SYNCHRONIZATION OF A PROLIFERATIVE POPULATION IN A CULTURED PLANT TISSUE

Kinetic Evidence for a G_1/S Population

CHARLES J. KOVACS and JACK VAN'T HOF. From the Biology Department, Brookhaven National Laboratory, Upton, New York 11973

INTRODUCTION

The use of synchronized populations of proliferative cells has been advantageous for the investigation of events of the cell cycle (4, 5, 15). Most investigations, however, have been carried out with procaryotic and eucaryotic single cells because dependable techniques for synchronization of cells in tissues were virtually unknown. It is therefore desirable, if not necessary, to develop procedures at the tissue level if the mitotic cycle is to be analyzed under conditions where metaphytic as well as metazoic cells are constrained anatomically and undergo normal differentiation.

The present note describes the methodology and the cell population kinetics of synchronized cells in the meristem of cultured isolated pea roots. The methodology takes advantage of the fact that the asynchronously dividing cells segregate exclusively into the G_1 and G_2 stages of the mitotic cycle when cultured aseptically in White's medium (14), devoid of carbohydrate (10, 11, 12). By sequential provision and deprivation of sucrose in conjunction with 5-fluoro-deoxyuridine (FUdR, Hoffmann-LaRoche, Inc., Nutley, N. J.), an inhibitor of DNA synthesis, the meristematic cells can be accumulated in G_1 and their distribution at the G_1/S boundary can be experimentally regulated.

RESULTS AND DISCUSSION

The manner in which labeled interphase cells appear when continuously supplied tritiated thymidine (T-^{3}H) describes cell distribution in G_1. In meristems starved of carbohydrate for 48 hr (hereafter termed S.P.), the distribution is rectangular and slightly offset from the G_1/S boundary. This is demonstrated by the constant linear increase of labeled cells with time following a 4–5 hr delay after the addition of 2% sucrose to the medium (Fig. 1 *a*, closed triangles). If, however, they are incubated in medium with 2% sucrose for 12 hr (a sucrose "spurt"), starved of carbohydrate for another 12 hr (recovery period), and then returned to medium with sucrose, the first cells enter S within 30 min (Fig. 1 *a*, open circles). The addition of 1×10^{-6} M FUdR to the medium during the 12 hr spurt, however, effectively accumulates the cells at the G_1/S boundary as evidenced by the altered pattern of entry into S (Fig. 1 *a*, closed circles). Initially 21% and subsequently 16% of the cells enter into the DNA synthetic stage. The plateau, observed at 4 hr, indicated that thereafter no additional cells initiate DNA synthesis and that the entire proliferative population of the meristem was in S. The rapid rise, initiated at 12 hr, signified the commencement of division (Fig. 1 *b*, solid circles), and represented the addition of labeled daughter cells to the tissue. Comparison of the manner in which the labeled cells divided (Fig. 1 *b*) demonstrated the efficacy of the technique, as only those treated with FUdR were synchronized at mitosis.

That the combined sucrose-FUdR spurt actually synchronized all the meristematic cells is seen from the data presented in Fig. 1 *c*. Here the accumulative per cent of labeled division figures is expressed as a function of time after the final provision of sucrose (zero hour on the graph). In 48 hr S.P., the percentage was 14 after 24 hr. For the meristems given a 12 hr spurt, it was 20, and in those treated with FUdR during the spurt, 38% was attained. In the latter case, the percentage was equivalent to the number of cells that entered into S (Fig. 1 *a*, solid circles), manifesting neither a net cell loss nor cell increase during the time interval between entry into S and mitosis.

The duration of the sucrose-FUdR spurt determined the manner in which the cells accumulated in G_1 and regulated the percentage of the total proliferative population that was positioned at the G_1/S boundary (Table I A). A 12 hr sucrose-FUdR spurt positioned 57% at G_1/S, an 18 hr treatment increased the percentage to 89, while 6 hr positioned only 8% at G_1/S. These statistics are from experiments with a 12 hr recovery period and are therefore comparable. If, however, the recov-

