Review Article

Cytometry of Cyclin Proteins

Zbigniew Darzynkiewicz, Jianping Gong, Gloria Juan, Barbara Ardelt, and Frank Traganos

The Cancer Research Institute, New York Medical College, Valhalla, New York

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 G_1 cyclins by cells in G_2/M and of G_2/M cyclins by G_1 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 G_1/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 G_1, the presentation of these proteins in many tumor cell lines also was seen during S and G_2/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 G_0 vs. G_1 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 such as in G_2 vs. M or G_2/M of a lower DNA ploidy vs. G_1 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.
© 1996 Wiley-Liss, Inc.

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

Cyclins are the key components of the cell cycle progression machinery. In combination with their respective cyclin-dependent protein kinases (CDKs), cyclins form the holoenzymes that phosphorylate different sets of proteins at consecutive stages of the cell cycle, thereby driving the cell through the cycle (reviews, see refs. 8,17,19,33,34,51,53,56–58,65,71). The role of cyclins is to activate their partner CDKs and possibly to target them to specific protein substrates. Nine types of cyclins, denoted cyclins A–I, have been identified thus far. Expression of cyclins B1, A, E, or D is cyclic and occurs at
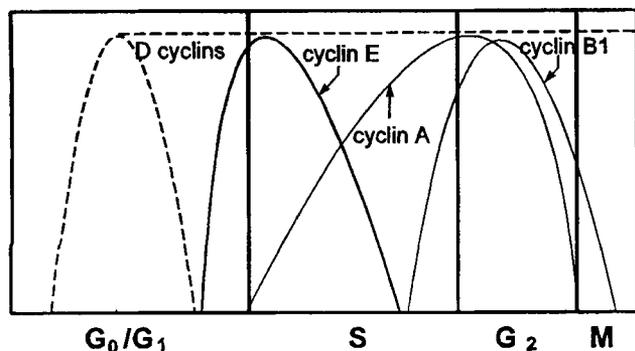
FIG 1. Cellular levels of D type cyclins, cyclin E, cyclin A, and cyclin B1 at different phases of the cell cycle. In normal cells and in some tumor cell lines during their exponential growth, the expression of D type cyclins is transient during G_1 and the cells entering S phase are cyclin D negative (e.g., see Fig. 2). Also, G_0 cells are cyclin D negative (see Fig. 3). In many tumor lines, however, the level of D-type cyclins remains high and invariable thorough 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 G_2 (21,42,61). Cellular accumulation of cyclin A starts in mid-S, is maximal at the end of G_2 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-G_1, 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 G_0 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 G_2 is shortened manyfold following the cell division, when the cell resides in G_1 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

Table 1
Cyclins and Their Partner CDKs During the Cell Cycle

Cyclin	Primary CDK partner(s)	Presumed role in cell cycle	Peak of expression	Localization
D type	CDK4 & CDK6	pRB phosphorylation, commitment to S phase	Early in G_1	Nucleus
E	CDK2	Initiation of S	G_1/S transition	Nucleus
A	CDK2 & CDC2	S and G_2 traverse	During G_2M	Nucleus
B1	CDC2	G_2 traverse entrance to M	Late G_2/M	Cytoplasm/nucleus[a]

[a]Cyclin B1 is localized in cytoplasm during G_2 and undergoes translocation to nucleus during prophase.

fixation with cold methanol (24). The choice of fixative thus apears 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 ethanol:acetone 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 immunoglobin) 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 G_2/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 G_2/M DNA content; most G_1 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 G_1 to S, (2) its level continuously decreases during cell progression through S, with the result that G_2 + M cells are essentially cyclin E negative, and (3) a distinct threshold in cyclin E expression is apparent at the G_1/S transition. As it is evident from the continuity of the cell clusters on scatter-plots (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 $G_{0/1}$ (Fig. 2). Most cells in S and G_2/M are cyclin D1 negative, with the exception of a very few cells with a G_2/M DNA content. The latter may be G_1 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 $G_{0/1}$ cells with most of the S and G_2/M cells being negative (24) (Fig. 3).

The expression of cyclin D1, as shown in Figure 2, is suggestive that only early G_1 may be cyclin D1 positive. Because G_2/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 G_1 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 G_1.

