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REVIEW

Analysis of the Cell Cycle and a Method Employing Synchronized Cells for Study of Protein Expression at Various Stages of the Cell Cycle

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Abstract—Study of protein expression during the cell cycle requires preparation of pure fractions of cells at various phases of the cell cycle. This was achieved by the development of methods for cell synchronization. Successful cell synchronization requires knowledge of the duration of all phases of the cell cycle. So, in the present review these interrelated problems are considered together. The first part of this review deals with basic methods employed for analysis of duration of cell cycle phases. The second summarizes data on treatments used for cell synchronization. Methods for calculation of percent of cells at various stages of the cell cycle in fractions of synchronized cells are considered in the third part. The fourth part of this review deals with a method of study of protein expression during the cell cycle by means of immunoblotting of synchronized cell fractions. In the Appendix, basic principles are illustrated with practical examples of analysis of the cell cycle, synchronization, and study of expression of some proteins at various stages of the cell cycle using synchronized XL2 (*Xenopus laevis*) cells.

Key words: cell cycle, proteins, cell synchronization

The nucleus became an object of research much earlier than other cell organelles. This explains why the notions "cell cycle" and "nuclear cycle" are used as synonyms. The whole cell cycle is subdivided into mitosis and interphase (a phase between two cell divisions). During mitosis, chromosomes are condensed and inactive, whereas at interphase chromosomes are decondensed and transcriptionally active. Mitosis is further subdivided into prophase, prometaphase, metaphase, anaphase, and telophase. Interphase is also subdivided into synthetic S-phase, characterized by DNA replication, G_1 (Gap) phase, which precedes S-phase, and G_2-phase, which follows S-phase and precedes mitosis. Such subdivision of the cell cycle into these phases was originally proposed by Howard and Pelc [1]. Some authors also subdivide G_1-phase, into two sub-phases: G_1-pm (G_1 postmitotic or early) and G_1-ps (G_1 presynthetic or late) [2]; this subdivision is based on sensitivity of cells to content of growth factors in a cultivation medium.

It should be noted that not all cells are constantly involved in the cell cycle in an organism or during *in vitro* cultivation. In organisms, the majority of terminally differentiated cells irreversible loose their ability to divide. Other non-divided cells may "be recruited" into the cell cycle by certain proliferative stimuli. Such cells are usually diploid; this means that they exit the cycle before onset of S-phase. The existence of reversible cell exit out of the cycle was proposed by Lajtha [3] and Quastler [4]. They also proposed the new term "G_0-phase" to describe such state of cells. All cells cultivated *in vitro* (except highly malignant tumor cells) are characterized by existence of some proportion of cells in G_0-phase. This should be taken into consideration during analysis of a cell population.

The terms "growth fraction" [5] or "proliferative pool" [6] are used to define the proportion of cells in the cell cycle (i.e., all cells except those in G_0).

Study of differential expression of various proteins during the cell cycle requires synchronically dividing cells. Under natural conditions, the early stages of embryonic development are characterized by a period of almost synchronous cell divisions known as cleavage. Study of cleavages resulted in the discovery of the key cell cycle regulators, which were defined as cyclins; this name reflects cyclic changes in the levels of these proteins in the cell [7-10].

Besides cyclins, the content of several other protein families cyclically changes. Interestingly, the term "cyclin" was originally used to define a protein that is now known as PCNA (Proliferating Cell Nuclear

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Antigen); according to modern nomenclature, it is not a cyclin [11, 12].

In contrast to proteins involved in regulation of cell division or DNA replication, the activities of many other proteins do not depend on the stage of the cell cycle; these include constantly needed cytoskeleton proteins, actin [13] or tubulins [14].

The cell cycle of the first synchronic divisions of embryonic cells almost lacks G_1- and G_2-phases. However, recent studies revealed that some principal events of the cell cycle of somatic cells, such as checkpoints, occur at G_1- and G_2-phases. For example, R point or G_1 restriction point crucial for cell entry to the next cycle is at the second half of G_1 [9, 10].

Formation of mitotic machinery, particularly the beginning of centriole replication [15-17] and also migration of centrosomes, future poles of the mitotic spindle [18], may also begin at the end of G_1-phase and the beginning of G_2-phase, respectively.

Thus, understanding of regulation of the cell cycle requires detailed study of cells at G_1- and G_2-phases. In the case of clear intracellular localization, changes in protein content in the cell may be roughly evaluated by morphologic studies. Subsequent biochemical study will give precise answer on the dependence of particular protein content on the stage of the cell cycle.

Quantitative analysis of protein expression in the cell requires a large amount of pure populations of these cells. However, in contrast to cell divisions in the beginning of early embryonic development, division of cultivated cells (in cell cultures) occurs asynchronously. Even sister cells originated from division of the mother cell may significantly differ in cell cycle duration [19, 20].

Various methods for cell synchronization were developed for preparation of populations of cells at the same stage of the cell cycle. In the present review, I have considered basic methods for the development of synchronized cultivated cell lines. Effective use of synchronization requires information on duration of all stages of the cell cycle of the investigated cell culture. The first chapter deals with methods of determination of duration of cell cycle phases.

1. DETERMINATION OF DURATION OF CELL CYCLE PHASES

1.1. Determination of cell cycle duration. This may be determined using the time required for duplication of cell number and proportion of cells in the G_0-phase.

The growth rate of a cell population is usually analyzed by changes in number of cells on a certain area (e.g., average cell number within one visual field of a microscope) up to the moment when cell isles begin to form a monolayer. This results in contact inhibition of proliferation.

In parallel, it is necessary to calculate number of cells simultaneously plated onto glass and incubated in the medium containing bromodeoxyuridine (BrdU). The time interval should be sufficient for determination of the proportion of cells at G_0-phase. Average time required for duplication of cell number is calculated using the formula: $t_d = t/\log_2 (N_t/N_0)$, where t_d is time required for duplication of cell number, t is time interval between initial and final calculation of cell number, N_0 and N_t are cell numbers at the beginning and the end of the experiment, respectively [21].

