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Immunocytochemical Study of Cell Cycle Control by Cytokinin in Cultured Soybean Cells

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Abstract. An immunocytochemical method was used to determine the proportion of cells in the DNA synthesis (S phase) of the mitotic cycle in suspension cultures of soybean (*Glycine max* (L.) Merr. cv. Acme) callus of cotyledonary origin, the stably cytokinin-dependent tissue used in the cytokinin bioassay devised by Carlos O. Miller. A standard cell synchronization protocol involving hydroxyurea was used to demonstrate the applicability of the immunocytochemical method to this cell culture. Cells were brought to mitotic arrest by cytokinin withdrawal, and the cell division cycle was restarted by the addition of cytokinin. We have followed the pattern of resumption of S phase after the readdition of cytokinin. This pattern reveals the existence of three subpopulations of cells in cytokinin-starved cultures, consistent with the occurrence of three cytokinin-requiring events in the cell cycle: one in mitosis, one in S phase, and one in the G_1 phase.

Key Words Cell cycle—Cytokinin—Soybean cells—*Glycine max*—Immunocytochemistry—S phase

The regulation of the switch from cell division to growth by expansion and differentiation is central to development. Current understanding of cell cycle control has been reviewed (Bayliss 1985, Francis and Halford 1995). Inducible variations in the duration of cycle phases suggest the existence of critical events, so-called *principal control* points. The length of G_1 varies most widely, pointing to a sensitive event in this phase. The other cell cycle phases can also vary in length, although within a narrower range, indicating that they may well also contain principal control points, however, of comparatively lower sensitivity to environmental changes. Changes in hormones required for growth appear to act on these control points. Auxin level affects the duration of G_1 and G_2 (Bayliss 1985, John et al. 1993a, 1993b). For example, the switch in a dividing cell culture to meristem-like growth after induction of embryogenesis by auxin withdrawal accelerated the cell cycle by shortening G_1. For cytokinins there is evidence that withdrawal and consequent cessation of proliferation do not result in cells accumulating at a single point in the cell cycle. Cells appeared to stop throughout the cell cycle; whether at random or as several subpopulations at discrete points was not established (Wang et al. 1981). The aim of the present study was to obtain more information on these cytokinin-requiring points. The immunofluorescence detection of cells in S phase uses a monoclonal antibody against the thymidine analog bromodeoxyuridine (Gratzner 1982) and provides an alternative to autoradiography, which, despite its drawbacks, is still a standard method in cell cycle studies. The application of immunocytochemistry is well established in animal cell cycle work, but it is still relatively new for plant systems (Grosskopf and Kroiher 1988, Levi et al. 1987, Pfosser 1989, Wang et al. 1989a, 1989b).

Materials and Methods

Suspension Culture of Soybean (Glycine max (L.) Merr., Cultivar Acme)

The cell line used was derived from callus initiated on cotyledon segments and has maintained a cytokinin requirement for growth over 20 years of subculturing. The medium described by Miller (1967) was used with the addition of 0.5 mg/liter (2.3 μM) kinetin and the omission of inositol. Subculturing was at weekly intervals to approximately six-fold dilution. The ratio of volume of suspension to volume of culture flask was 1:4. Cultures were kept on an orbital shaker (100 rpm) in the dark at 25°C.

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DI, deionized water; FITC, fluorescein isothiocyanate; HU, hydroxyurea; L-AOPP, L-α-aminooxy-β-phenylpropionic acid; LI, labeling index; PA, polyamine; PI, propidium iodide.

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For experiments, batches of cells in the exponential phase of growth, 3 days after subculturing, were used. In experiments on kinetin starvation, kinetin was removed by three washes with kinetin-free medium by centrifugation (1,500 ×*g*, 5 min) and resuspension. Cell density was adjusted to about 2×10^5 cells/mL. Determination of cell number was carried out according to Brown and Rickless (1949) by hemocytometer (modified Fuchs Rosenthal, depth 0.2 mm).

For the effect on cell number, kinetin or adenine was added to kinetin-starved cultures to a final concentration of 0.5 mg/liter. Addition of the same volume of deionized water (DI) was included as a control. For the effects of kinetin starvation and readdition on DNA synthesis, L-α-aminooxy-β-phenylpropionic acid (L-AOPP) was added to the kinetin-free medium to a final concentration of 10^{-7} M.

