination between G₂/M cells of lower DNA ploidy vs. G₁ cells of higher ploidy enables one to estimate the kinetics of cell progression through the cell cycle under conditions where cell division (cytokinesis) is prevented by such drugs as cytocholasin or staurosporine (STS) (76).

As mentioned, cyclin B₁ is degraded during the transition from metaphase to anaphase whereas cyclin A is degraded earlier, namely, during prometaphase. The mitotic cells (within the time window between prometaphase and anaphase) thus can be discriminated from G₂ cells by the absence of cyclin A (32, 66). This approach (Fig. 8), which complements other flow cytometric methods to distinguish mitotic cells (15), can be applied to estimate the mitotic index or in stathmokinetic experiments to estimate the kinetics of cell entrance to mitosis.

It should be noted, however, that both the discrimination between G₂/M diploid cells vs. G₁ tetraploid as well as between mitotic and G₂ cells cannot be accomplished when the cells being studied exhibit unscheduled expression of the required cyclins.

New Subcompartments of the Cell Cycle Distinguished by the Bivariate Cyclin vs. DNA Content Distribution

The landmarks provided by the expression of cyclins together with the traditional landmarks, i.e., DNA replication and mitosis, can be used to map the cell cycle. The following subcompartments can be identified based on the bivariate analysis of cellular DNA content vs. expression of cyclins D, E, A and B₁ (Table 2):

1. The precyclin D (prD) compartment represents G₀ cells that are either cyclin D negative or have such a low level of this protein that it is undetectable by flow cytometry (D⁻). An example of such cells are peripheral...
FIG. 8. Discrimination between mitotic (postprometaphase) and G$_2$ cells based on differences in expression of cyclin A. Exponentially growing MOLT-4 cells were treated with Vinblastine for up to 9 h. The rapid breakdown of cyclin A during prometaphase results in a cyclin A negative population that can be distinguished from G$_2$ cells following arrest in metaphase by Vinblastine. The percent of mitotic cells in the Vinblastine treated cultures as a function of time of the treatment measured by this approach is similar to that measured by the alternative assay based on DNA denaturability (15).

Table 2

Subdivision of Cell Cycle Based on Differential Expression of Cyclins D, E, A, and B in Relation to Cell Traverse Through S and Mitosis

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Cyclin expression, DNA replication</th>
<th>Location in cell cycle</th>
<th>Examples, description</th>
</tr>
</thead>
<tbody>
<tr>
<td>prD</td>
<td>D$^-$</td>
<td>Prior to rp-D</td>
<td>Nonstimulated lymphocytes, quiescent fibroblasts (G$_0$ cells)</td>
</tr>
<tr>
<td>poD-prE</td>
<td>D$^+$/E$^-$</td>
<td>Between rp-D and rp-E</td>
<td>Lymphocytes 4-12 h after PHA, STS arrested normal cells, postmitotic cells from tumor lines</td>
</tr>
<tr>
<td>poE-prS</td>
<td>E$^+$/S$^-$</td>
<td>Between rp-E and entrance to S</td>
<td>G$_2$ cell subpopulation with cyclin E above the threshold</td>
</tr>
<tr>
<td>poE-S</td>
<td>E$^+$/S$^+$</td>
<td>Between entrance to S and loss of cyclin E</td>
<td>Cyclin E positive S phase cells (Fig. 2)</td>
</tr>
<tr>
<td>S-prA (?)</td>
<td>S$^+$</td>
<td>Between entrance to S and rp-A</td>
<td>Early S cells prior to onset of cyclin A expression</td>
</tr>
<tr>
<td>poA-prB</td>
<td>A$^+$/B$^-$</td>
<td>Between rp-A and rp-B</td>
<td>S phase cells expressing cyclin A but not B1</td>
</tr>
<tr>
<td>poB-S (?)</td>
<td>B$^+$/S$^-$</td>
<td>Between rp-B and exit from S</td>
<td>S phase cells that have initiated cyclin B1 expression</td>
</tr>
<tr>
<td>poB-M</td>
<td>B$^+$/M$^-$</td>
<td>Between rp-B and entrance to M</td>
<td>Cyclin B1 positive G$_2$ cells, cells arrested in G$_2$ by ionizing radiation, m-AMSA (25,28)</td>
</tr>
<tr>
<td>MA$^+$</td>
<td>M$^+$/A$^+$/B$^+$</td>
<td>Between prophase and prometaphase</td>
<td>Early mitotic cells that are both cyclin A and cyclin B1 positive (32)</td>
</tr>
<tr>
<td>MA$^-$</td>
<td>M$^+$/A$^+$/B$^+$</td>
<td>Between prometaphase and anaphase</td>
<td>Late mitotic cells that are cyclin A negative but cyclin B1 positive (32)</td>
</tr>
</tbody>
</table>

*As explained in the text.

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phase ($pOE$: $prs$). The cells represented on the cyclin E vs. DNA content scatterplots as in G, but with cyclin E values above the threshold level (Fig. 2) belong to this subcompartment.

4. Early in S, one can identify cells that still are cyclin E positive but are replicating DNA ($E^+/S^-$ cells). These cells belong in the compartment $pOE$: $S$.