For cell cultures characterized by low proportion of cells in G_0-phase (0-5%), duplication time insignificantly differs from duration of the cell cycle (when the proportion of cells in G_0 at zero time absolutely coincides). If the proportion of cells in G_0 is higher, duration of cell cycle should be calculated using the formula: $T = t_d/\log_2 [(2 - y)/(1 - y)]$, where T is duration of cell cycle, t_d is average time of duplication of cell number, and y is proportion of cells in G_0-phase [21]. Such analysis allows precise calculation of average duration of cell cycle (see Appendix 1.1). Its variability for individual cells may be determined using vital observations (e.g., periodic video recording) [2].

1.2. Determination of S-phase duration and the whole cell cycle in one experiment. Short incubation of asynchronous cell culture (for 15-30 min) in a medium containing BrdU allows determining the relative duration of S-phase in the cell cycle. Using only cells of a proliferative pool, a researcher should calculate proportion of cells in G_0-phase and calculate proportion of cells in S-phase. This pool is equivalent to the proportion of cells incorporating BrdU during the incubation time, which is much higher than the duration of the whole cell cycle. For most cultures of vertebrate cells the proportion of S-phase is ~40% of the cell cycle [20, 22, 23].

Duration of S-phase and the whole cell cycle can be determined more precisely if this experiment is continued and cells grown in the medium containing BrdU are periodically fixed. In culture, cells divide asynchronously, and so at each time interval the same proportion of new cells enter S-phase (deviations of this rule will be discussed below). Thus, using BrdU incorporation into DNA as a probe it is possible to observe proportional dependence of the increase of labeled cells on incubation time.

It should be noted that during first hours of incubation of cells in the medium containing BrdU the rate of accumulation of labeled cells was somewhat lower than was found later. Duration of this period was equivalent to duration of G_2-phase and mitosis. While labeled cells did not pass through mitosis, their number was actually proportional to the time of their incubation with BrdU. However, when cells labeled in the end of S-phase pass G_2-phase and mitosis their division yields two labeled cells (from one labeled cell). Thus, analyzing time course

of accumulation of labeled cells it is possible to determine total duration of G_2-phase and mitosis. However, more frequently it is determined by the proportion of labeled mitoses (see section 1.3) rather than by total proportion of labeled cells because it is difficult to determine rather small change in the rate of accumulation of labeled cells during such a short time interval. When the last cells, which were at G_2-phase in the beginning of their incubation with BrdU, pass through mitosis a new rate of accumulation of labeled cells will set up. This new rate will reflect total dynamics of entry of new cells from G_1-phase into S-phase and duplication of the number of labeled cells after mitosis.

At a certain time interval the rate of accumulation of the proportion of labeled cells reduces. This is due to entry of cells labeled at the end of S-phase of the previous cell cycle into S-phase of the next cell cycle. Change in the slope of the curve on the plot will be smooth due to differences between cells in duration of S-phase and the whole cell cycle. The intersect of rectilinear extensions of the middle and terminal parts of the curve of labeled cell accumulation correspond to duration of cell cycle with deduction of S-phase duration. This is the period "T minus S". Since a proportion of cultivated cells are in G_0-phase, the proportion of labeled cells does not reach 100%. On the other hand, it seems unlikely that the increase in proportion of labeled cells will be completely stopped, because under conditions of unlimited growth each proliferating cell forms two labeled cells from one during mitosis; this causes twofold reduction in the proportion of unlabeled (non-dividing) cells per cell cycle. This process is partially compensated by exit of some proportion of cells from the cell cycle, and therefore the value of the proliferative pool may be stabilized during a certain time interval [21].

Thus, knowing proportion of S-phase of the whole cell cycle (from the first part of the experiment) and duration of the period "T minus S" (from the second part of the experiment), it is possible to calculate duration of the cell cycle (T) and S-phase by solving a system of equations (see Appendix 1.2).

1.3. Determination of G_2-phase duration. The only reliable method for determination of G_2-phase duration is known as the method of labeled mitoses [24]. Until recently replicating DNA was usually labeled with [^{3}H]thymidine and the labeled cells were recognized by autoradiography. Now cells are labeled at S-phase by BrdU incorporation followed by subsequent treatment with specific antibodies. Labeling of mitosis requires cells in S-phase at the beginning of the incubation. Incubation time characterized by appearance of the first labeled mitosis (the first labeled early prophase) corresponds to minimal duration of G_2-phase. Since it is impossible to determine the duration of each stage of mitosis in fixed cells, it is usually assumed that the fixed cell is in the middle of the particular stage where it has been fixed. For more accurate determination of minimal duration of G_2-phase, it is necessary to take into consideration the stage of first labeled mitoses. For example, if the first labeled cell was found in metaphase after the incubation of cells with BrdU for 2 h, minimal duration of G_2-phase will be 2 h minus duration of prophase, prometaphase, and half metaphase. Incorporation of BrdU into a cell requires the presence of this cell in S-phase for some minimal time interval. Consequently, actual minimal duration of G_2-phase is even lower than the calculated value. Usually this time interval is not taken into consideration, however, due to probability we can assume that it represents half-time of cell incubation in BrdU.

The data are plotted as the dependence of proportion of labeled cells on the time of cell incubation in the medium containing BrdU. Average value of G_2-phase duration is the time required for labeling of 50% mitoses minus halftime of duration of mitosis. More accurate value of average duration of G_2-phase can be calculated considering percent of labeled prophases only. In this case, average G_2-phase duration will represent the time required for labeling of 50% of prophases minus halftime of prophase duration.

Maximal duration of G_2-phase corresponds to incubation time required for detection of the last unlabeled mitosis minus time from the beginning of mitosis up to the middle of this stage of mitosis. The other mode of calculation of maximal duration of G_2-phase includes determination of minimal time required for labeling of all mitoses minus duration of mitosis (see Appendix 1.3).