For synchronization, hydroxyurea (HU) was added to a final concentration of 4 mM. The cells were released from the blockage by three washes with fresh medium. To detect S phase at the first time point, washing out HU was coincident with the beginning of the incorporation of 5-bromo-2-deoxyuridine (BrdU), and so the washing medium contained 15 μM BrdU.

Immunocytochemical Detection of DNA Synthesis

Cells in S phase were labeled in a 30-min pulse with BrdU. BrdU was added to the suspension to give a final concentration of 15 μM.

Preparation of Protoplasts. To determine the proportion of labeled cells (the labeling index, LI), cell clusters were separated by isolating and fixing protoplasts from labeled material. The selective incorporation of thymidine in preference to BrdU (Dudits et al. 1979, Vyskot and Bezdek 1982) was used to minimize further BrdU incorporation during protoplast isolation. Excess thymidine (15 mM final concentration) was added while washing out BrdU (once with DI, twice with osmoticum) by centrifugation (2,000 ×*g*). The osmotic requirement for optimal release of protoplasts varied between 0.25 and 0.35 M sorbitol with fluctuations in the physiologic state of the culture, for example, in the degree of cell expansion due to different treatments. The osmoticum contained in addition 0.25 mM $CaH_4(PO_4)_2 \cdot H_2O$.

Protoplast release was achieved in a 1-h incubation at 25°C on an orbital shaker (20–35 rpm), in the dark, in 4% (w/v) Onozuka R-10 and 0.4% (w/v) pectolyase Y-23, dissolved in osmoticum and clarified by centrifugation. Remaining cell clusters were filtered out by rinsing through four layers of muslin. Protoplasts were separated from the enzyme solution by centrifugation (200 ×*g*, 5 min). Washing with osmoticum followed prior to fixation.

Protoplast Fixation. Before fixation, protoplasts derived from suspensions containing elongated cells, as found under conditions of kinetin withdrawal, had to be plasmolyzed further (0.7 M sorbitol osmoticum) to make them less fragile. The fixative used by Hammat and Davey (1988) was modified to improve membrane stability by inclusion of 5 mM $CaH_4(PO_4)_2 \cdot H_2O$ and gave better results in the subsequent immunofluorescent staining than fixatives containing glutaraldehyde or formaldehyde. The duration of the treatment was a minimum of 2 h at room temperature or overnight at 4°C.

Immunocytochemical Staining. Slide preparation was as Geber and Schweizer (1988). DNA was denatured either by heat (Beisker et al. 1987, Pfosser 1989), or hot acetic acid (10 min in 45% (v/v) at 60°C, prior to fixation by washes with ice-cold ethanol (twice in 70% and twice in 96%, 5 min each), method from Pfosser). The hot acetic treatment extracted RNA and so eliminated the staining of cytoplasm by propidium iodide (PI); compare Fig. 1, *A* and *B*.

Slides were immersed in Tris-Triton buffer (Levi et al. 1987) for at least 15 min prior to application of 50 μL of primary antibody (mouse anti-BrdU, Becton and Dickinson, 1:10 dilution in Tris-Triton buffer). Cellophane squares were used as covers. The incubation period was 1 h in a humidified chamber at 37°C in the dark. Anti-BrdU was removed in three washes. The secondary antibody, 50 μL of goat anti-mouse FITC conjugate, Sigma F-5262, 1:10 dilution in Tris-Triton buffer, was applied and washed off in the same way. PI (0.04 μg/mL, 15–30 s) was used to counterstain all nuclei.

In mounting the slides, an antifade (Johnson and de Nogueira Araujo 1981) was used to reduce the loss of fluorescence on exposure to UV light.

The LI was determined using an epifluorescence microscope (Vickers M17) equipped with an FITC filter set (2× Leitz KP490/495, GG 455, pass band 455–495 nm, dichroic mirror cutoff 550 nm, barrier filter transmitting from 515 nm). A minimum of 800 nuclei was counted on each slide.