5. It is unclear at present how early during S the cell starts to accumulate cyclin A, and therefore, whether it is possible to distinguish a $pFA$ compartment in $S$ ($S^+/A^-$ cells). The flow cytometric data suggest that the onset of cyclin A accumulation is very early in $S$, perhaps at the time of initiation of DNA replication, or at least very shortly after it (Fig. 2).

6. It also is unclear as to whether cyclin B1 starts to be expressed before DNA replication is completed, as is generally accepted, or whether its expression begins in $G_2$. The latter can be inferred from the bivariate DNA vs. cyclin B1 scatterplots (Fig. 2). If the onset of cyclin B1 expression occurs in $S$, the early $S$ phase cells that do not express cyclin B can be discriminated from the very late $S$, cyclin B positive cells ($S^+/B^-$ vs. $S^+/B^+$).

7. Based on the timing of expression of cyclins B1 and A as discussed earlier, one can identify mitotic cells within the narrow time window between prometaphase and proanaphase as cells with a $G_2/M$ DNA content that express cyclin B1 but not cyclin A ($A^-/B^+$), which are distinct from the earlier mitotic and $G_2$ cells, which are $A^+/B^+$.

The major advantage of these subdivisions, compared with cell cycle mapping based on metabolic cell features such as RNA content or chromatin structure (15,16), is that the cyclin landmarks have well-defined roles in regulation of the cell cycle: they activate their respective partner CDKs and thus are essential for cell advancement through the particular checkpoints and phases of the cycle. The compartments based on cyclin expression thus are more representative of the cell cycle progression status and predictive of the cell kinetic potential, compared to rather nonspecific metabolic parameters related to rRNA metabolism or gross chromatin structure (15).

The rate of cellular accumulation of each of the cyclins during the cycle, although relatively rapid, is not instantaneous. Therefore, the discrimination of cells in the above compartments solely by cyclin level may be difficult in some cases. It was observed, however, that when the cells were arrested in a particular compartment, the cyclin that was expressed in the preceding subcompartments was generally increasing, whereas the appearance of the subsequent cyclin was prevented. Such a situation was observed in the case of cells arrested in $G_{0/1}$, e.g., by $n$-butyrate, STS or genistein (28,29). Identification of the site of arrest in such a case was straightforward. It is unknown, however, whether the same holds for cells arrested later in the cycle. The data on cells arrested at the entrance to $S$ by inhibitors of DNA replication indicate that the expression of both cyclin A and cyclin B1 is not prevented in these cells (31,77). Inhibition of DNA replication thus does not provide a signal that would preclude accumulation of cyclins A and B1.

Because each cyclin type appears to be essential for the traverse of a portion (checkpoint) of the cell cycle, the valuable concept of a cell cycle restriction point introduced by Pardee (62) can be extended taking into consideration individual cyclin types. Inactivation of a particular cyclin type is expected to halt the cell transit through the section of the cell cycle during which a particular set of proteins is phosphorylated by the CDK(s) activated by this cyclin. This constitutes the restriction point executed by this particular cyclin type. One can identify thus at least four restriction points: the restriction point of the D type cyclins ($rp-D$), of cyclin E ($rp-E$), cyclin A ($rp-A$), and cyclin B ($rp-B$). It is likely that the restriction point prior to $S$ that is sensitive to cycloheximide (61) represents two restriction points one executed by cyclin D and another by cyclin E ($rp-D$ and $rp-E$). Thus, when protein synthesis is inhibited by cycloheximide, the cells that were in the cycle prior to $rp-D$ are expected to be halted at that point, whereas the cells that were between $rp-D$ and $rp-E$ will be blocked at $rp-E$.

The most typical arrest of cells in $G_2$, induced by DNA topoisomerase II inhibitors such as amsacrine ($m$-AMSA), or by ionizing radiation (28) as well as by alkylating agents and hyperthermia (59) appears to be past the cyclin B1 restriction point (Table 3).

### Analysis of the Cell Cycle Point of Action of Antitumor Agents

The knowledge of the cell cycle phase specificity of antitumor drugs is of importance in oncology in developing clinical treatment protocols and designing antitumor strategies, especially involving drug combinations (13). Thus far the point of action of many drugs has been estimated in relation to the traditional four phases of the cell cycle, $G_1$, $S$, $G_2$, and M. The subdivision of the cell cycle based on the cyclin-related subcompartments and the restriction points, as proposed above, allows one to map cell arrest in the cycle with greater accuracy.

The actual mapping is very straightforward. When the drug that arrests the cell prior to the onset of expression of the message of a particular cyclin is administered into the culture, it prevents the appearance of this cyclin in the cell. The point of arrest thus is prior to this particular cyclin restriction point (Table 3). Conversely, when the point of arrest by the drug is past the point of expression of cyclin message (the cyclin restriction point), that cyclin accumulates in the arrested cell. Actually, the arrested cell often expresses more of this cyclin, showing "unbalanced" growth (31,77), compared to its expression during unperturbed growth.