1.4. Determination of mitosis duration. Duration of mitosis can be evaluated by duration of the whole cell cycle, proportion of cells in G_0-phase, and mitotic index. Usually, in cultivated vertebrate cell mitosis lasts ~1 h and it represents about 3-5% of the total time of the cell cycle.

The duration of various mitotic stages is determined during observation of vital cells. It should be noted that normal passage through mitosis depends on temperature and so all vital observations should be carried out at temperature corresponding to the cultivation temperature (see Appendix 1.4).

1.5. Determination of G_1-phase duration. Duration of G_1-phase can be calculated on the basis of data on duration of other stages of the cell cycle (see Appendix 5) or determined in experiments. The most accurate but the most laborious method includes study of vital cells during various time intervals after mitosis; such cells are shortly preincubated (for 15-30 min) in medium containing BrdU and then fixed. Using this method, it is possible to determine both average duration of G_1-phase and its variability.

Duration of G_1-phase can also be determined by time interval since BrdU incorporation into cells, which were initially synchronized in mitosis.

1.6. Determination of duration of all stages of the cell cycle in one experiment. Reviewing all results considered

in this section, we can propose a scheme of experiment designed for simultaneous determination of all parameters of the cell cycle. Asynchronous cell culture should be incubated in medium containing BrdU; cells of this culture should be fixed and stained with anti-BrdU antibodies.

For determination of cells in G_0-phase, they are incubated in the medium containing BrdU during a time interval that is longer than the duration of the whole cell cycle. The proportion of cells that did not incorporate BrdU will correspond to the proportion of cells in G_0-phase. Duration of S-phase and the whole cell cycle are determine by data on the proportion of cells in G_0-phase, proportion of labeled cells in the beginning of experiment, and at time interval corresponding to the bend-point (point "T minus S") on the curve of time-dependent accumulation of labeled cells. Mitotic index (proportion of cells in mitosis) shows the ratio of duration of mitosis and the whole cell cycle. Only cells of the proliferative pool should be taken into consideration. Duration of G_2-phase corresponds to the time interval required for 50% labeling of mitosis (after incubation of cells in the medium containing BrdU; see section 1.3 for details). Duration of G_1-phase is calculated by subtraction of the duration of all other stages from the total duration of the cell cycle.

2. REVIEW OF SYNCHRONIZATION METHODS

Many methods have been employed for preparation of fractions of cells at certain stages of the cell cycle [25-33]. However, for each cell line synchronization conditions should be individually optimized. The time required for the effect of a synchronization agent is determined by duration of phases of cell cycle in a particular cell type. The concentration of such substances is the other principal parameter. In various cell lines, the same concentration may reversibly arrest cell cycle, induce apoptosis, or be completely ineffective in blockade of transition of cells via the cell cycle.

It is impossible to get cells at different phases of the cell cycle using any single treatment: because of variability of all phases, progressive desynchronization occurs during cell cultivation. Thus, it is important to use combined treatments of cells by several synchronizing agents.

In the next section, I consider substances that are frequently used for cell synchronization and also physical methods employed for separation of cells at various phases of the cell cycle. Since many principles of cell synchronization were originally developed using "thymidine block" as the synchronization method, it is described first.

2.1. Chemical substances used for cell synchronization. Excess of **thymidine** in the cultivation medium (2-2.5 mM) increases the intracellular pool of dTTP by ~5-fold. This results in inhibition of dCDP formation from CDP followed by inhibition of DNA replication and arrest of the cell cycle in S-phase [34, 35]. Cells that were in mitosis, G_2-, or G_1-phase at the beginning of the incubation in the medium with high thymidine content are accumulated in early S-phase, whereas cells being in S-phase at the beginning of this incubation are arrested in this phase. Thus, the incubation period corresponding to the duration of the period "T minus S" results in the presence of two groups of cells: cells synchronized in early S-phase and asynchronous cells in early, middle, or late S-phases. Use of "double thymidine block" can be used to obtain a more synchronized cell population [36, 37]. After the first incubation in the medium with thymidine cells are washed with fresh medium and cultivated in it during the time interval required for transition of cells accumulated in early S-phase during the first thymidine block to G_2-phase. (This period is equivalent to the maximal duration of S-phase.) Since in almost all cell lines total duration of G_2, mitosis, and G_1 (even minimal) exceeds the maximal duration of S-phase, cells arrested in late S-phase during the first block cannot reach subsequent S-phase during washout. Cells are subsequently incubated in the medium containing 2-2.5 mM thymidine for the incubation time equivalent to "G_2 + mitosis + G_1". Thus, in the ideal case all cells of the proliferating pool will be accumulated at the G_1–S boundary or in early S-phase. For complete and rapid removal of the thymidine block cells are further cultivated (after washing) in medium containing 10-24 μM deoxycytidine [26, 38].

However, it should be noted that synchronization of cells by "thymidine block" influences not only DNA replication but also RNA synthesis [39]. So it is not recommended to incubate cells with high thymidine content for more than 16 h [38]. This allows the use of this method for synchronization of cells characterized by relatively short cell cycle.

Aphidicolin, an inhibitor of DNA polymerase-α [27, 40-42], is used for synchronization of cells in S-phase; the working range of concentrations 1-10 μg/ml is characterized by low toxicity for cells and easy removal by washing [43-45]. The optimal concentration of aphidicolin can differ for various cell cultures. For example, inhibition of DNA synthesis in human melanoma up to 20% of initial level requires aphidicolin concentration of 10 μg/ml [46]. In other cell lines concentration of 1-3 μg/ml is sufficient for total inhibition of DNA synthesis [42, 44, 45, 47-49]. Since cells being at the moment of incubation at S-phase delay their movement over the cell cycle, effective synchronization requires either double treatment with aphidicolin [42] (with intermediate washing during the period equivalent to maximal duration of S-phase) or pilot cell synchronization by another method (e.g., serum depletion [44, 45] or isoleucine deprivation [50]).