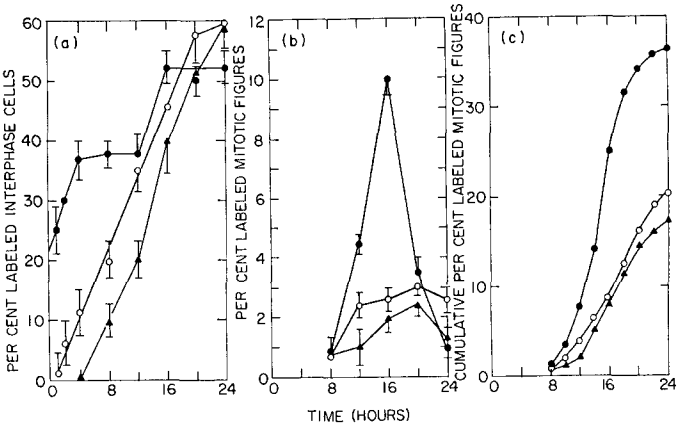

FIGURE 1 The resumption of cell progression in the mitotic cycle following the final transfer to medium containing sucrose and tritiated thymidine. (*a*) entry of cells from G_1 into S; (*b*) the division of former G_1 cells; (*c*) the total number of former G_1 cells that divided. Per cent labeled interphase cells obtained by scoring at least 2×10^3 cells; per cent labeled mitotic figures obtained by scoring 4×10^3 cells; cumulative per cent from 1 *b* and assuming 2–2.5 hr for mitosis (10). Closed circles, 48 hr S.P. → 12 hr sucrose-FUdR spurt → 12 hr without sucrose; open circles, 48 hr S.P. → 12 hr sucrose spurt → 12 hr without sucrose; closed triangles, 48 hr S.P. Cytological preparations and radioautography performed as previously published (1, 11). Tritiated thymidine; 1 μCi/ml; S.A. 6.0 Ci/mmole; Schwarz BioResearch Inc., Orangeburg, N. Y.

ery period is changed, the number of cells at the G_1/S boundary is altered as shown in Table I A. The highest percentage (89%) was obtained with an 18 hr sucrose-FUdR spurt and a 12 hr recovery period. Further increases in the duration of the sucrose-FUdR spurt virtually positioned all the cells at the G_1/S boundary, but proved deleterious to subsequent cell progression in the cycle even when the recovery period was altered accordingly.

Confirmation of the cytological measurements was obtained when incorporation of T-^{3}H into DNA of cells treated with increased durations of sucrose-FUdR spurts was determined (Fig. 2). Incorporation was measured for 1 hr beginning with the termination of the 12 hr recovery period. As noted in Fig. 2, the percentage of labeled cells and the amount of T-^{3}H incorporated into DNA form almost parallel curves that varied directly with the duration of the sucrose-FUdR spurt.

The ratio of labeled to total number of mitotic figures, LMF/TMF (Table I B), was used to estimate the degree of synchrony. When the ratio was unity, the population at G_1/S consisted of all the meristematic cells in the root tip and indicated optimum homogeneity. Both 12- and 18-hr sucrose-FUdR spurts followed by 12-hr recovery periods produced ratios of 1. In experiments where the recovery period was either more or less than 12 hr, a ratio less than unity was obtained. It is necessary, therefore, to achieve the proper balance between the durations of the sucrose-FUdR spurt and the recovery period, if a high degree of homogeneity is desired.

In a tissue that has the inherent potential for both proliferative and differential activity, the question arises as to how the onset of differentiation and the transition of cells through the mitotic cycle are coordinated. The physiological state during which a cell may either continue in the mitotic cycle or enter into differential activity has not been defined. Wilson and Morrison (16) suggested the existence of a "null" period in the mitotic cycle, occurring between the M and G_1 stages of the cycle. During this null period, cells may leave the cycle and differentiate, but do not necessarily lose their mitotic competency. In a comprehensive review of the resting stage, Epifanova and Terskikh (2) suggested that, even if some of the requirements for differentiation are produced during the cycle, the point at which cells leave the cycle occurs during the G_0 stage, a stage corresponding in position with Wilson and Morrison's null period (16). They suggested, however, that G_0 did not complete the possible regions of the mitotic cycle where transition into a resting stage can occur. The results from this investigation, however, suggest that cells

TABLE I

*Homogeneity of G_1 Population after Various Culture Conditions**

A. $G_1 \rightarrow S$

Culture conditions‡	Labeled interphase cells at G_1/S	Total proliferative population at G_1/S
hr	%	%
0	0	0
12 (+S spurt)	0	0
6 (+S, FUdR spurt) 12 (−S recovery)	3	8
18 (+S, FUdR spurt) 18 (−S recovery)	3	8
12 (+S, FUdR spurt) 12 (−S recovery)	21	57
18 (+S, FUdR spurt) 12 (−S recovery)	33	89

B. $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$

Culture conditions‡	Labeled mitotic figures (LMF)	Total mitotic figures (TMF)	LMF/TMF
hr	%	%	%
0	13.76	18.48	0.75
12 (+S spurt)	20.22	23.40	0.86
6 (+S, FUdR spurt) 12 (−S recovery)	24.20	31.93	0.76
18 (+S, FUdR spurt) 18 (−S recovery)	19.19	27.40	0.70
12 (+S, FUdR spurt) 12 (−S recovery)	37.50	37.50	1.00
18 (+S, FUdR spurt) 12 (−S recovery)	37.50	37.50	1.00

* Based on the rate of entry into S and the cumulative mitotic figure frequency after 24 hr.

‡ Following establishment of a stationary phase meristem (S.P.).

after successfully completing G_1 do not leave the cycle and enter into a resting stage during their transition through the cycle. If cells were to leave the cycle after G_1, the observed one-to-one relationship between the number of G_1/S cells that initiate DNA synthesis and those that eventually divide would not hold.

