Reversible Accumulation of Plant Suspension Cell Cultures in G₁ Phase and Subsequent Synchronous Traverse of the Cell Cycle

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ABSTRACT

The induction of DNA synthesis in Datura innoxia Mill. cell cultures was determined by flow cytometry. A large fraction of the total population of cells traversed the cell cycle in synchrony when exposed to fresh medium. One hour after transfer to fresh medium, 37% of the cells were found in the process of DNA synthesis. After 24 hours of culture, 66% of the cells had accumulated in G₂ phase, and underwent cell division simultaneously. Only 10% of the cells remained in G₀ or G₁. Transfer of cells into a medium, 80% (v/v) of which was conditioned by a sister culture for 2 days, was adequate to inhibit this simultaneous traverse of the cell cycle. A large proportion of dividing cells could be arrested at the G₀ + G₁/S boundary by exposure to 10 millimolar hydroxyurea (HU) for 12 to 24 hours. Inhibition of DNA synthesis by HU was reversible, and when resuspended into fresh culture medium synchronized cells resumed the cell cycle. Consequently, a large fraction of the cell population could be obtained in the G₀ phase. However, reversal of G₁ arrested cells was not complete and a fraction of cells did not initiate DNA synthesis. Seventy-four percent of the cells simultaneously reached 4C DNA content whereas the frequency of cells which remained in G₀ + G₁ phase was approximately 17%. Incorporation of radioactive precursors into DNA and proteins identified a population of nondividing cells which represents the fraction of cells in G₀. The frequency of cells entering G₁ was 11% at each generation. Our results indicate that almost 100% of the population of dividing cells synchronously traversed the cell cycle following suspension in fresh medium.

Regulation of genetic expression in plants is under the control of interacting processes and involves the biosynthesis of several different classes of molecules. The sequence of these reactions is not well known or understood. These regulatory mechanisms are difficult to study using entire plants because of the cell heterogeneity within different plant organs. However, the maintenance of uniform plant cells in suspension cultures will allow these studies. Such cultures are particularly relevant when large populations of cells are obtained in the same, stable biochemical state. Therefore, efforts are required to induce synchrony of proliferating cells in a specific stage of the cell cycle.

Understanding the cell cycle and factors which control it, should provide information about control of these processes in whole plants. Data generated elsewhere with cultivated plant cells in suspension indicate the repeated passage of the cells through the cell cycle (5, 13, 16). While cytologic observations using microscopy or biochemical measurements provide static analysis of the cell cycle, these methods do not allow continuous monitoring of the cell cycle. As a consequence, such analyses limit the conclusions which can be drawn concerning the effect of different environmental perturbations upon the cycle. They also do not permit studies of the responses to culture modifications beyond one cell cycle. Nevertheless, it has been shown that cell cycle parameters can be accurately quantified for both plant and mammalian cells using image analysis or flow cytometry to measure the nuclear DNA content of single cells within a given population after staining with DNA-specific fluorochrome (3, 22, 25, 29). Fluorescence intensities are correlated with the position of the corresponding cell within the cell cycle (2, 11). Moreover, the sorting capabilities of flow cytometers make possible the isolation of large samples of cells. Such populations can be used to provide an analysis of biochemical properties specific to cells within a specific stage of the cell cycle.

Reported here are the results of investigations carried out to determine the capacity of a population of cultured plant cells in suspension to undergo DNA synthesis after synchronization in the first phase (G₁) of the cell cycle. Accumulation of Datura innoxia cultured cells in G₁ phase and/or early S phase was obtained by reversible suppression of DNA synthesis with HU. Cell cycle kinetics have been examined biochemically and using a single parameter flow microfluorometer (20, 30). In vitro cell proliferation can be stimulated or inhibited by chemical agents. However, it would be preferable to conduct studies of the biochemical processes that occur in the cells without addition of any synchronizing agent. These chemicals present potential stresses that could modify the results. Therefore, techniques have been developed with mammalian cells to induce synchronous traverse of the cell cycle by limiting the amount of an essential growth factor in the culture medium (27). In this paper, we have developed a

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† Abbreviations: HU, hydroxyurea; CV, coefficient of variation.
technique in which D. innoxia cells can be accumulated in a reversible state of G1. Large quantities of cells can be synchronized in S phase without using any chemical perturbing agent.