It should be emphasized, however, that any change in

Fibroblasts

Stimulated Lymphocytes
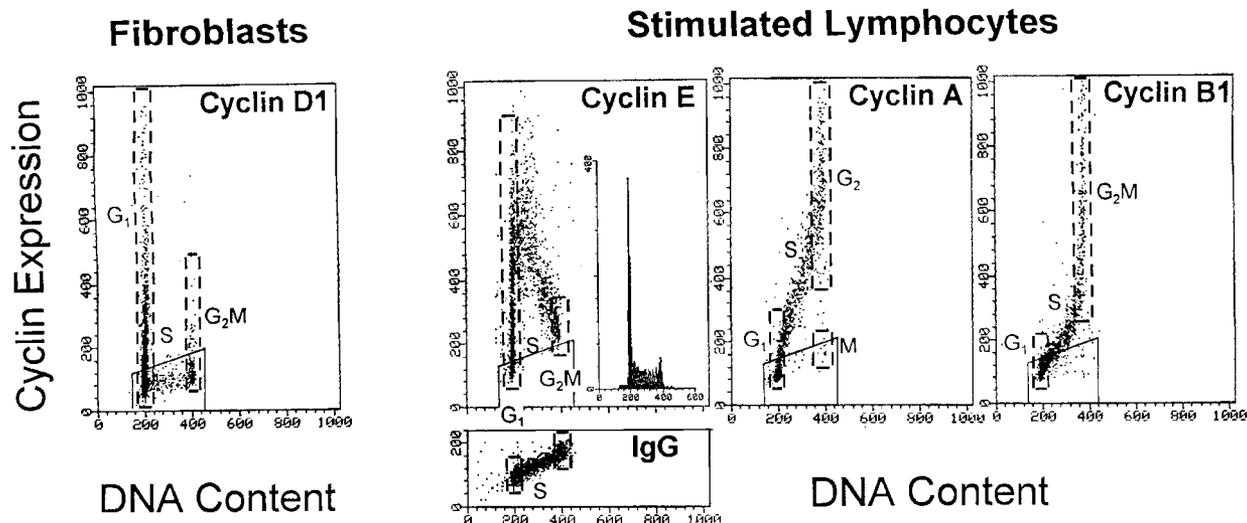
FIG. 2. Typical bivariate cyclin vs. DNA content distributions (scatterplots) showing expression of cyclin D1 in human normal fibroblasts and cyclins E, A, and B1 in PHA-stimulated human lymphocytes. The trapezoid windows represent the level of fluorescence of the respective control cells stained with the isotype IgG rather than the respective cyclin MoAb, prior to fluoresceinated secondary antibody. The $G_{0/1}$ and G_2/M populations gated based on DNA content are marked by broken lines.
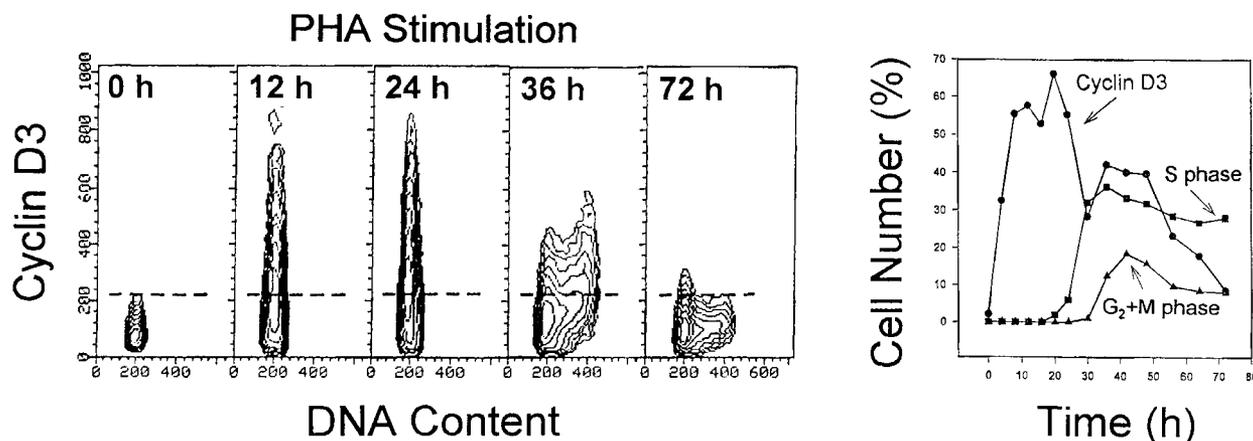
PHA Stimulation

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 G_1 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).

the growth rate of the cells from exponential phase, such as sub-confluence, addition of fresh medium with serum, inhibition of cell growth by antitumor drugs, etc., markedly alters expression of D type cyclins, both in terms of the absolute level of these proteins, as well as the pattern of their expression vis-a-vis cell cycle position.

Expression of Cyclin D Discriminates G_0 vs. G_1 Cells

Normal quiescent cells (G_0 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 G_0 to G_1 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

DNA Content
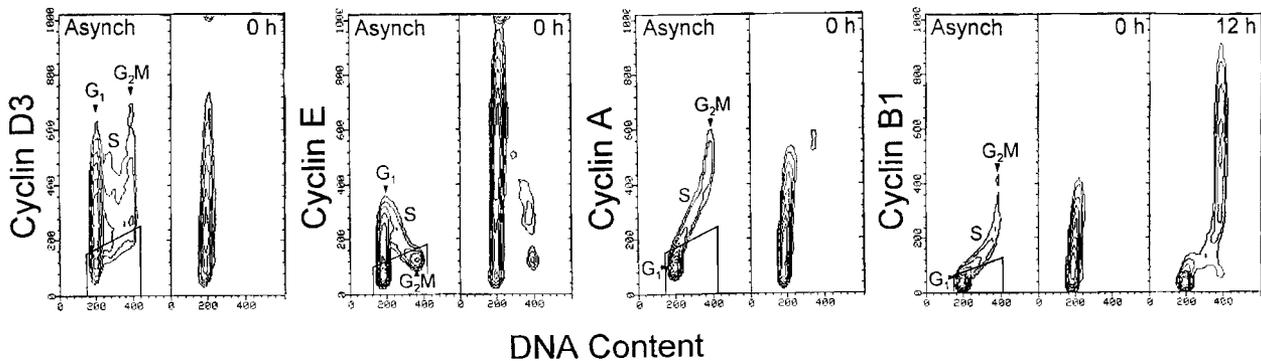
FIG. 4. Overexpression of cyclin E and unscheduled expression of cyclins A and B1 in MOLT-4 cells synchronized at the G_1/S boundary by inhibition of DNA replication. Expression of cyclins D3, E, A, and B1 was measured in exponentially growing, asynchronous cell population (Asynch) and the cells synchronized at G_1/S by double thymidine block, at the time of release from the block (0 h), or 12 h after the release (cyclin B1, 12 h). For details, see (31).

of D type cyclins is transient in G_1 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 G_0 status. Its presence, however is the positive evidence that the cell has exited G_0.