Hydroxyurea is an inhibitor of ribonucleotide diphosphate reductase [51]. At concentrations of 0.5-1 mM [33, 52], it arrests cells in early S-phase due to exhaustion of the deoxyribonucleotide pool.

Isoleucine deprivation. This method originally developed for Chinese hamster ovary (CHO) cells [26] was subsequently applied with various efficacies for other cell cultures [53]. Since after cell transfer into complete cultivation medium cells entered S-phase after ~4 h, we can conclude that absence of isoleucine blocks cell movement over the cell cycle in the middle of G_1-phase.

Calpain inhibitor I (N-acetyl-leucyl-leucyl-norleucinal, ALLN). This inhibitor of cysteine and serine proteases (including calpain) [32, 54] inhibits cyclin degradation. At 40-μg/ml concentration, it arrests XL2 cells in metaphase for several hours [45]. (The mitotic spindle has normal structure.)

Lovastatin and its analogs **(mevastatin, compactin)** are 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors. Treatment of cells with lovastatin arrested cells in G_1-phase up to a restriction point (checkpoint) [54-57]. For cell synchronization, 5-μM lovastatin is usually used, but 10-μM lovastatin can cause induction of apoptosis [56].

Methotrexate (MTX) is a thymidine analog inhibiting tetrahydrofolate reductase, which prevents dUMP methylation by thymidylate synthetase [58]. In the concentration range 0.88-5 μM [43, 52, 59, 60], methotrexate arrests the cell cycle and causes accumulation of cells at G_0/G_1. It should be noted that cultivation medium used for cell synchronization by methotrexate should not contain thymidine.

Mimosine is a rare plant amino acid, acting as chelating agent, which prevents formation of new replication forks by inhibiting ribonucleotide reductase and reduction of dATP and dGTP pools [61-63]. Mimosine impairs deoxyribonucleotide metabolism and therefore it acts at the phase of DNA elongation. For synchronization of cells in early S-phase, it is used at concentrations of 200-600 μM [30, 33, 64].

Nocodazole and also two other substances (**colchicine** and **colcemide**) known as anti-microtubule poisons significantly increase critical concentration of tubulin polymerization within the cell; this leads to microtubule depolymerization and block of mitosis [28, 43, 52, 54, 65, 66]. Although microtubules play an important role in interphase cells, their depolymerization is not accompanied by arrest of movement of most cells over the cell cycle from G_1 to mitosis. Only transient delay of cells in G_2-phase of cell cycle was reported [67]. Even low concentrations of nocodazole can arrest cells in mitosis and so minimally required concentration (usually 0.1-0.5 μg/ml) is experimentally determined for each cell line. During prolonged incubation, treatment of most cell types with nocodazole can result in cell transition into interphase without anaphase chromosome separation (i.e., polyploidy) [68].

Staurosporine is an alkaloid that inhibits cyclin-dependent kinase (cdk) by binding to retinoblastoma protein [69, 70]. For cell synchronization in G_1-phase, it is used at 100 nM concentration, but long-term treatment may cause apoptosis [71-73].

Serum depletion. Movement of nonmalignant cells during the cell cycle requires the presence of exogenous growth factors. These factors are usually present in fetal serum, which is added to the cultivation medium. Absence of serum in the medium results in arrest of cells in G_1-phase or their exit from the cell cycle into G_0-phase [2, 74-76].

Trichostatin A (TSA) is an inhibitor of histone deacetylases [77]. At concentrations 0.1-5 μM it reversibly arrests cells in G_1- and G_2-phases [52, 77, 78].

5-Fluorodeoxyuridine inhibits thymidylate synthase and arrests cells at early S-phase of the cell cycle [79]. For synchronization, 0.05-μM concentration is used [80].

Hoechst 768159 ([2-(4-hydroxytoluene-3-yl)-4,5-dihydro-4-carboxythiazole]) inhibits posttranslational changes of the rare amino acid hypusine; at 40-μM concentration, it arrests cells in late G_1-phase of the cell cycle [81].

Cyclopyroxolamine (CPX) is an inhibitor of initiation of DNA replication [54, 82]. It blocks cells at the G_1/S boundary. For synchronization, 10-μM concentration is used [64].

Etoposide (VP16), inhibitor of topoisomerase 2, is a widely used anticancer drug. It delays cells at S-phase of the cell cycle and arrests cells at the G_2/M boundary. For synchronization of cells, it is used in the concentration range from 0.7 to 10 μM [83, 84]. Etoposide causes specific inactivation of kinase activity (CDK2) associated with cyclin A. This results in activation of S-phase and G_2/M-check-point control. Prolonged incubation of cells with etoposide induces apoptosis.

2.2. Physical methods of synchronization and selective separation of cells. Cooling of cells reduces all cellular processes and leads to an increase in the proportion of cells in G_1-phase and their exit from cell cycle into G_0-phase. However, when cells are under normal cultivation conditions they follow the cycle asynchronously. Thus, cooling of cells can be used for preparation of cell population with high proportion of cells in G_0-phase only. Cooling is also used for prevention of cell exit into G_1-phase during accumulation of mitotic cells synchronized by other methods [43].

Centrifugal elutriation is used for suspension cell cultures or cells detached from substrate. This method is based on cell separation by size and different flotation densities during centrifugation [85-87]. It requires a special centrifugation rotor equipped with tubes for liquid flow. This method produces only fractions of cells enriched with cells in G_1, S, or G_2 (but not pure fractions of cells at various phases of cell cycle). In combination

with chemical synchronization, use of this method yields relatively pure populations of synchronized cells [83].

Shake-off of mitotic cells is effective for cell lines characterized by rounding shape of metaphase cells and weak attachment to substrate (e.g., mouse fibroblasts [88]). This method is employed after incubation of cells in medium with anti-mitotic drugs for increase of percent of mitoses due to blockade of mitotic spindle formation (see nocodazole). In the case of epithelial cells, this method is less effective. For increase in yield of mitotic cells cultivation is carried out in special slowly rotating vials (0.5 rpm), which periodically (every 10 min) are rotated at 200 rpm for shake-off of weakly detached mitotic cells. For prevention of cell exit into G_1-phase of accumulated mitotic cells, they are cooled [43]. For better attachment of interphase cells, cultivation vials are treated with magnesium acetate [89].