MATERIALS AND METHODS

Maintenance of Datura innoxia Cell Suspension Cultures

The Datura innoxia Mill. cell line was originally obtained from the laboratory of Dr. O. F. Gamborg (University of Saskatchewan, Saskatoon, Canada). Suspension cell cultures were grown in the dark in a modified Gamborg's 1BS medium (15) and maintained as already described (19).Suspensions were transferred as exponentially growing cultures by a fourfold dilution into fresh culture medium preheated to 30°C at 2 d intervals. Cell cultures were maintained as one-fifth volume in 250 mL baffled Delong flasks.

Preparation of Conditioned Medium

Two d after subculture, D. innoxia cells were removed from suspension by centrifugation at 300g for 2 min. The supernatant (conditioned medium) was passed through a 0.2 μm cellulose nitrate membrane using a 115 mL presterilized filter unit (Nalgene). The resultant sterile conditioned medium could be stored at room temperature for several weeks.

Synchronization of Cell Suspension Cultures

Two synchrony-induction techniques were examined. (a) Modification of the growth conditions of the culture by transfer of rapidly dividing, 2 d old cells into a solution containing 20% (v/v) new medium and 80% (v/v) conditioned medium. (b) Reversible arrest of DNA synthesis using HU (26). Rapidly dividing cell cultures were grown in different concentrations (1, 5, 10, 20, 100 mM) of HU (Sigma Chemical Co.). At 3 h intervals, aliquots of cells were removed from each culture, protoplasts were prepared and nuclei isolated for further determination of relative DNA content distributions. To release the cells from HU inhibition, the cells were centrifuged at 800g, washed twice, and resuspended into fresh medium.

Protoplast Isolation and Staining

Protoplasts were isolated from suspension cultures by mixing an equal volume of cell suspension with a solution containing 600 mM KCl, 2 mM NH₄NO₃, 3 mM CaCl₂, 1 mM KH₂PO₄, 1% (w/v) Cellulysin (Cal-Biochem), and 0.2% (w/v) Rhizyme HP150 (Rohm and Haas, Philadelphia, PA). The pH of the solution was adjusted to 5.5 with KOH, and sterilized by passage through a 0.2 μm nylon membrane (Corning) before use. Suspensions were incubated with agitation (150 rpm) at 30°C for 90 min. Protoplasts were separated from cells by passage of the digested material through a 30 μm nylon sieve. Protoplasts were washed twice in an ice-cold solution containing one part culture medium and one part of the above solution without enzyme. All steps following these washes were carried out at 4°C. Protoplasts were then either fixed or resuspended in the lysis medium for cell cycle analysis.

Fixation was accomplished by passing 3 mL of the above protoplast suspension through a 30 μm nylon sieve directly into 7 mL of ice-cold 95% ethanol. Samples were stored at 4°C for at least 1 h prior to further treatment. Nuclei were released by resuspending protoplasts in lysis buffer (5 mM Hepes [pH 8 with KOH], 50 mM KCl, 10 mM MgSO₄) (6). Triton X-100 (Sigma Chemical Co.) was added to a final concentration of 0.25% (w/v), and the suspension was allowed to set for 10 min at room temperature. Remaining protoplast membranes were then mechanically disrupted by three repeated passages of the suspension through a Pasteur pipet. Samples of either fixed or lysed protoplasts were stained with mithramycin (25 μg/mL, final concentration) and filtered through one layer of Miracloth (Calbiochem-Behring Corp.) before being subjected to flow cytometric analysis. Mithramycin was a generous gift from Pfizer, Inc. to the National Flow Cytometry Resource. Flow cytometric analysis proceeded following exposure to mithramycin for at least 30 min at 4°C.