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 G_1/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 G_1/S boundary. Thus cyclin E is grossly overexpressed (5-fold increase, ref. 30) in comparison with unperturbed G_1/S cells. Overexpression of cyclin E may be expected considering that the cells were being held arrested at G_1S, 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 G_1/S boundary are cyclin B1 and A negative, whereas the G_1/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 G_2 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 G_1 cyclins (i.e., cyclin E) by the cells in G_2/M, or alternatively, G_2/M cyclins (i.e., cyclins A and/or B1) by G_1 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 expres-

sion of cyclins B1 and A in G_1 may be associated with the stabilization of these proteins (whose message is present during G_1) 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 G_2/M cells, this protein also is detected in G_1 and in early S phase in some tumor lines. This is the case for human promyelocytic leukemic 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 G_1/early S but also in G_2/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 G_1 cells (which are expected to be cyclin E positive) are mistak-

DNA Content
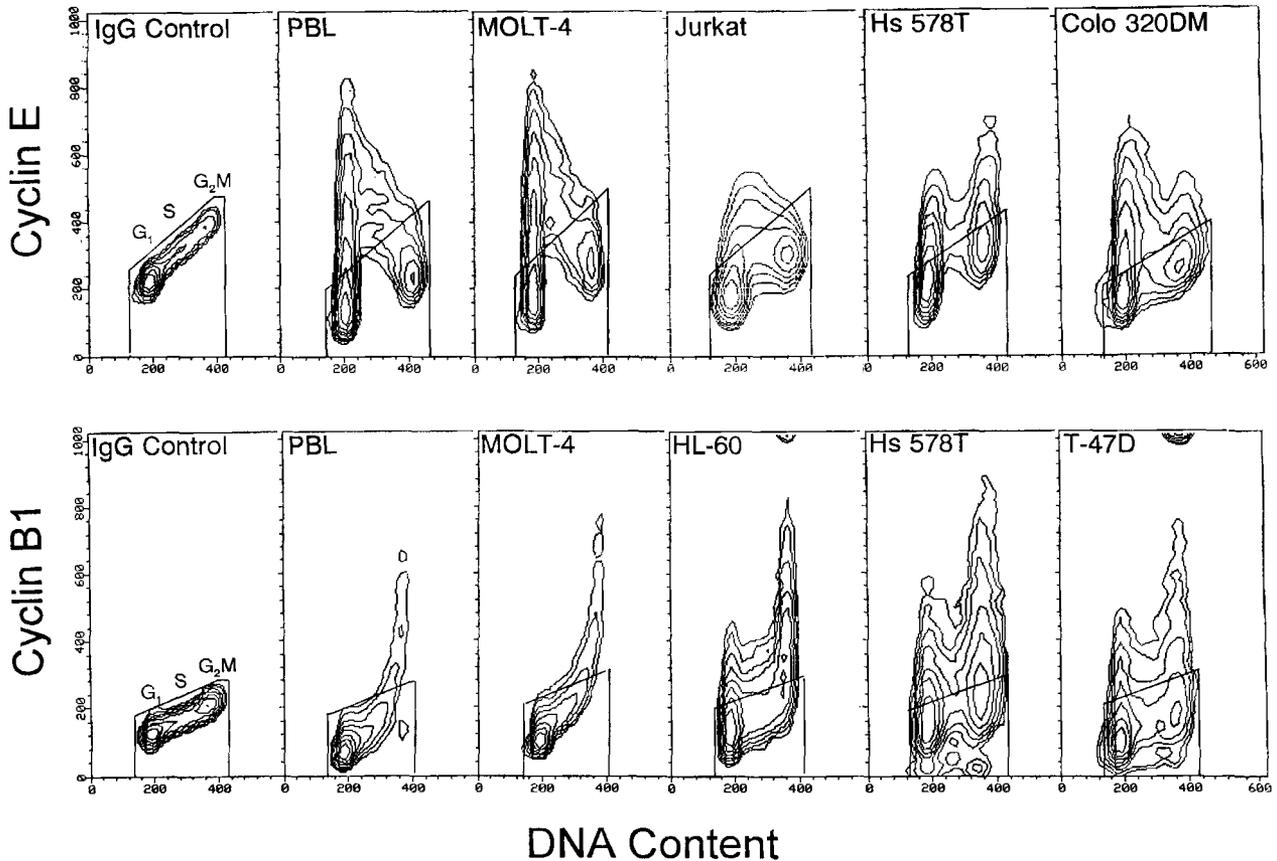
FIG. 5. Examples of the scheduled and unscheduled expression of cyclins E and B1 in different cell lines. PHA stimulated (3 days) normal peripheral blood lymphocytes (PBL) and lymphocytic leukemic MOLT-4 cells provide an example of the scheduled expression of these cyclins. Another leukemic line, Jurkat, expresses cyclin E at minimal levels and shows no characteristic threshold in G_1. The breast (Hs578T) and colorectal (Colo 320DM) carcinoma lines show some expression of cyclin E by G_2/M cells; Colo 320DM also demonstrates the lack of a threshold in G_1. Unscheduled expression of cyclin B1 is shown by promyelocytic leukemic (HL-60) and breast carcinoma (Hs578T, T-47D) cells.