Cell separation in a flow cytofluorimeter. This method is based on automated separation of cells that were vitally stained with fluorescent dyes (e.g., Hoechst 33342) stoichiometrically bound to DNA [90]. However, this method (as well as centrifugation methods) cannot separate cells in G_2-phase from mitotic cells or tetraploid cells in G_1-phase.

The appendix to the second chapter shows an example of optimization of a protocol for synchronization of XL2 cells.

3. CALCULATION OF PERCENT OF CELLS IN VARIOUS PHASES OF THE CELL CYCLE IN FRACTIONS OF SYNCHRONIZED CELLS

For analysis of expression of various proteins during the cell cycle, it is necessary to analyze the composition of cell fractions obtained after synchronization. Two principle methods are known.

1. Composition of cell fractions can be investigated by means of automated analysis of fluorescently labeled cell nuclei using flow cytofluorimeter (FCM-analysis) [44, 91-93]. In its original variant, such analysis was based on quantitative determination of a fluorescent dye (usually propidium iodide or DAPI, DiAmidino-Phenyl-Indole) stoichiometrically bound to DNA in each cell (univariant analysis on amount of DNA). Later multivariant methods for analysis of cells by several parameters (e.g., size and amount of DNA-incorporated BrdU) were developed [94].

FCM-analysis provides general information on dynamics of changes in ratio of cells in G_1-, S-, and G_2/M-phases of cell cycle. However, in contrast to cells in the organism, the amount of chromosomes (and consequently, DNA) in cell cultures may vary over a wide range: some cells are aneuploid and some cells are tetraploid. However, FCM-analysis cannot separate cells in G_2 and mitosis (and both these groups are inseparable from tetraploid cells in G_1) and cells in G_1 and G_0. This significantly reduces applicability of this method for accurate analysis of ratio of various cells in different phases in fractions. Analysis of experimental data obtained by various authors suggests that use of results of FCM-analysis without cytological control of morphology of synchronized cells results in incorrect interpretation of results [68, 95].

For cells attached to substrate a combined method using high rate of FCM-analysis and accuracy of immunocytochemistry, known as laser scanning cytometry (LSCM-analysis), was developed. Automated analysis of cells uses a microcytofluorimeter connected to a microscope; using this microcytofluorimeter it is possible to analyze up to 100 cells per 1 sec with high sensitivity [96]. This method has significant advantages compared with FCM-analysis, because sample preparation does not require cell centrifugation, which inevitably leads to their damage and partial loss. As in the case of cytological study, it is possible to monitor cell morphology visually [97].

2. The most reliable and accurate method for analysis of composition of a cell population is the cytological study of cells vitally labeled with compounds that are incorporated into DNA during the S-phase of the cell cycle. Using this approach Howard and Pelc [1] discovered the synthetic (S) phase of the cell cycle. These authors predetermined all subsequent development of studies of the cell cycle. They found that radioactive phosphorus (^{32}P) was incorporated into interphase chromosomes, and this incorporation was detected only during a certain time interval. This observation revealed that duplication of genetic material of chromosomes occurred during interphase, not prophase as it was thought earlier on the basis of observation of chromatid movement. However, even now, fifty years after this discovery, the authors of some (especially embryologic) reviews still indicate that before mitosis (or the first meiotic division) cells are diploid and not tetraploid as they actually are. Later ^{32}P labeled precursors incorporated into DNA were replaced by more specific markers such as [^{3}H]thymidine. Use of radioautography (autoradiography) revealed all principal stages of the cell cycle [9, 98]. Now, the development of immunocytochemical methods has resulted in substitution of [^{3}H]thymidine as the marker for non-radioactive markers such as BrdU.

After cell fixation, BrdU incorporated into DNA is stained by specific monoclonal antibodies [99]. Thus, after pulsed labeling with 20-40 μM BrdU (for 15-30 min) the proportion of cells in S-phase is recognized. The proportion of cells in G_0-phase is evaluated by the index of non-labeled cells after their incubation in medium with BrdU during a time interval reliably exceeding the duration of the cell cycle. The proportion of cells in mitosis is directly calculated by direct observation using a phase-contrast microscope or after DNA staining with DAPI or any other dye staining chromosomes.

The proportion of cells in G_1- and G_2-phases of the cell cycle in asynchronous populations can be determined using Quastler equations [24]. According to these equations, the ratio between the proportion of cells in any phase of the cell cycle and duration of this phase is a constant value. However, after treatments with synchronizers, such proportional ratios change and the Quastler equations become inapplicable. In such case, it is necessary to study the dynamics of changes in the proportion of cells in mitosis and S-phase starting from the set point when cells in G_1- or in G_2-phase were absent from the cell population. For example, during cell synchronization with aphidicolin, hydroxyurea, or thymidine cells are accumulated in S-phase or at the G_1/S boundary. Simultaneously, all cells being at G_2-phase and mitosis at the beginning of the treatment passed these stages. Thus, at the moment of washing of synchronizer cells in G_2-phase and mitosis are absent in the cell population and the proportion of cells in G_1-phase can be calculated using the proportion of cells in S- and G_0-phases. Change in proportion of cells in various phases of the cell cycle is calculated using data on dynamics of changes of the cell proportion in S-phase and mitosis.

Since the majority of cells were at the G_1/S boundary at the moment of aphidicolin washout, this means that after a time interval roughly equivalent to the duration of S-phase these cells will enter G_2-phase. It should be noted that under these conditions S-phase is somewhat shorter than in normal asynchronous cell culture [44, 45]. It is possible that during synchronization cells accumulate some factors promoting accelerated DNA replication.