Figure 9 presents an example of the analysis of the point of action of $n$-butyrate, cycloheximide, quercetin, mimosine, and aphidicolin in relation to the time of expression of cyclin E. All these drugs are known to arrest cells in $G_1$ or at $G_1/S$ boundary. The data in Figure 9 clearly indicate that the point of arrest by $n$-butyrate and cycloheximide is prior to the onset of cyclin E expres-
of MOLT-4 cells to different agents that arrest cells in G, or at the G, S border results either in a decrease (the arrest point is prior to the onset of cyclin E synthesis), or an increase (the arrest is past the initiation of cyclin E synthesis) of cyclin E in the arrested cells. See (25,28,29) for details.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Presumed target</th>
<th>Compartment/ restriction point</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-butyrate</td>
<td>Histone acetylation</td>
<td>prE</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>Protein isoprenylation</td>
<td>prE</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Protein synthesis</td>
<td>prE</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Protein kinases</td>
<td>prE,D-prE</td>
</tr>
<tr>
<td>Genistein</td>
<td>Protein kinases</td>
<td>prE</td>
</tr>
<tr>
<td>Mimosine</td>
<td>DNA polymerase</td>
<td>poE</td>
</tr>
<tr>
<td>Quercetin</td>
<td>DNA polymerase</td>
<td>poE</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>DNA polymerase</td>
<td>poE</td>
</tr>
<tr>
<td>m-AMSA</td>
<td>DNA topoisomerase II</td>
<td>poBI</td>
</tr>
<tr>
<td>γ radiation</td>
<td>DNA</td>
<td>poBI</td>
</tr>
<tr>
<td>Melphalan</td>
<td>DNA</td>
<td>poBI</td>
</tr>
</tbody>
</table>

*Refs. 28, 29, 60.

Administration of the drug to the cultures containing G, cells at the time of addition of the mitogen allows one to discriminate the point of action with respect to cyclin D expression. Thus, e.g., when the protein kinase inhibitor and G, blocker STS was added into the culture of quiescent lymphocytes together with the mitogen PHA, the induction of cyclin D expression by PHA was unaffected but the induction of cyclin E was prevented. The point of cell arrest by STS thus is in G, somewhere between the time of expression of cyclin D and cyclin E (29). It is possible that one of the targets of STS may be the holoenzyme cyclin D-CDK4 itself. Table 3 summarizes the results of analysis of the point of arrest induced by different agents in relation to cyclin expression.

**Future Directions**

Our knowledge of the molecular clockwork of the cell cycle is rapidly expanding. Cyclins, the list of which continues to grow, consist of only one element of the regulatory mechanisms of the cycle. Another key element are the inhibitors of CDKs (reviews, 8,33,37). The passage of the cell through a particular section of the cycle is regulated by the mass action law involving particular cyclin/CDK holoenzymes and their respective set of inhibitors. Because the level of CDKs remains rather constant throughout the cell cycle, it is the ratio of cyclins to the respective inhibitors that controls cell passage through particular sections of the cycle. The balance between the cyclins and the inhibitor(s) can be measured by multicolor, immunocytocchemical staining of these components, followed by their ratiometric analysis. Such analysis is expected to provide more complete information about the cell cycle status of the cell than the analysis of cyclins alone. Furthermore, the ratio analysis generally offers a higher sensitivity compared to analysis of each of the components alone.

Another essential element of the cell cycle drive machinery is the status of phosphorylation of the critical amino acids at the active sites of the CDKs, cyclins, and their inhibitors. Phosphorylation of these sites provides either an on or off switch of the activation mechanism. Antibodies are being developed that discriminate between the phosphorylated and nonphosphorylated epitopes of these regulatory molecules. Their ratiometric analysis (e.g., ratio of the phosphorylated CDK to total, or to unphosphorylated CDK) would then provide an estimate of the degree of phosphorylation of the component and thus the status of its activity.

A word of caution is necessary, however, in drawing conclusions regarding the activity of the complexes of proteins (CDKs, cyclins, and CDK inhibitors) regulating cell transitions through the respective cell cycle phases or check points, based on the quantity of these proteins detected by flow cytometry. As mentioned, their activity is regulated by timely phosphorylations and dephosphorylations at critical sites. CDKs themselves are activated by CDK7 (MO15), a CDK-activating kinase; the latter is activated by cyclin H (20). It is likely that yet additional levels of regulation will be discovered. The content of individual regulatory proteins, therefore, represents only one element of the complex multifaceted regulatory machinery and may not always correlate with the activity of the whole complex. Multiparameter flow cytometric analysis of cyclins, CDKs, or their inhibitors may provide a plethora of information as shown, but complementary biochemical kinetic studies are necessary to assess their activity.

The cell cycle regulatory mechanisms are also coupled to the regulation of the alternative pathway for the cell,
namely, death by apoptosis. The explosive growth of applications of cytometry in the field of apoptosis in recent years is expected to be followed in the future by simultaneous analysis of both pathways, cell proliferation and cell death. Once the molecular mechanisms that determine the cell choice of either pathway are elucidated, cytometric method will be developed to identify the cells that make the decision to proliferate or to die. The prognostic value of these methods, especially in oncology, will be of importance.

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LITERATURE CITED