Radioactive Labeling of the Cells

Exponentially growing D. innoxia cells were grown for one generation (24 h) in media containing radioactive protein and DNA precursors. Newly synthesized proteins were labeled by addition of 10 μCi/0.1 μg/mL of [3H]leucine; DNA was labeled by addition of 10 μCi/10 μg/mL [3H]thymidine. All radioactively labeled compounds were purchased from DuPont-New England Nuclear, Inc. HU (10 mM final concentration) was added to the cultures for 24 h. Cells were then released from HU inhibition by washing and suspension in a culture medium containing 0.05% (v/v) colchicine (Sigma Chemical Co.). Further incubation was for 24 h at 30°C. Radioactively labeled and synchronized cells were then converted to protoplasts as described above. Labeled protoplasts were fixed by suspension in a solution containing 25% glacial acetic acid and 75% methanol (v/v) for 24 h.

Flow Cytometric Analysis

The flow analysis of alcohol-fixed protoplasts and isolated nuclei was carried out using the flow microfluorometer (FMF) of the National Flow Cytometry Resource (Los Alamos, NM). The beam power of the argon ion laser (Coherent Innova, 90) was 350 mW at 457.9 nm. The yellow fluorescence of the DNA-mithramycin complex was collected through a 512 nm long wave pass filter.

Cell cycle profiles were analyzed utilizing computer programs supplied by the National Flow Cytometry Resource (11).

Sorting of Radioactively Labeled Protoplasts

Radioactively labeled protoplasts were stained with mithramycin and analyzed using flow cytometry. Sorting 'windows' were set to sort fixed protoplasts according to their relative DNA content as determined by fluorescence intensity. Protoplasts in the G₀ + G₁ phase and those in the G₂ + M phase of the cell cycle were collected separately. Sorted protoplasts were concentrated by subsequent centrifugation at
Cetic acid slide applied to a the nucleus with a 10% trichloroacetic acid solution. Suspensions were then placed onto microscope slides.

**Autoradiography Analysis**

Samples of sorted radioactively labeled protoplasts were applied to microscope slides and squashed by application of pressure to a cover slip. Cover slips were removed by freezing the slide on dry ice and dried slides were immersed in Kodak nuclear track emulsion (Kodak Co., Rochester, NY) in total darkness. Emulsion was allowed to expose for a minimum of 4 d. Slides were then developed in Kodak Microdol-X, washed in running water, then fixed in Kodak Rapid Fixer. Dried slides were stained with a solution containing 50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 30 mM MgCl₂, and 100 µg/mL mithramycin for 5 min. Slides were then observed using an epifluorescent microscope (Carl Zeiss, West Germany), fitted with a combination of 450 to 490/FT510/LP520 filters and both 40× and 100× neofluor lenses. In each single protoplast the nucleus was identified according to the fluorescence of the DNA-mithramycin complex. The protoplasts were then scored for the presence or absence of silver grains over the entire cellular content or over only the nucleus.

**RESULTS**

**Cell Cycle Parameter Analysis**

Figure 1 represents the DNA profile of a 2 d old asynchronous population of cultured *D. innoxia* cells. Figure 1a corresponds to the DNA distribution obtained from a suspension of isolated nuclei. The left peak has been arbitrarily positioned at channel 80. This peak (CV: 3.6%) corresponds to cells in the G₀ and G₁ phases of the cell cycle with a 2C DNA content (35.1% of the total cells). The nuclei in S phase are plotted from channel 80 to channel 160. The computer program indicated that 15.2% of the nuclei comes from cells in S phase. The peak at channel 160 corresponds to cells in G₂ with a 4C DNA content (frequency: 49.7%), but also includes cells in late phases of nuclear DNA replication.