Since an increase in cell proportion in G_2-phase occurs due to transition of cells from S-phase and a decrease in cell proportion in G_2-phase occurs due to their entry into mitosis, the formula for calculation of cell proportion in G_2-phase will be as follows: $G_2(n) = G_2(n - 1) + [S(n - 1) - S(n)] - M(n)$. Since mitosis lasts ~1 h, study of time course of changes of cell proportion in G_2-phase requires cell fixation hourly after the beginning of aphidicolin washout. In the formula above given the point $(n - 1)$ corresponds to the fixation point 1 h before the calculated one. Since the proportion of cells in G_1-phase increases due to cells that passed mitosis during the previous hour, the formula for calculation of cell proportion in G_1-phase will be: $G_1(n) = G_1(0) + 2\,M(n - 1)$.

For cell population synchronized in mitosis by combined treatment with nocodazole and ALLN (after synchronization by aphidicolin) the composition of the cell population is calculated as follows: since cells synchronized earlier by aphidicolin are blocked in mitosis at G_1/S boundary, the cells entering S-phase under these conditions represent the asynchronous part of this population. So, Quastler equations are applicable for calculation of cell proportion in G_2-phase [24]. In other words, in accordance with duration of these phases the portion of cells at G_2-phase will be proportionally less than the portion of cells in S-phase. The proportion of mitotic fraction cells in G_1-phase may be calculated by deducing the proportion of cells in known phases.

The following method can be used for calculation of composition of cell population in a fraction with maximal content of cells in G_1-phase; this fraction is obtained by incubating cells in complete medium for 10 h after washout of a mixture from nocodazole–ALLN. During this period, synchronized cells enter G_1-phase from mitosis. However, this time interval is not sufficient for reaching S-phase. Cells remaining at the end of this interval in mitosis and G_2-phase represent the asynchronous part of the cell population. So, it is possible to calculate accurately the proportion of cells in G_2-phase using the proportion of cells in mitosis: in accordance with G_2-phase duration exceeding mitosis duration their proportion will be higher than that of mitotic cells.

Appendix 3 provides the composition of synchronized XL2 cell fractions calculated by the rules that have been considered in this chapter.

4. STUDY OF COMPARATIVE PROTEIN EXPRESSION IN THE CELL CYCLE

Quantitative analysis of protein expression in the cell cycle by immunoblotting requires the use of an equal number of cells in all samples. For calculation of suspension culture cells, various counting chambers (Goryaev's or its analogs) are used. The number of cells growing on the substrate may be calculated by average cell density (e.g., number of cells is calculated in 10-20 vision fields of a microscope with measured object-micron diameter) using the area of the culture flask for calculation of total number of cells.

However, even careful calculation of cell number cannot guarantee absence of errors originating in the process of protein isolation. So, it is better to compare relative amount of proteins in cell fractions at various cell cycle phases versus cell cycle dynamics in content of known proteins such as β-tubulin. Tubulin concentration increases proportionally to the cell mass; it does not differ between interphase and non-dividing cells [12, 100]. Measuring relative amount of protein versus β-tubulin, it is possible to determine deviation from proportional increase in the protein under study in the cell cycle. Actin and cyclin-dependent kinase 2 (cdc2) are other proteins used as standards [45].

Using data obtained from analysis of cells with high synchronization level, it is possible to find changes in expression of various proteins during the cell cycle. However, using fractions with lower synchronization such analysis of system with four variables is rather complicated. It is impossible to determine whether a protein detected in the fraction of synchronized cells is typical for this

particular phase of the cell cycle or it is attributed to contamination of this fraction with cells in other phases. It should be also noted that the ratio of cells in various phases of the cell cycle varies during every synchronization, and so it is necessary to standardize results of analysis for correct comparison of results.

In my previous paper [101], I described a method of mathematical treatment of data that determines protein expression level in hypothetical cell populations that consist of cells in one cell cycle phase. In real cell culture, it is impossible to obtain such fractions; the proportion of synchronized cells in G_1- and S-phase never exceeds 80-95%; for G_2-phase or mitosis, this value is even lower (60-75%). My method, which can solve this problem, consists in use of these four fractions of partially synchronized cells for determination of expression of studied proteins. Solving the system of equations based on data of protein content and proportion of cells in each phase we can get the calculated level of the protein in fractions with 100% cell content in each phase [101]. In that study the calculation was based on analysis of highly synchronized cell fractions. However, this method is also applicable for analysis of dynamics of protein content in partially synchronized cell populations provided that the content of cells in various cell cycle phases has been accurately determined. Using this approach, it is possible to reduce the time of cell synchronization and to minimize the toxic effect of the synchronizing treatment and imbalanced cell growth.

In the Appendix to chapter 4, I demonstrate calculation of expressions of several proteins based on the results obtained in studies of real synchronized cell fractions with data calculated for hypothetical "pure" fractions.

In planning experiments on cell synchronization, it is ultimately important to select a correct set of synchronizing treatments. Synchronization method depends on the goals of the study. However, a study of any process on synchronized cells requires lack of direct effect of the synchronizer arresting cells at a certain stage of cell cycle on the investigated process.

Selective blockade of some processes of the cell cycle may result in so-called imbalanced cell growth [22, 54]. This phenomenon is related to the fact that synchronization of various processes occurs only at certain points of the cell cycle. If some processes are artificially inhibited, others may remain. For example, after the restriction point at G_1-phase cell growth does not depend on DNA replication. After long-term synchronization (e.g., with thymidine) cells may reach sizes typical for cells at G_2-phase, but the DNA content will correspond to cells at G_1- or S-phase [22]. It should be also noted that synchronization-induced protein accumulation in cells may shorten subsequent stages of the cell cycle after removal of the synchronizer [44, 45]. All these points should be taken into consideration during interpretation of results obtained using synchronized cells.

On the other hand, many experiments employing synchronizers are often designed for uncoupling of various intracellular processes; such uncoupling may emphasize the interrelationship of such processes. In these experiments, results obtained during comparison of effects of various synchronizing treatments are used for reason-causal connections between various processes of the cell cycle.