Results

Synchronization by Addition and Withdrawal of Hydroxyurea

Data presented in Fig. 2, show the synchronizing effect of HU supplied over different time periods (20, 24, 32, and 36 h). The proportion of cells in S phase after release from HU blockage followed basically the same pattern: two consecutive peaks. This biphasic response points to the existence of a second cell cycle event sensitive to HU. As a result of HU treatment, the cells segregated into two subpopulations. The larger subpopulation entered S phase immediately following the removal of HU, indicating that these cells were arrested in S phase, as expected from the known inhibition of DNA synthesis by HU. The timing of S phase in the smaller subpopulation was later, between 10 and 20 h after removing HU. This suggests that the second HU-sensitive cell cycle event was 10 h before the G_1–S boundary, i.e. in mitosis. The occurrence of a dip in labeling within the first peak in all four experiments is consistent with the occurrence of a wave of mitosis shortly after HU removal. Likewise, the second peak contains a dip in the proportion of cells labeled around the time that cells that began S phase on HU removal would be expected to undergo mitosis. The cell cycle analysis (Fig. 2) illustrates how coinciding cell cycle events in the two subpopulations of synchronized cells lead to the distribution of DNA synthesis observed following washout of the inhibitor in the sample given a 36 h-HU treatment.

With prolonged exposure to HU the first peak only was shifted toward an earlier time point, increasing in height and narrowing in duration, resulting in a clearer