Figure 1b illustrates the DNA profile of chemically fixed protoplasts isolated from the same cell population. Computer analysis of the cell cycle profiles indicates that 29.0% of the cells are in G₀ or G₁ phase; the CV of the corresponding peak is 7.1%. Twenty-one and six-tenths percent of the cells appear to be synthesizing DNA (S), and 49.4% are found in the G₂ + M phases. The peak corresponding to the cells with a 4C DNA content is usually referred to as 'G₂ + M.' This nomenclature indicates that using single parameter flow cytometry both the cells in the G₂ phase and those in mitosis is classified in a composite peak. When analyzing isolated nuclei (Fig. 1a, Fig. 2, Fig. 3), no mitotic cells would be observed if one assumes that chromosomes are released individually into suspension because of the lack of the nuclear membrane. Therefore, in this paper, the peak corresponding to the nuclei with a 4C DNA content has been referred to as 'G₂' unless cell cycle analysis was performed using fixed protoplasts (Fig. 1b) or after colchicine treatment (Table 1).

**Nondividing Cells (G₀)**

Table 1 reports the cell cycle parameters of cells cultured in the presence of radioactive DNA and protein precursors, followed by sequential 24 h treatments with HU and colchicine. Colchicine was added to prevent cells from completing mitosis, and returning from the G₂ phase to the G₁ phase. Consequently, only nondividing cells (G₀) were left in the G₀ + G₁ channels. Only 15.5% of the protoplasts remained in the G₀ + G₁ phase. Among these protoplasts, only 11% synthesized DNA as determined by the incorporation of [³H] thymidine into nuclei, whereas more than 92% of the protoplasts contained labeled proteins. The frequency of protoplasts containing labeled proteins was identical for both G₀ + G₁ and G₂ + M channels. The percentage of protoplasts synthesizing both DNA and proteins is approximately 90% among the sorted G₂-phase protoplasts. These results indicate that a proportion of living cells with a 2C DNA content do not synthesize DNA. However, a large majority of both dividing
and nondividing cells are synthesizing proteins. The population of quiescent cells which were not progressing from 2C DNA content through S to G2 phase and mitosis corresponds to the G0 stage. In order to maintain a constant percentage of cells in G0, some cells from dividing populations must enter G0 each generation. The 11% of cells from the G0 + G1 population which had actively synthesized DNA within the previous 24 h might represent such cells.

**Modification of Growth Conditions**

Figure 2 demonstrates the change in distribution of cells throughout the cycle in response to transfer into fresh medium. Kinetic events associated with cell activation were measured after suspension of a 2-d old culture into fresh medium. Aliquots of cells were collected from the culture at 2 h intervals. Protoplasts were obtained and nuclei isolated as for Figure 1a. Cell cycle analysis revealed that at the time of subculture, only a small percentage of nuclei (14.8%) could be assigned to the S phase. At t = 0, 35.1% were found in the G0 + G1 phase, and 50% were in G2. One h following suspension in fresh culture medium, a wave of cells simultaneously began DNA synthesis (arrow). Frequencies of cells in each phase of the cell cycle determined at regular intervals throughout 48 h of culture demonstrate that suspension of 2-d old cells in fresh medium is sufficient to promote DNA synthesis. After 14 h in fresh culture medium, 10% of the cells were found in G0 + G1 phase, 21.7% in S, and 68.3% in G2. Later, cells simultaneously underwent cell division and the population of G2 cells returned to the G0 + G1 phase. Increased background values are the result of contamination of the suspension with chromatin structures from the mitotic nuclei. In the absence of nuclear membrane, chromosomes in different stages of condensation (prophase through telophase) are released as isolated chromosomes or chromosome clumps. The corresponding fluorescence intensities form a peak at channel numbers below channel 80 (Fig. 2, 36 h).

Flow cytometric analysis of cell cycles revealed the presence of two distinct populations within the culture (Fig. 2); one containing twice the DNA content of the other. Therefore, the channels corresponding to the diploid cells in G2 phase include some tetraploid cells in the G0 + G1 phase. The D. innoxia cell line which were used in these studies exhibited a low frequency of tetraploid cells. However, when suspended in fresh medium, tetraploid cells also initiate DNA synthesis as shown by the peak to the right of the G2 peak diploid cells (channel numbers greater than 160). It has not been possible to determine whether or not the total population of tetraploid cells is constant and established in the suspension cultures, or if some of those cells result from an escape of the mitosis at each generation of the diploid cells.