Many compounds used for cell synchronization are widely employed in clinical medicine for chemotherapy of malignant tumors. This is based on selective treatment of highly proliferative malignant cells. Analysis of new anticancer drugs requires detailed study of this effect on cells (including malignant ones) *in vitro*; this allows investigating cytotoxicity of the compounds used in dependence of stage of the cell cycle. The search for optimal combination of such preparations and/or combination with other (chemo)therapeutic treatments also implies pilot study on cell cultures. So, the development of synchronization methods has not only basic but also clinical importance for design of the most effective and selective methods for treatment of malignant tumors.

Studies of cell cycle regulation often employ cell cultures from the clawed frog *Xenopus laevis*. In contrast to mammalian cells, the use of *Xenopus* cell cultures allows investigation in parallel of proteins during oogenesis and early stages of embryogenesis by means of *in vitro* stimulated oocytes. Using this experimental model it is possible to study the full cell cycle of cultivated cells with reduced (without G_1- and G_2-phases) cell cycle at early cleavage divisions. It is also important that cultivation temperature (25°C) optimal for these cells is also comfortable for the researcher. The latter significantly facilitates all manipulations with cells, particularly vital observations. All these advantages have made XL2 culture with well-characterized cell cycle and synchronization conditions the most popular and convenient research object.

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Appendix to Chapter 1

1.1. Determination of cell cycle duration in XL2 cells. XL2 cells were seeded onto glasses, and after 48 h of cultivation their density was 1463 cells/mm^2. After 72 h of experiment (120 h after cell seeding), cell density reached 7161 cells/mm^2. Thus, the number of cells increased by 4.89 times and average duplication time was 31.44 h ($\log_2$ 4.89). During this experiment, the proportion of cells in G_0 was 0.179. Hence, average cell cycle duration was T = 31.44/$\log_2$ [(2 – 0.179)/(1 – 0.179)] = 31.44/1.16 = 27.1 h (27 h 6 min).

1.2. Determination of S-phase duration and the whole cycle of XL2 cells in one experiment. In one experiment, the proportion of XL2 cells pulse-labeled with BrdU was 0.300, whereas the proportion of cells in G_0 was 0.215. Thus, the proportion of cells in S phase was 0.3/(1 – 0.215) = 0.382. Cells incubated in the medium containing BrdU were fixed after 3, 6, 9, 12, 15, 18, 21, 24, 28, and 33 h, and at each point, labeling proportion was determined for 5000 cells. The bend-point on the plot of time dependence of number of labeled cells was at 17.4 h. Consequently, S/T = 0.382 and "T – S" = 17.4 h; hence, T = 17.4/(1 – 0.382) = 28.2 h (28 h 12 min), S = 0.382·28.2 = 10.77 h (10 h 46 min).

Using data of these experiments, S-phase duration can also be calculated using another formula [98]: $S = N_0 t/(N_t - N_0)$, where N_0 and N_t are proportions of cell labeled at the beginning of experiment and after t-time interval, respectively. This formula does not take into consideration the proportion of cells at G_0, because the coefficient of proportion of dividing cells is the same for numerator and denominator. In this experiment after incubation for 9 h with BrdU the proportion of labeled XL2 cells was 0.563, and so S-phase duration was calculated as follows: S = 0.304 · 9/(0.563 – 0.304) = 10.56 h (10 h 34 min). Using this value it is possible to calculate duration of the whole cycle: T = 10.56/0.382 = 27.64 h (27 h 38 min).

Thus, duration of the whole cycle, calculated using three different methods was 27.1 (see Appendix 1.1), 28.2, and 27.64 h or on average 27.65 h (27 h 39 min). Average S-phase duration calculated by two different methods was 10.77 and 10.56 h or on average 10.67 h (10 h 40 min).

1.3. Determination of G_2-phase duration in XL2 cells. For this cell line minimal, average, and maximal values of G_2-phase duration calculated by the method of "labeled mitoses" were 1 h 38 min, 2 h 4 min, and 3 h 42 min, respectively. Average G_2-phase duration calculated by the method of "labeled prophases" was 2 h 27 min (2.45 h).

1.4. Determination of mitosis duration in XL2 cells. Vital observation revealed that total mitosis duration in XL2 cells was 54.1 min (0.9 h). Corresponding values of prophase, prometaphase, metaphase, anaphase, and telophase were 5.7, 6.7, 19.4, 5.7, and 16.6 min, respectively [44].

1.5. Determination of G_1-phase duration in XL2 cells. For XL2 cells, G_1-phase duration was determined by subtracting total duration of all other stages of the cell cycle from total cell cycle duration. So, G_1-duration was $G_1 = T - S - M - G_2 = 27.65 - 10.56 - 0.90 - 2.1 = 14.09$ h.

Appendix to Chapter 2

Synchronization of XL2 cells. For presynchronization (reduction of cell proportion in S-phase before cell synchronization with aphidicolin) several methods were tested: cell cooling to 9°C, cell incubation in medium without serum, and preliminary additional incubation with aphidicolin.

Cell cooling (for 180 h) significantly reduced the proportion of cells in S-phase (from 38.2 to 9.4%) but increased the proportion of cells in G_0 (38.6% 31 h after cell transfer to normal cultivation conditions at 25°C). This indicates that cell cooling cannot be used for presynchronization because cell entry into the cell cycle after their transfer to normal conditions was slow and asynchronous.

Lack of serum in cultivation medium resulted in progressive cell exit from the cycle and the proportion of cells in S-phase was reduced in an undulating manner. The latter can be attributed to different sensitivity to such treatment of cells at various phases of the cell cycle. It was found that the first minimum of cell proportion in S-phase (~10%) was observed only 24 h after their transfer into medium without serum. Thus, incubation for 24 h in the serum-free medium resulted in partial synchronization of cells in G_1-phase (or reversible exit of some of them to G_0-phase).