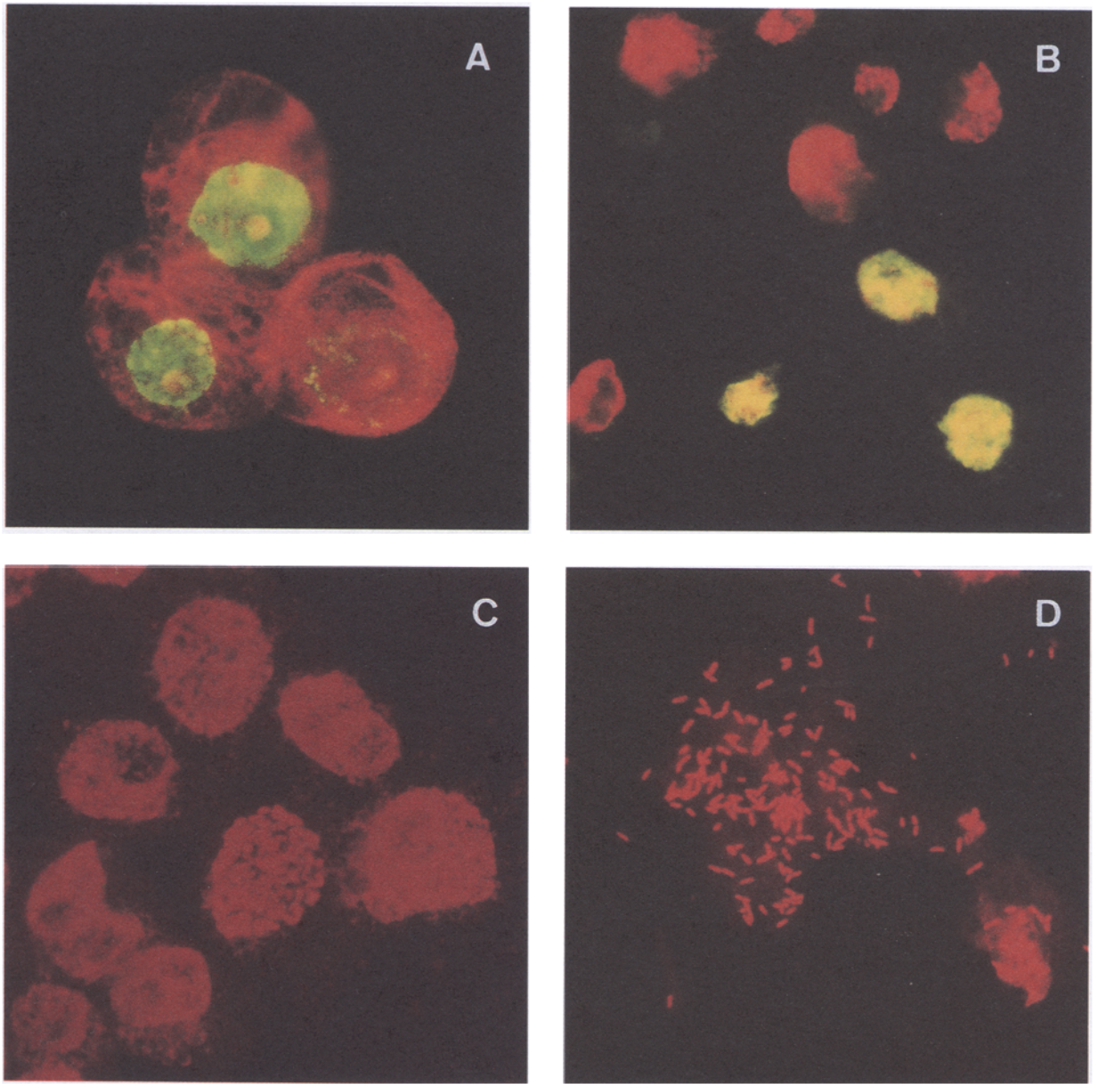

Fig. 1. Immunocytochemical detection of DNA synthesis in soybean: protoplasts from BrdU pulse-labeled suspension cultures, FITC-Ab labeled, and counterstained with PI. **(A)** DNA denaturation by heat only and **(B)** heat-acid treatment. PI staining of the cytoplasm is eliminated in **(B)**. **(C)** Protoplasts prepared 15 h after readdition of kinetin to kinetin-starved culture. A high percentage of nuclei contain condensed chromosomes. **(D)** as **(C)**.

separation from the following peak. This shift indicates that cells were entering S phase and progressing through it, even though very slowly, despite the presence of HU. This is seen best by comparing the 32- and 36-h treatments, in which the exposure time to HU was close to and exceeding the duration of a complete cell cycle, respectively. Synchronization was apparently not by total inhibition of DNA synthesis but by slowing the rate of synthesis, allowing cells in other phases of the cell cycle to catch up. Flow cytometric detection of cells in S phase during HU treatment (Pfosser 1989) is consistent with this observation.

These results demonstrate the applicability of the immunocytochemical technique for studies of partial mitotic synchrony in suspension cultures of soybean callus. As an antimitotic HU has a well characterized action, inhibition of ribonucleotide reductase, and the data presented confirm that the method is capable of revealing

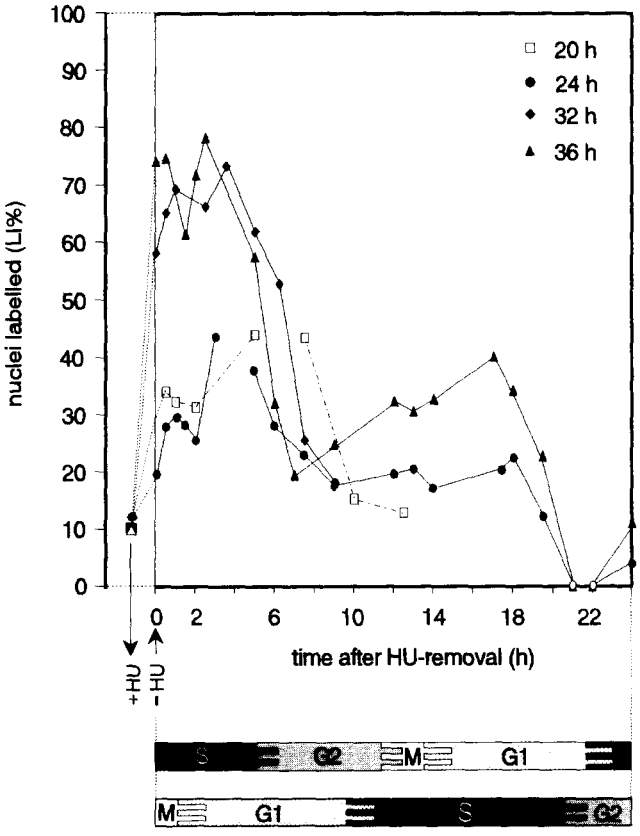

Fig. 2. Pattern of synchronization after treatment with 4 mM HU for different periods of time. The time course of the proportion of cells in S phase after release from HU blockage is shown, accompanied by a cell cycle analysis diagram. *+HU* marks the proportion of cells in S phase at the time of the HU addition.