enly included in the G_2/M population. This possibility can be ruled out if a high proportion of cells with a G_2/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 G_1 cells can be mistakenly identified as diploid G_2/M. Such an eventuality can be excluded based on the absence of DNA tetraploid cells with an S and G_2/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 G_1: 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 disregulation of the cell cycle drive machinery. Namely, the presence of G_1 cyclins during G_2/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-
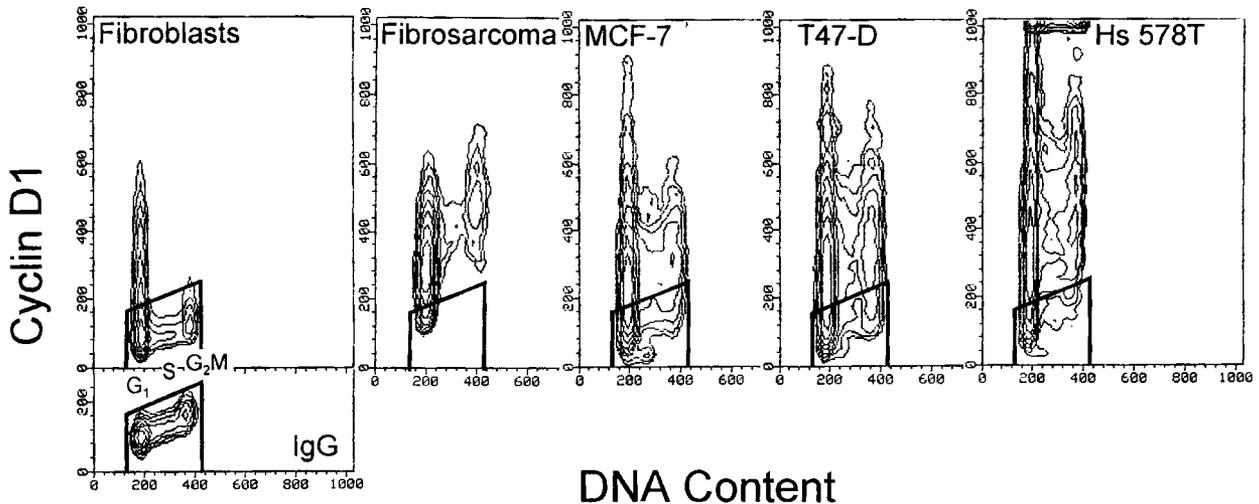
FIG. 6. Expression of cyclin D1 in normal fibroblasts, fibrosarcoma, and breast carcinoma (MCF-7, T47-D, and Hs 578T) cell lines. In contrast to normal fibroblasts that express cyclin D1 transiently during G_1 and show absence of this protein during S, the cells of tumor lines show high levels of cyclin 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).

tained under ideal conditions, in exponential phase of cell growth. For example, addition of IL2 to cultures of PHA stimulated proliferating lymphocytes changes both the bivariate pattern of its expression (more cells progressing 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 cells growth, e.g., for exponentially growing cells during asynchronous, unperturbed growth, and with full access to growth factors.

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, as well as during G_2/M, are cyclin D1 negative, most tumor cell lines contain significant proportions of S and G_2/M cells with high levels of cyclin D1. This is perhaps best illustrated by comparison of normal fibroblasts and fibrosarcoma cells, or normal lymphocytes and MOLT-4 cells (Fig. 6). 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 pat-

tern is consistent with the status of phosphorylation of pRB in normal cells; namely, pRB is underphosphorylated early in G_1, prior to cell commitment to S. It should be mentioned, however, that pRB also can be phosphorylated by CDK2 and CDK1 during S and G_2, 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 G_1 cells (ref. 30, Fig. 6). This would suggest that phosphorylation of pRB by CDK4 is not restricted to an early portion of G_1 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-

DNA
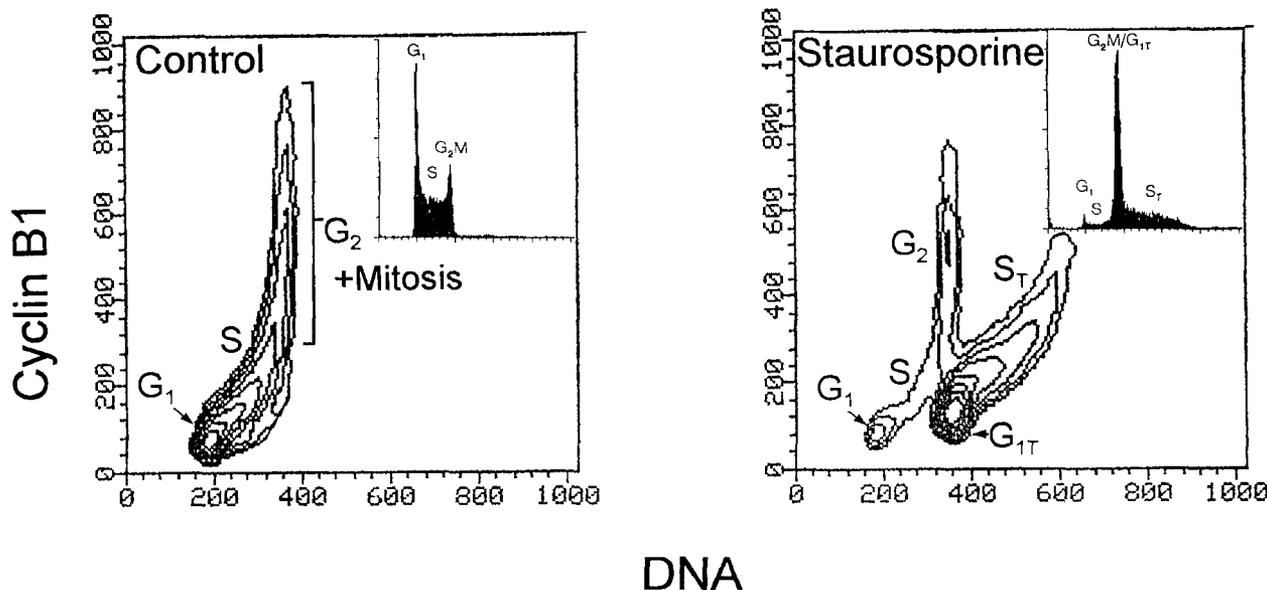
Fig. 7. Distinction of the G_1 DNA tetraploid (G_{1T}) from G_2/M diploid cells based on the differences in expression of cyclin B1. Cytokinesis of MOLT-4 cells was prevented by the protein kinases inhibitor staurospo-rine and analysis of the cell cycle progression at two DNA ploidy levels was accomplished by the bivariate analysis of cyclin B1 and DNA content (76).

vides for the detection of the unscheduled expression of these proteins will be essential for evaluating their prognostic potential. It should be mentioned, however, that when the primary oncogenic changes are downstream of the cyclin D-CDK4 complex (e.g., at the level of pRB or E2F, resulting in constitutive activation of the genes associated with the cell's commitment to S), expression of D-type cyclins may not be critical for tumor growth, and actually a decrease in expression of cyclin D may be observed (e.g., 64).