**Reversibly Arresting Cells in G1 Using Hydroxyurea**

Figure 3 shows cell cycle distributions at 3 h intervals when exponentially growing cells were suspended in a conditioned:fresh medium (8:2, v/v). Cells were suspended in the same medium plus HU to induce synchrony. In order to obtain large quantities of cells at the G1/S boundary, cells were grown for 24 h in media containing 1, 5, 10, 20, or 100 mM HU. The efficiency of arresting cells at the G0 + G1/S boundary is dependent upon both the concentration of HU added and the time of treatment. In Figure 3, data collected from cultures exposed to 10 mM are shown.

A final concentration of 1 mM HU did not promote a significant increase of the cell population in any segment of the cell cycle. Arrest at the G0 + G1/S boundary was maximized in 9 h of exposure to 5 mM HU. Cells with a 4C DNA content increased rapidly thereafter. Following 12 h of culture, cell divisions occurred and chromatin structures from the mitotic nuclei resulted in a broad histogram at channel numbers lower than 80.

In the presence of 10 mM HU the population of cells which
initiate genome replication moves from the $G_0 + G_1$ phase to the $G_2$ phase much slower than the controls. A maximum of cells arrested at the $G_0 + G_1/S$ boundary was obtained after 12 h of treatment using 10 mM HU (Fig. 4). The frequency of cells in the $G_2$ phase decreased until 12 h, then remained stable since no more cells underwent cell division. After 12 h, the cells progressed through the S phase (Fig. 3, Fig. 4). Therefore, after 24 h a large proportion of the cells were in $G_1$, $S$, and late $S$ phases. However, no increase in the number of $G_2$ cells was observed. By comparison, the cell cycle distributions obtained from cells growing in the absence of HU are very consistent and reproducible throughout the entire experiment. The average percentage of cells in $G_0 + G_1$ was 48.4 ± 5%, cells in $S$ represented 19.1 ± 8%, and cells in $G_2$ represented 32.5 ± 5% of the entire population (Fig. 3, control).

Cells exposed to 20 mM HU responded similar to cells growing in 10 mM HU. However, cells in late S phase were observed after 15 h of culture instead of 18 h. When using 100 mM HU, cell cycle analysis was reliable to 12 h, a maximum of cells in $G_2$ appeared from 9 to 12 h. After 12 h of culture, the cell cycle parameters and segments could not be determined with confidence, probably due to a loss of culture viability.

**Traverse of Synchronized Cells Through the Cycle**

The preceding results indicate that a large number of cells can be synchronized at the $G_0 + G_1/S$ phase boundary by combining growth in conditioned medium and the use of HU. Such a cell population is suitable for studying the efficiency of simultaneous DNA synthesis after release from HU inhibition. Once washed and suspended in fresh medium, cells that had accumulated in $G_1$ and early $S$ phases completed DNA synthesis. Consequently, a large number of cells accumulated in $G_2$, a fraction of which simultaneously begin mitosis, and return to $G_1$. Figure 3 shows an increase of events per channel in channels 0 to 80 (arrows) 3 h after washing the synchronized cells. This peak results from the dispersion of the chromosomes and is larger 14 h after washing the HU-treated cells, indicating that the number of cells undergoing mitotic division increased significantly. At this time a large percentage of cells are in $G_2$ (73.9%), whereas only 17.2% remain in the $G_0 + G_1$, and 8.8% in $S$.