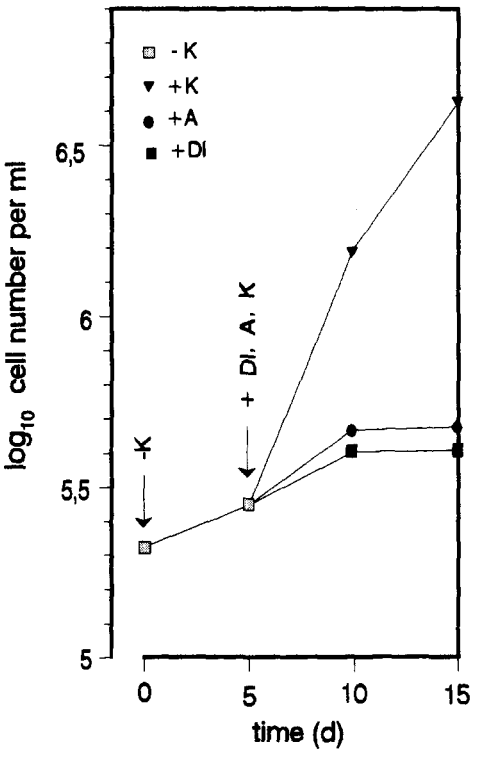

Fig. 3. The effect of kinetin (*K*) and its analog adenine (*A*) on cell division. Changes in cell number/mL of suspension following transfer to kinetin-free medium and subsequent readdition of kinetin (2.3 μM) or adenine (3.7 μM) or DI. A representative experiment is shown.

the expected pattern of changes in the proportion of cells in S phase with time following the reinitiation of mitotic cycling. This justifies our moving with confidence to an examination of the pattern of changes in the proportion of cells in S phase following the reinitiation of mitotic cycling by the addition of cytokinin, with a view to generating some evidence that will help circumscribe the nature of cytokinin action in the cell cycle.

Effects of Kinetin: Cell Division Reinitiated by Kinetin but Not by Adenine

The specificity of the dependence of cell division on cytokinin was investigated by comparing the effect of kinetin and adenine, as an analog of kinetin. Fig. 3 shows the cytokinin dependence of cell division. After the withdrawal of kinetin a proportion of cells completed one more division, putatively due to carryover of kinetin, before the cell number finally plateaued. This is in line with the findings of Wang et al. (1981). Differences in the timing of the cessation of cell division may be attributable to the fact that cultures were in different growth phases. Wang et al. used suspensions in the late linear phase. This study was done with cultures in the early linear phase. The readdition of kinetin reinitiated cytokinesis, whereas the addition of adenine did not. In the presence of adenine cell division occurred only marginally more than in the DI control.

DNA Synthesis following Withdrawal and Readdition of Kinetin in the Presence of L-*AOPP*

Initially no protoplasts could be obtained from batches of cells deprived of cytokinin. Following cytokinin withdrawal, cultures browned, and cell wall-located autofluorescence increased, indicating accumulation of phenolic compounds. To prevent this, L-AOPP, an inhibitor of phenylalanine ammonia-lyase, the enzyme catalyzing the first step in the biosynthesis of phenylpropanoids (Jones 1984), was added to the medium.

In the short term, browning was prevented by L-AOPP, and cultures gave high yields of protoplasts over several days of kinetin starvation, allowing DNA synthesis to be monitored. L-AOPP treatment had a slightly