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 G_2 diploid and G_1 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 G_2/M diploid from G_1 tetraploid cells in such cases can be accomplished only when an additional marker of G_1 or G_2/M is available. Cyclins E or B1 are such markers for G_1 and G_2/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 G_2/M cells of lower DNA ploidy vs. G_1 cells of higher ploidy enables one to estimate the kinetics of cell progression through the cell cycle under conditions where cell division (cy-tokinesis) 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 G_2 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 G_2/M diploid cells vs. G_1 tetraploid as well as between mitotic and G_2 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 G_0 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
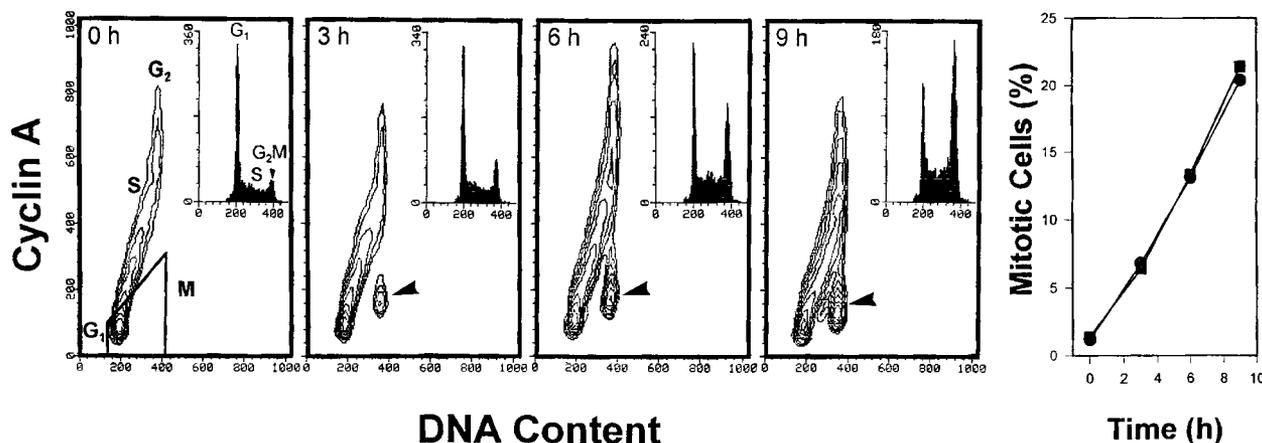
Fig. 8. Discrimination between mitotic (postprometaphase) and G_2 cells based on differences in expression of cyclin A. Exponentially growing MOLT-4 cells were treated with Vinblastine for up to 9 h. The rapid breakdown of cyclin A during prometaphase results in a cyclin A negative population that can 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[a]

Compartment	Cyclin expression, DNA replication	Location in cell cycle	Examples, description
prD	D^-	Prior to *rp-D*	Nonstimulated lymphocytes, quiescent fibroblasts (G_0 cells)
poD-prE	D^+/E^-	Between *rp-D* and *rp-E*	Lymphocytes 4–12 h after PHA, STS arrested normal cells, postmitotic cells from tumor lines
poE-prS	E^+/S^-	Between *rp-E* and entrance to S	G_1 cell subpopulation with cyclin E above the threshold
poE-S	E^+/S^+	Between entrance to S and loss of cyclin E	Cyclin E positive S phase cells (Fig. 2)
S-prA (?)	S/A^-	Between entrance to S and *rp-A*	Early S cells prior to onset of cyclin A expression
poA-prB	A^+/B^-	Between *rp-A* and *rp-B*	S phase cells expressing cyclin A but not B1
poB-S (?)	B^+/S	Between *rp-B* and exit from S	S phase cells that have initiated cyclin B1 expression
poB-M	B^+/M^-	Between *rp-B* and entrance to M	Cyclin B1 positive G_2 cells, cells arrested in G_2 by ionizing radiation, *m*-AMSA (25,28)
M,A$^+$	$M/A^+/B^+$	Between prophase and prometaphase	Early mitotic cells that are both cyclin A and cyclin B1 positive (32)
M,A$^-$	$M/A^-/B^+$	Between prometaphase and anaphase	Late mitotic cells that are cyclin A negative but cyclin B1 positive (32)

[a]As explained in the text.

blood lymphocytes prior to and during the initial 4 h of PHA stimulation. Also, fibroblasts maintained for extended time at confluency lose cyclin D1. Because there is no formal marker of G_0 cells, it is tempting to use the absence of expression of D type cyclins (i.e., prior to its induction in G_1) as such a marker. This marker may help to resolve the long-standing controversy as to whether cells in exponentially growing cultures enter G_0 or G_1 following mitosis. Because cyclin D appears to be absent in normal cells in G_2/M (Fig. 2), one must assume it is absent in immediately postmitotic cells. By this criterium, therefore, even exponentially growing nontumor cells transiently reside in G_0 prior to entrance to G_1. In contrast, most tumor cell lines are characterized by high

level of cyclin D in S and G_2/M (ref. 30) (Fig. 6), which suggests that these postmitotic cells do inherit this protein, thereby by-passing the G_0 state.

2. The postcyclin D and precyclin E (*poD-prE*) compartment represents the cells that are cyclin D positive but yet are not expressing cyclin E (D^+/E^- cells). An example of such cells are PHA-stimulated lymphocytes between 4 and 12 after stimulation with PHA (24) or lymphocytes arrested in G_1 by STS. The postmitotic, early G_1 cells of most tumor cell lines can be characterized as the cells in the *PoD-prE* subcompartment.