Presynchronization of cells with aphidicolin for 30 h followed by subsequent washout for 15 h in the complete medium before synchronization by the second aphidicolin treatment gave the same result.

Using two principal criteria, the minimal proportion of cells in S-phase and rapid cell return into cycle, cell incubation for 24 h in the serum-free medium was the best presynchronization method for XL2 cells. Double synchronization with aphidicolin also gave satisfactory result, but this approach was time consuming.

There is one important problem of when cell incubation in serum-free medium should be started: right after seeding, in logarithmic phase, or in monolayer? After reseeding, the proportion of dividing cells increases and reaches equilibrium at logarithmic phase of growth. At the stage of confluent monolayer the proportion of cells in G_0 increases. Thus, each cell reseeding is accompanied by cyclic changes in the ratio of dividing and non-dividing cells. The stage of confluent monolayer is the optimal time for the beginning of incubation in serum-free medium after cell reseeding [44]. In that

study cells were initially incubated in complete medium for 3 h; this was required for normal cell attachment and also for total inactivation by serum of versene used for reseeding.

The following protocol of XL2 cell synchronization was developed for preparation of cell populations enriched with cells in G_1-, S-, and G_2-phases and in mitosis.

Cells grown up to confluent monolayer were detached with a mixture of trypsin and versene, shaken off the plates, resuspended in full medium, and simultaneously seeded onto plastic plates and cover glasses at concentration ~600 cells/mm^2. Three hours later, when cells were attached to the substrate, the medium was washed out three times with warm PBS (25°C) and replaced with serum-free medium. After 24 h, cells were transferred into full medium containing 40 μM aphidicolin. Cells were incubated for 30 h, and then aphidicolin was washed out and cells were incubated in full medium. After that period, aphidicolin was washed out with warm PBS (25°C) and cells were then incubated in full medium. Populations of cells with maximal proportion of cells in S-phase and G_2-phase were obtained 2 and 9-11 h after aphidicolin washout. In the latter case, purity of this fraction was microscopically monitored to prevent possible contamination of this fraction with mitotic cells. (Cells were immediately used for biochemical study right after detection of the first mitosis.)

Cells cultivated on cover glasses were fixed at all principal points of the experiment and every hour after aphidicolin washout. Percent of cell in S-phase and mitotic index (1000-5000 cells per point) were calculated after immunofluorescence staining with antibodies against BrdU.

Duration of S- and G_2-phases is rather variable and aphidicolin washout causes cells to become rather asynchronous, and preparation of a cell population with high proportion of mitotic cells requires additional treatments. Eight hours after the beginning of aphidicolin washout (i.e., before the first synchronized cell entry into mitosis) nocodazole or ALLN were added into the cultivation medium at final concentration of 0.5 or 40 μg/ml, respectively. Both substances cause cell arrest in mitosis, but the mechanisms of this effect differ (see chapter 2). The best method was two stage synchronization by the following scheme: initial cell incubation in the presence of nocodazole (0.5 μg/ml) for 3 h and then subsequent incubation in the presence of both nocodazole (0.5 μg/ml) and ALLN (40 μg/ml) for 4 h. Such complex treatment prevented cell polyploidy typical for synchronization with nocodazole and totally blocked mitosis (which was impossible during the use of ALLN alone).

After nocodazole and ALLN washout in fresh medium for 20 min (4 times for 5 min each), a cell fraction with predominance of mitotic cells (70% of proliferative pool) was obtained.

The population with maximal proportion of cells in G_1-phase was obtained by incubating cells in full medium for 10 h after washout of nocodazole and ALLN mixture [45, 101].

Appendix to Chapter 3

Calculation of cell proportions of various cell cycle phases in fractions of synchronized XL2 cells. Use of the synchronization protocol (see above) yielded four populations of synchronized cells: "max G_1" (85.6% G_1; 11.9% S; 1.9% G_2; 0.7% M); "max S" (7.7% G_1; 92.3% S; 0% G_2; 0% M); "max G_2" (0% G_1; 18.2% S; 77.2% G_2; 4.6% M); "max M" (12.1% G_1; 13.5% S; 3.4% G_2; 71.7% M) (see [101] for details).

Appendix to Chapter 4

Study of comparative expression of β-tubulin, DNA topoisomerase 2α, pEg2, XlEg5, pEg7 (XCAP-D2), and XCAP-E at various phases of the cell cycle using synchronized XL2 cells. Using quantitative immunoblotting we have been investigating comparative content of six various proteins: β-tubulin, DNA topoisomerase 2α, kinase of Aurora A family (pEg2), kinesin-like protein motor XlEg5 and proteins of condensing complex pEg7 (XCAP-D2) and XCAP-E [101, 102]. Levels of other cell proteins in cell cycle were normalized versus β-tubulin level in a corresponding fraction. The ratio in fraction with maximal content of cells in G_1-phase ("max G_1") was defined as the unit. The relative level of condensing complex protein insignificantly changes over the whole cycle, whereas levels of DNA topoisomerase 2α, XlEg5, and especially pEg2 tended to increase from G_1 to mitosis. Comparison of the "mitotic" fraction of mixed composition with "max G_1" fraction revealed 15.44-fold difference in content pEg2 protein [102]. However, it was clear that under such differences in protein content even small contamination of "max G_1" cell fraction with cells at other cell cycle phases could significantly influence the final result. This was directly demonstrated after recalculation of comparative content of this protein for hypothetically "pure" fractions. Actual difference in pEg2 levels between mitosis and G_1 was 40.08 [101]. These data corresponded better to results of immunocytochemical analysis of this centrosome protein in interphase and mitosis [103-105]. The level of pEg2 changed cyclically during the cell cycle: this protein is rapidly degraded within 1 h after mitosis [104] and its accumulation in cells begins at the end of S-phase of the cell cycle [105].

For proteins characterized by relatively stable level during the cell cycle, differences during analysis of mixed and pure fractions were rather small (e.g., XCAP-E and pEg7 proteins of the condensin complex) [101].

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