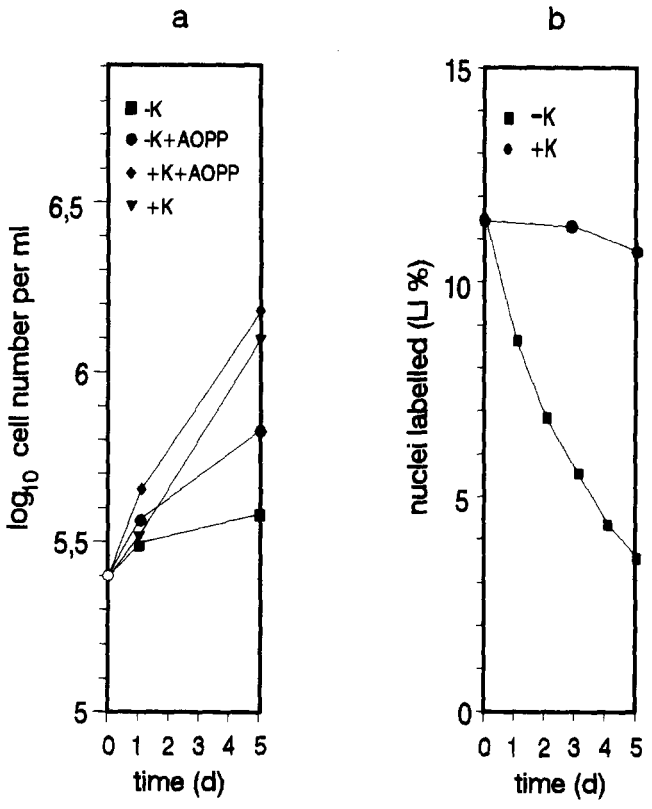

Fig. 4. The suspension culture in the presence of L-AOPP. **(a)** Effect of L-AOPP on cell number; response of cell number after transfer to media containing combinations of L-AOPP (10^{-7} M) and kinetin (2.3 μM) for 5 days. **(b)** Effect of kinetin withdrawal on DNA synthesis in the presence of L-AOPP (10^{-7} M). The percentage of nuclei in S phase in the absence (*–K*) and presence (*+K*) of kinetin over a 5-day period is shown. Representative experiments are presented.

stimulatory effect on cell number (Fig. 4*a*). This effect was more distinct in medium lacking kinetin. Although cell division stopped, cell expansion continued. Increased size of vacuoles together with accumulation of starch made protoplasts fragile, and they burst on addition of ethanol-containing fixatives. If protoplasts were condensed by further plasmolysis prior to fixation and the fixative was modified by the addition of Ca^{2+} as in Burgess et al. (1973), protoplasts were stabilized, and it was possible to follow the pattern of DNA synthesis over up to 5 days of kinetin starvation.

Withdrawal of Kinetin. Fig. 4*b* illustrates the results of kinetin starvation in the presence of L-AOPP. During kinetin deprivation the fraction of cells in S phase declined steadily, although it did not reach zero within the period of observation.

Readdition of Kinetin. On readdition of kinetin the proportion of cells in S phase oscillated between 15 and close to 0% (Fig. 5). The conclusion is that cytokinin starvation of 4.5 days does not effectively synchronize the cell cycle. The minima of cells in S phase at 15 and 25 h coincided with the occurrence of condensed chromosomes in a very high percentage of nuclei (Fig. 1, *C* and *D*). This and the distinctness of the minima make it feasible graphically to align each minimum in the pattern of labeling with the end of mitosis and each peak with S phase and then to unroll the rest of the cell cycle to either side (Fig. 5). The duration of the cell cycle phases (at 25°C: G_1, 12.2 h; S, 11.8 h; G_2, 6.3 h; and mitosis, 1.8 h) was interpolated from the data of Chu and Lark (1976). The fit between the calculated and observed values suggests that the oscillation pattern observed in this study is due to fractionation of the cell population into three subsets as a result of kinetin withdrawal. This analysis (Fig. 5) suggests that there may be three cytokinin-sensitive events in the cultured soybean cell cycle. As a result, three partially synchronized fractions of cells are generated and resume cycling after resupply of kinetin, one in mitosis (I), one in G_1 (II), and one in S (III).

Discussion

One consequence of cytokinin deficiency was browning of the cultures. L-AOPP, an inhibitor of the synthesis of phenolic compounds, cured browning without detectable side effects. A stimulatory effect of L-AOPP on cell number, especially under cytokinin-starved conditions, might be attributable to changes in the relative proportions of free and bound endogenous polyamines (PAs). Phenolic