3. The next distinct subcompartment, in sequence of the cell cycle progression, represents cells that express cyclin E (cyclin E positive) but have not yet entered S

phase (*poE-prS*). The cells represented on the cyclin E vs. DNA content scatterplots as in G_1 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 *prA* 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 subcompartment was generally increasing, whereas the appearance of the subsequent cyclin was prevented. Such a situation was observed in the case of cells arrest 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 rep-

lication 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-
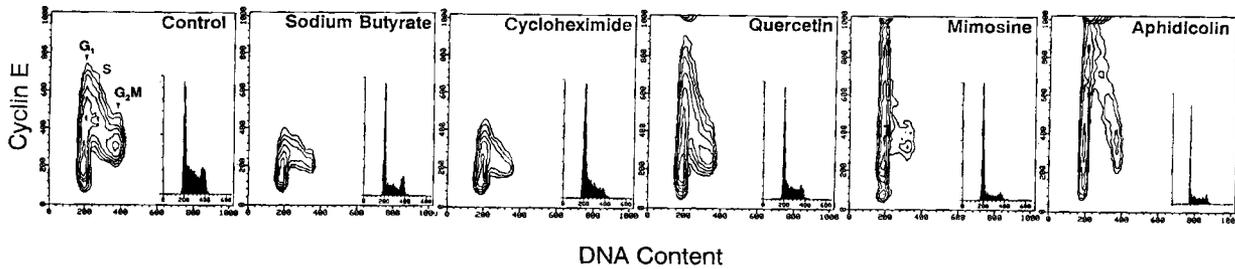
Fig. 9. Effect of different G_1 blockers on cyclin E expression. Exposure of MOLT-4 cells to different agents that arrest cells in G_1 or at the G_1/S border results either in a decrease (the arrest point is prior to the onset of cyclin E synthesis), or an increase (the arrest is past the initiation of cyclin E synthesis) of cyclin E in the arrested cells. See (25,28,29) for details.

Table 3
Cell Cycle Point of Arrest Induced by Different Agents With Respect to Expression (po-post; pr-prior) of Cyclins D, E, or B1[a]

Drug	Presumed target	Compartment/restriction point
n-butyrate	Histone acetylation	*prE*
Lovastatin	Protein isoprenylation	*prE*
Cycloheximide	Protein synthesis	*prE*
Staurosporine	Protein kinases	*poD-prE*
Genistein	Protein kinases	*prE*
Mimosine	DNA polymerase	*poE*
Quercetin	DNA polymerase	*poE*
Aphidicolin	DNA polymerase	*poE*
m-AMSA	DNA topoisomerase II	*poB1*
γ radiation	DNA	*poB1*
Melphalan	DNA	*poB1*

[a]Refs. 28, 29, 60.

sion, whereas the remaining three agents act past the *rp-E.*

Administration of the drug to the cultures containing G_0 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_1 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_1 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 inhibi-

tor(s). 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, immunocytochemical 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 asses 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.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Ajchenbaum F, Ando K, DeCaprio JA, Griffin JD: Independent regulation of human D type cyclin gene expression during G1 phase in primary human T lymphocytes. J Biol Chem 268:4113–4119, 1993.
2. Amon A, Irniger S, Nasmyth K: Closing the cell cycle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. Cell 77:1037–1050, 1994.
3. Ando K, Ajchenbaum-Cymbalista F, Griffin JD: Regulation of G_1/S transition by cyclins D2 and D3 in hematopoietic cells. Proc Natl Acad Sci USA 90:9571–9575, 1993.
4. Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G: Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev 7:812–821, 1993.
5. Bartkova J, Lukas J, Strauss M, Bartek J: Cell cycle-related variation and tissue-restricted expression of human cyclin D1 protein. J Pathol 172:237–245, 1994.
6. Bartkova J, Lukas J, Strauss M, Bartek J: Cyclin D1 aberrantly accumulates in malignancies of diverse histogenesis. Oncogene 10:775–778, 1995.
7. Bauer KD, Jacobberger JW: Analysis of intracellular proteins. Meth Cell Biol 41:352–373, 1994.
8. Cardon-Cardo C: Mutations of cell cycle regulators. Biological and clinical implications for human neoplasis. Am J Pathol 147:545–560, 1995.
9. Clevenger CV, Epstein AL, Bauer KD: Quantitative analysis of nuclear antigen in interphase and mitotic cells. Cytometry 8:280–286, 1987.
10. Clurman BE, Roberts JM: Cell cycle and cancer. JNCI 87:1499–1501, 1995.
11. Crissman HA, Darzynkiewicz Z, Tobey RA, Steinkamp JA: Normal and perturbed CHO cells: Correlation of DNA, RNA and protein by flow cytometry. J Cell Biol 101:141–147, 1985.
12. Daksis JI, Lu RY, Faccini LM, Marhin WW, Penn LJZ: Myc induces cyclin D1 expression in the absence of de novo protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. Oncogene 9:3635–3645, 1994.
13. Darzynkiewicz Z: Apoptosis in antitumor strategies: Modulation of cell cycle or differentiation. J Cell Biochem 58:151–159, 1995.
14. Darzynkiewicz Z, Traganos F, Gong J: Expression of cell cycle specific proteins cyclins as a marker of the cell cycle independent of DNA content. Meth Cell Biol 41:422–435, 1994.
15. Darzynkiewicz Z, Traganos F, Melamed MR: New cell cycle compartments identified by multiparameter flow cytometry. Cytometry 1:98–108, 1980.
16. Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR: Lymphocyte stimulation: A rapid multiparameter analysis. Proc Natl Acad Sci USA 73:2881–2884, 1976.
17. Draetta FG: Mammalian G1 cyclins Curr Opin Cell Biol 6:842–846, 1994.
18. Dulic V, Lees E, Reed SI: Association of human cyclin E with a periodic G1-S phase protein kinase. Science 257:1958–1961, 1992.
19. Filmus J, Robles AI, Shi W, Wong MJ, Colombo LL, Conti CJ: Induc-