**DISCUSSION**

If flow cytometry is to be applied to the study of the cell cycle, samples, either isolated nuclei or fixed protoplasts, should correspond to a true representation of the cultured cells themselves. Cell cycle analysis obtained from both iso-

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**Table I. Frequency of Cells Incorporating DNA and Protein Radioactive Precursors after Successive Incubations into Hydroxyurea and Colchicine**

<table>
<thead>
<tr>
<th></th>
<th>Cell Cycle Distribution*</th>
<th>[3H]thymidine</th>
<th>[3H]leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_0 + G_1$</td>
<td>15.5</td>
<td>10.9</td>
<td>92.4</td>
</tr>
<tr>
<td>$G_2 + M$</td>
<td>62.9</td>
<td>89.4</td>
<td>93.5</td>
</tr>
</tbody>
</table>

* Results given in %.

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The discrimination program close the cells of stages station fluorescence intensities staining (data monitored significantly better and a means provide an analysis (CV: 3.6%, Fig. 1a) and a means have been extensively described by others (5, 16). Those methods lead to the determination of the growth rate by cell counting after protoplast production, by measurement of turbidity and packed volume of cells, and by the radioactive labeling of the cells. The usefulness of flow analysis to study cell cycle distributions of plant protoplasts has already been demonstrated for several other plant species (7, 18, 24), and have been reviewed (4, 14). Single parameter flow cytometry has the advantage of simplicity and reliability, making series of investigations possible over extended periods of time. Biparametric analysis using two sources of fluorescence may provide more information. However, this kind of flow cytometry is more expensive and the preparation of the samples is significantly more complex. High resolution cell cycle studies could be obtained by combining DNA with RNA measurements (9, 28). Moreover, immunocytochemical techniques have been combined with flow cytometry to allow the distinction between replicating and nonreplicating cells (10, 17). For example, in mammalian research and more recently in plant sciences, dual parameter flow cytometry has been successfully applied to cell cycle analysis after incorporation of 5-bromo-2'-deoxyuridine (BrdU) into the genome during DNA replication (12, 21, 23).

Modification of growth medium conditions was effective for accumulating plant cells from different plant species at a specific point in the cell cycle. Synchronization is usually related to the addition or removal of a particular growth regulator (8). The reduction in the number of cell divisions in D. innoxia cell cultures suspended in 2-d old conditioned medium can result from either the consumption of one crucial growth factor or the generation of an inhibitor synthesized by the cells. Addition of fresh media results in further cell division suggesting that the former is most likely.

Suspension cell cultures of D. innoxia are reversibly arrested at the G_{1}/S boundary of the cell cycle after inhibition of the DNA synthesis with 10 mM HU (Fig. 3). It has been shown in mammalian cultures that HU reduces the rate of cell progression from G_{1} to S, preventing initiation of DNA replication (27). D. innoxia cells were also suspended in a 80% conditioned medium containing HU to avoid the fresh medium-dependent activation of the DNA synthesis. Consequently exposure time to HU could be minimized, avoiding...
cytotoxicity effects of HU upon non-S phase cells (27). Synchronous cell cycle traverse of the D. innoxia cells both after HU treatment and after suspension in fresh culture medium, involved most of the population of dividing cells. However, cell cycle distributions (Figs. 2 and 3) revealed some cells remaining in the G₀ + G₁ phase (typically 10–17%). Studies incorporating radiolabeled precursors of DNA and proteins (Table I) demonstrated the presence of a fraction of cultured cells (frequency about 11%) which are not actively dividing but are still metabolically active.

Preliminary data demonstrates a maintenance of a synchronous population of dividing cells 2 d after release from HU inhibition (our manuscript in preparation). Therefore, such a cell suspension should be useful for the study of periodic biochemical events or synthesis of macromolecules and regulatory processes related to specific stages of the cell cycle. The ability of D. innoxia cell suspensions to respond to perturbing agents of the cycle, and the ability to synchronize cultures with fresh culture medium, with no need of chemical agent, makes this cell line a good model for further investigations. Such a cell culture should provide information regarding cellular biochemistry and molecular synthesis related to the cell regulation and should allow the development of a better understanding of mitotic processes in plant cells.

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LITERATURE CITED