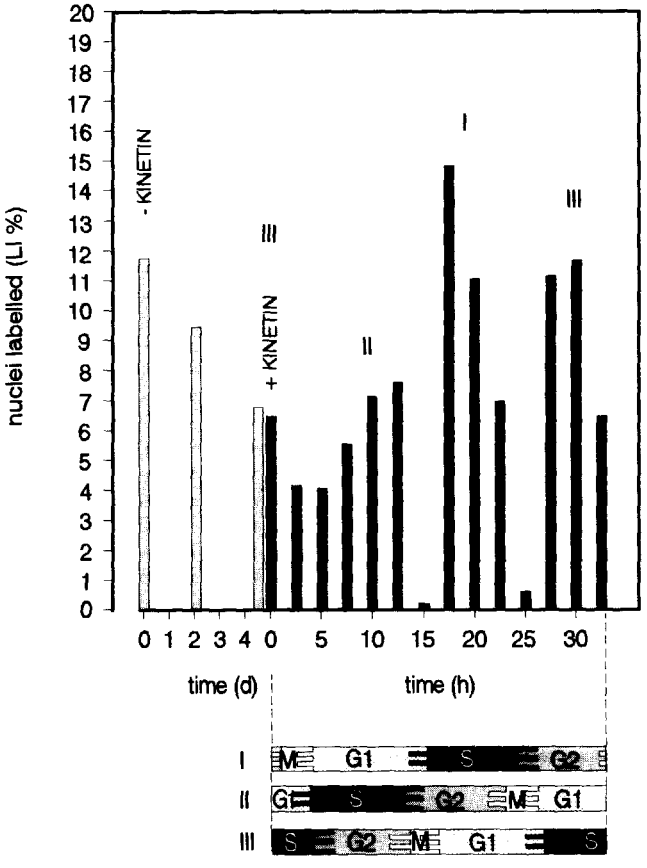

Fig. 5. Effect of kinetin on DNA synthesis: distribution of cells in S phase after kinetin readdition subsequent to a 4.5-day kinetin starvation. An individual experiment is presented. The cell cycle diagram illustrates the coexistence of three subpopulations of cells.

acids form conjugates with PAs. Therefore, decreasing the availability of phenolic acids may reduce the diversion of free PAs into conjugates. The dynamics of endogenous PA concentrations has been related to progress through the cell cycle (for reviews see Del Duca and Serafini-Fracassini 1993, Serafini-Fracassini 1991).

Developmental processes in plants that are, in part, controlled by cytokinin, may also be affected by PAs, and the promotive effect of cytokinins on endogenous PAs levels, especially on free putrescine, is pronounced (Cho 1983, Hemanataranjan and Vaishampayan 1984, Walker et al. 1988). Putrescine is the most abundant PA in cycling cells (Maki et al. 1991, Pfosser 1993, Pfosser et al. 1990). The precise part a lowering of endogenous PA levels may play in the response to cytokinin starvation has yet to be investigated.

We have interpreted the successive waves of S phase which follow release from HU inhibition or kinetin starvation as synchronized subpopulations resulting from action by these chemical agents at more than one point in the cell cycle. Alternative explanations are possible, e.g. if the cell culture contains variants differing in responsiveness to inhibitors or hormones. The results of Patau et al. (1957) indicated that tobacco pith cells with lower ploidy levels enter S phase more readily than cells that have undergone endoreduplication. After careful consideration of the timing and duration of the waves of S phase detected here we have had to conclude that such alternative explanations are less likely, even if they cannot be entirely ruled out.

For HU, the time shift between the two peaks, consistently 15 h for all exposures, was too short to be the duration of a total mitotic cycle. Thus, the second peak could not represent the S phase in the daughter cells originating from the first peak. It seems to us a highly improbable coincidence that there should be two different cell types in the original culture which by chance are separated so clearly by HU treatment. Difference in cell cycle length would not be enough. A fraction of slow cycling cells, failing to reach S phase during the exposure to HU, would not be synchronized and so would not result in a distinct second peak. More complex theories, for which there is no basis in the results, would need to be developed, such as differential sensitivity of cell populations to HU. Therefore, that HU affects two separate points in the cell cycle, S phase and mitosis, remains the most plausible explanation of the biphasic response. The dip in peak I, indicative of a wave of mitosis diluting the population in S phase, is at least consistent with that model. Certainly, there is support in the literature for the conclusion that mitosis is affected by HU. Mitotic nuclear division was reported to be inhibited by HU

(Kwon and Hoch 1991). According to Navarrete et al. (1979) cells in G_2 were delayed in entry to mitosis. Findings by Takegami and Ito (1982) point to the existence of a HU effect on division, mitotic as well as meiotic. The presence of HU during the late S phase and early G_2 interfered with the progress of mitosis. The fact that a fraction of G_2/M cells remained during HU treatment (Pfosser 1989) is also consistent with this explanation.