20. Fisher RP, Morgan DO: A novel cyclin associates with MO15/CDK7 to form CDK-activating kinase. Cell 78:713–724, 1994.
21. Girard F, Strausfield U, Fernandez A, Lamb NJC: Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell 67:1169–1179, 1991.
22. Glotzer M, Murray AW, Kirschner MW: Cyclin is degraded by the ubiquitin pathway. Nature 349:132–138, 1991.
23. Gong J, Ardelt B, Traganos F, Darzynkiewicz Z: Unscheduled expression of cyclin B1 and cyclin E in several leukemic and solid tumor cell lines. Cancer Res 54:4285–4288, 1994.
24. Gong J, Bhatia U, Traganos F, Darzynkiewicz Z: Expression of cyclins A, D2 and D3 in individual normal mitogen stimulated lymphocytes and in MOLT-4 leukemic cells analyzed by multiparameter flow cytometry. Leukemia 9:983–899, 1995.
25. Gong J, Li X, Traganos F, Darzynkiewicz Z: Expression of G1 and G2 cyclins measured in individual cells by multiparameter flow cytometry: a new tool in the analysis of the cell cycle. Cell Prolif 27:357–371, 1994.
26. Gong J, Traganos F, Darzynkiewicz Z: Expression of cyclins B and E in individual MOLT-4 cells and in stimulated human lymphocytes during their progression through the cell cycle. Int J Oncol 3:1037–1042, 1993.
27. Gong J, Traganos F, Darzynkiewicz Z: Simultaneous analysis of cell cycle kinetics at two different DNA ploidy levels based on DNA content and cyclin B measurements. Cancer Res 53:5096–5099, 1993.
28. Gong J, Traganos F, Darzynkiewicz Z: Use of cyclin E restriction point to map cell arrest in G_1 induced by n-butyrate, cycloheximide, staurosporine, lovastatin, mimosine and quercetin. Int J Oncol 4:803–808, 1994.
29. Gong J, Traganos F, Darzynkiewicz Z: Staurosporine blocks cell progression through G1 between the cyclin D and cyclin E restriction points. Cancer Res 54:3136–3139, 1994.
30. Gong J, Traganos F, Darzynkiewicz Z: Threshold expression of cyclin E but not D type cyclins characterizes normal and tumour cells entering S phase. Cell Prolif 28:337–246, 1995.
31. Gong J, Traganos F, Darzynkiewicz Z: Growth imbalance and altered expression of cyclins B1, A, E and D3 in MOLT-4 cells synchronized in the cell cycle by inhibitors of DNA replication. Cell Growth Differ 6:1485–1493, 1995.
32. Gong J, Traganos F, Darzynkiewicz Z: Discrimination of G2 and mitotic cells by flow cytometry based on different expression of cyclins A and B1. Exp. Cell Res 220:226–231, 1995.
33. Hartwell LH, Kastan MB: Cell cycle control and cancer. Science 266:1821–1823, 1994.
34. Hartwell LH, Weinert TA: Checkpoints: controls that ensure the order in cell cycle events. Science 246:629–634, 1989.
35. Hinds PW, Dowdy SF, Eaton EN, Arnold A, Weinberg RA: Function of a human cyclin gene as an oncogene. Proc Natl Acad Sci USA 91: 709–713, 1994.
36. Hirama T, Koeffler HP: Role of cyclin-dependent kinase inhibitors in the development of cancer. Blood 86:841–854, 1995.
37. Hunter T: Braking the cycle. Cell 75:839–841, 1993.
38. Jacobberger JW, Fogelman D, Lehman Analysis of intracellular antigens by flow cytometry. Cytometry 7:356–364, 1986.
39. Keyomarsi K, Pardee AB: Redundant cyclin overexpression and gene amplification in breast cancer cells. Proc Natl Acad Sci USA 90: 1112–1116, 1993.
40. Koff A, Giordano A, Desai D, Yamashita K, Harper JW, Elledge S, Nishimoto T, Morgan DO, Franza BR, Roberts JM: Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science 257:1689–1694, 1992.
41. Knoblich JA, Sauer K, Jones L, Richardson H, Saint R, Lehner CF: Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell 77:107–120, 1994.
42. Krek W, Xu G, Livingston DM: Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. Cell 83:1149–1158, 1995.
43. Kung AL, Sherwood SW, Schimke RT: Differences in the regulation of

protein synthesis, cyclin B accumulation, and cellular growth in response to the inhibition of DNA synthesis in Chinese Hamster Ovary and HeLa S3 cells. J Biol Chem 268:23072–23080, 1993.

44. Landberg G, Tan EM: Characterization of a DNA-binding nuclear autoantigen mainly associated with S phase and G2 cells. Exp Cell Res 212:255–261, 1994.

45. Lukas J, Aagaard L, Strauss M, Bartek J: Oncogenic aberrations of p16$^{INK/CDKN2}$ and cyclin D1 cooperate to deregulate G1 control. Cancer Res 55:4818–4823, 1996.

46. Lukas J, Bartkova J, Welcker M, Petersen OW, Peters G, Strauss M, Bartek J: Cyclin D2 is a moderately oscillating nucleoprotein required for G1 phase progression in specific cell types. Oncogene 10:2125–2134, 1995.

47. Lukas J, Jadayel D, Bartkova J, Nacheva E, Dyer MJS, Strauss M, Bartek J: BLC-2/cyclin D1 oncoprotein oscillates and subverts the G1 phase control in B-cell neoplasms carrying the t(11;14) translocation. Oncogene 9:2159–2167, 1994.

48. Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ, Kato J-Y: D-type cyclin D kinase activity in mammalian cells. Molec Cell Biol 14:2066–2076, 1994.

49. Matsushime H, Roussel MF, Ashmun RA, Sherr CJ: Colony stimulating factor 1 regulates novel cyclins during G$_1$ phase of the cell cycle. Cell 65:701–713, 1991.

50. McIntosh CG, Anderson JJ, Milton I, Steward M, Parr AH, Thomas MS, Henry JA, Angus B, Lennard TWJ, Horne CHW: Determination of the prognostic value of cyclin D1 overexpression in breast cancer. Oncogene 11:885–891, 1995.

51. Morgan DO: Principles of CDK regulation. Nature 374:131–134, 1995.

52. Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM, Arnold A: A novel cyclin encoded by bcl-1 linked candidate oncogene. Nature 350:512–515, 1991.

53. Murray AW: Creative blocks: cell cycle checkpoints and feedback controls. Nature 359:599–604, 1992.

54. Musgrove EA, Hamilton JA, Lees CS, Sweeney KJE, Watts CK, Sutherland RL: Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. Mol Cell Biol 13:3577–3487, 1993.

55. Musgrove EA, Lee CSL, Buckley MF, Sutherland RL: Cyclin D1 induction in breast cancer shortens G$_1$ and is sufficient for cells arrested in G$_1$ to complete the cell cycle. Proc Natl Acad Sci USA 91:8022–8026, 1994.

56. Nigg EA: Cyclin-dependent protein kinases:key regulators of the eukarotic cell cycle. BioEssays 17:471–480, 1995.

57. Norbury C, Nurse P: Animal cell cycles and their control. Annu Rev Biochem 61:441–470, 1992.

58. Nurse P: Ordering S phase and M phase in the cell cycle. Cell 79:543–550, 1994.

59. Ohtsubo M, Roberts JM: Cyclin-dependent regulation of G$_1$ in mammalian fibroblasts. Science 259:1908–1912, 1993.

60. Orlandi L, Zaffaroni N, Bearzatto N, Costa A, Supino R, Vaglini M, Silvestrini R: Effect of melphalan and hyperthermia on cell cycle progression and cyclin B1 expression in human melanoma cells. Cell Prolif 28:617–630, 1995.

61. Pagano M, Pepperkok R, Verde F, Ansorge W and Draetta G: Cyclin A is required at two points in the human cell cycle. EMBO J 11:961–971, 1992.

62. Pardee AB: G1 events and regulation of cell proliferation. Science, 246:603–608, 1989.

63. Peters GJ: The D-type cyclins and their role in tumorigenesis. Cell Sci 18:98–96, 1994.

64. Peterson SR, Gadbois DM, Bradbury EM, Kraemer PM: Immortalization of human fibroblasts by SV large T antigen results in the reduction of cyclin D1 expression and subunit association with proliferating cell nuclear antigen and Waf1. Cancer Res 55:4651–4657, 1995.

65. Pines J: Arresting developments in cell-cycle control. Trends Biochem Sci 19:143–145, 1994.

66. Pines J, Hunter T: Human cyclin-A and cyclin B are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J. Cell Biol 115:1–17, 1991.

67. Prosperi E, Scovassi AI, Stivala LA, Bianchi L: Proliferating cell nuclear antigen bound to DNA synthesis sites: Phosphorylation and association with cyclin D1 and cyclin A. Exp Cell Res 215:257–262, 1994.

68. Raffeld M, Jaffe ES: bcl-1 t(11;14), and mantle-cell derived lymphomas. Blood 78:259–263, 1991.

69. Quelle DE, Ashum RA, Shurtleff SA, Kato J-Y, Bar-Sagi D, Roussel MF, Sherr CJ: Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev 7:1559–1571, 1993.

70. Sharpless TK, Traganos F, Darzynkiewicz Z, Melamed MR: Flow cytofluorimetry: Discrimination between single cells and cell aggregates by direct size measurements. Acta Cytol 19:577–581, 1975.

71. Sherr CJ: G1 phase progression: Cycling on cue. Cell 79:551–555, 1994.

72. Sherwood SW, Rush DP, Kung AL, Schimke RT: Cyclin B1 expression in HeLa cells studied by flow cytometry. Exp Cell Res 211:275–281, 1994.

73. Shirodkar S, Ewen M, DiCaprio JA, Morgan J, Livingston DM, Chittenden T: The transcription factor E2F interacts with the retinoblastoma gene product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell 68:157–166, 1992.

74. Sicinski P, Donaher JL, Parker SB, Li T, Fazell A, Gardner H, Haslam SZ, Bronson RT, Elledge SJ, Weinberg RA: Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell 82:621–630, 1995.

75. Tam SW, Theodoras AM, Shay JW, Draetta GF, Pagano M: Differential expression and regulation of cyclin D1 in normal and tumor cells: Association with Cdk4 is required for cyclin D1 function in G1 progression. Oncogene 9:2663–2674, 1994.

76. Traganos F, Gong J, Ardelt B, Darzynkiewicz Z: Effect of staurosporine on MOLT-4 cell progression through G$_2$ and on cytokinesis. J. Cell Physiol 158:535–544, 1994.

77. Urbani L, Sherwood SW, Schimke RT: Dissociation of nuclear and cytoplasmic cell cycle progression by drugs employed in cell synchronization. Exp Cell Res 219:159–168, 1995.

78. Weinberg RA: The retinoblastoma protein and the cell cycle control. Cell 81:3232–330, 1995.

79. Zhou P, Jiang W, Weghorst CM, Weinstein IB: Overexpression of cyclin D1 enhances gene amplification. Cancer Res 56:36–39, 1996.