As the results obtained with HU gave no reason to doubt the homogeneity of the original culture, the periodic fluctuation of LI (this study) and oscillations in the mitotic index (Wang et al. 1981) following the resupply to cytokinin after a period of starvation point to the existence of several cytokinin-sensitive events in the cell cycle.

There is evidence that DNA synthesis is not dependent on cytokinins in the same way as cell division. Miller et al. (1955) recorded stimulation of cell division by cytokinin in cases in which otherwise cells were likely to become multinucleate. Cytokinin starvation of soybean cultures stopped cell division; the DNA content, however, continued to increase two to three times (Fosket and Short 1973). This is all evidence for successive rounds of DNA replication, endoreduplication, and endomitosis in the absence of mitosis due to the absence of cytokinin (for review, see Bayliss 1985). The present study is consistent with all that. Cell division was shown to be the most cytokinin-dependent cell cycle event. Cell number increased only slightly after the removal of kinetin. The LI, however, took 3–4 days to decrease by only half (Fig. 5). Cytokinin starvation appeared to act as a switch from mitotic to amitotic cycles. On resupply of cytokinin after starvation, there was a wave of cell divisions, which would account for dilution of the LI as the cell cycle was restored following the release of the different cell populations from the cytokinin-sensitive points at which they had gathered. Data from this study suggest that the S phase most likely contains one of these cytokinin-sensitive points. This is in line with reports of cytokinin speeding up DNA synthesis (Houssa et al. 1990), suggesting that cytokinin can be limiting to progress through S phase. The observed pattern of changes in the LI further indicates that the cell cycle was reinitiated in a third population functionally in G_1 (in addition to the fractions gathered at the G_2/M transition and in a slowed down S phase). This is consistent with the findings by Blaschke et al. (1978) that G_1 can be accelerated by cytokinins.

The results from starvation and readdition of kinetin leave open the possibility of additional explanations centering around the effects of cell expansion and endoreduplication of DNA on the restart of cycling.

Cell size-dependent control mechanisms operating in cells triggered by a resupply of cytokinin to switch from expansion back to stabilization of the optimal size for cycling cells may complicate the picture (compare John et al. 1993a and 1993b).

The fractionation of the cell population could be accompanied by changes in cell cycle parameters, such as the duration of cell cycle phases in cells that have undergone endoreduplication. If that was the case, unchanging duration of peaks in S phase (Fig. 5) implies that only phases other than S phases are likely to be affected. Clarification of the extent of endoreduplication in this system would be of interest. For example, following on from the early results of Patau et al. (1957) that cells with lower DNA content can be stimulated more easily to enter S phase, one question would be whether a correlation between particular peaks of S phase and DNA content can be found after resupply of cytokinin in this system.

In conclusion, cytokinin does not seem to act exclusively at cytokinesis but also in other cell cycle controlling events, although these may be less limited by low concentrations as the endogenous carryover appears to allow some amitotic cycles before final arrest takes place. In reviewing evidence from other systems Jacqmard et al. (1994) arrived at a similar conclusion. Thus, based on the data presented here and supported by evidence from the literature, the existence of several cytokinin-sensitive events in the cell cycle remains the most likely working hypothesis until it is proven, disproven, or modified in further studies.

A further aspect of cell cycle control in connection with cytokinins is the close involvement of $p34^{cdc2}$-like protein kinases, related to the G_1/S and G_2/M transitions and S phase (Magyar et al. 1993). These are the same cell cycle events that this study suggests show a cytokinin requirement. In fact, the $p34^{cdc2}$-like protein kinases involved in cell division proved to be dependent on cytokinin for function (for review see John et al. 1993a). Cell division activity did not correlate with the amount of $p34^{cdc2}$ present but with its activity. Auxin alone induced $p34^{cdc2}$ synthesis. Cell division, however, required activation by cytokinin. For G_1/S and S phase the cytokinin requirement for activation of the $p34^{cdc2}$-like protein kinases has not been reported. However, the results of the present study on cytokinin starvation strongly suggest that possibility. Further studies are needed to verify the mechanisms involved in the cell cycle regulatory activities of cytokinins.

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