Webster and Van't Hof (13) have observed that for S.P. G_1 cells to enter into S, both RNA and protein synthesis are required, the requisite RNA being synthesized 4–8 hr prior to the protein. Although FUdR inhibits the synthesis of RNA and protein, as well as DNA, by mammalian cells after prolonged incubation (3), it has not been found to interfere with the synthesis of RNA and protein required for initiation of DNA synthesis by proliferative cells of S.P. It may, however, affect progress through the cycle by inhibiting the synthesis of functional RNA or protein required for transit through G_2 and M. G_1/S cells, however, are able to complete DNA synthesis with a high degree of synchrony, indicating that any presumed effect on macromolecular synthesis, other than DNA, may result in an alteration of the duration of the G_2, M, or the second generation G_1 stage of the cycle. RNA synthesis, during the G_2 stage of *Physarum*, has been reported to be greatly reduced by FUdR, provided that the amount of uridine present was inadequate (6). During the S period, however, a reduction in RNA synthesis by FUdR could not be overcome by providing uri-

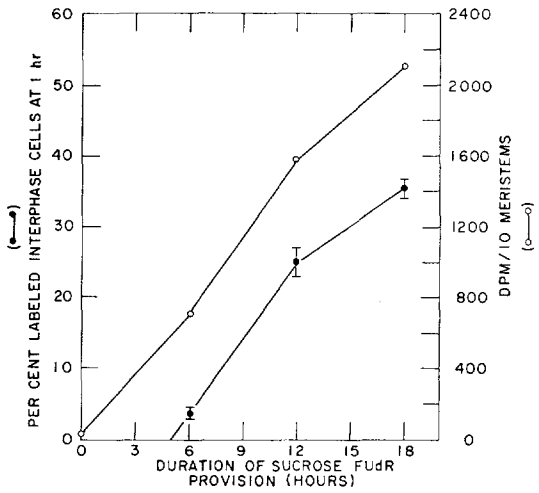

FIGURE 2 Incorporation of tritiated thymidine into DNA of cultured meristems as measured by radioautography and scintillation counting. The format was 48 hr S.P. → sucrose-FUdR spurt → 12 hr without sucrose or FUdR → 1 hr incubation in medium with sucrose and 1 μCi/ml, S.A. 6.0 Ci/mmole, tritiated thymidine. DNA extracted according to Smillie and Krotkov (8) and collected on glass filters (7) for counting.

dine, and it has been suggested that at least part of the newly synthesized DNA may be involved in RNA synthesis required for progression through G_2 and M. Variable S + G_2 periods have been reported in lymphocytes synchronized by inhibition of DNA synthesis (9). However, this observation could be ascribed to both the heterogeneity of the population and inherent dispersion during the cell cycle as well as to an FUdR effect on the kinetics of the cell cycle. The possibility that FUdR effects occurring in G_1, but realized in the S and G_2 stages of the cycle, are currently being investigated.

SUMMARY

The utilization of sequential and discrete cultural conditions consisting of prolonged carbohydrate starvation, provision of sucrose and FUdR, and a second starvation followed then by replenishment of carbohydrate has effectively synchronized the proliferative cells of root meristems in the G_1 stage of the mitotic cycle. The distribution of G_1 cells and their passage through the cycle were measured with tritiated thymidine and radioautography. The results indicated that all proliferative cells had accumulated at the G_1/S boundary and, depending on the relative durations of the culture conditions, initiated DNA synthesis either instantaneously and uniformly or at a constant rate over a 5 hr period.

Research was supported in part by the United States Public Health Service (Fellowship 1FO2 CA42865-01 from the National Cancer Institute to Dr. Kovacs) and in part by the United States Atomic Energy Commission.

Received for publication 3 March 1970, and in revised form 20 May 1970.

REFERENCES

1. CONGER, A. D., and L. M. FAIRCHILD. 1953. A quick-freeze method for making smear slides permanent. *Stain Technol.* **28**:281.
2. EPIFANOVA, O. I., and V. V. TERSKIKH. 1969. On the resting period in the cell life cycle. *Cell Tissue Kinet.* **2**:75.
3. PAUL, J., and A. HAGIWARA. 1962. A kinetic study of the action of 5-fluoro-2′-deoxyuridine on synthetic processes in mammalian cells. *Biochim. Biophys. Acta.* **61**:243.
4. PETERSEN, D. F., R. A. TOBEY, and E. C. ANDERSON. 1969. Synchronously dividing mammalian cells. *Fed. Proc.* **28**:1771.
5. PETERSEN, D. F., R. A. TOBEY, and E. C. ANDERSON. 1969. Essential biosynthetic activity in synchronized mammlian cells. *In* The Cell Cycle. G. M. Padilla, G. L. Whitson, and I. L. Cameron, editors. Academic Press Inc., New York. 341.
6. RAO, B., and M. GONTCHAROFF. 1969. Functionality of newly synthesized DNA as related to RNA synthesis during the mitotic cycle in *Physarum polycephalum*. *Exp. Cell Res.* **56**:269.
7. SCHILDKRAUT, C. L., C. C. RICHARDSON, and A. KORNBERG. 1964. Enzymic synthesis of deoxyribonucleic acid. XVII. Some unusual physical properties of the product primed by native DNA templates. *J. Mol. Biol.* **9**:24.
8. SMILLIE, R. M., and G. KROTKOV. 1960. The estimation of nucleic acids in some algae and higher plants. *Can. J. Bot.* **38**:31.
9. STEFFEN, J. A., and W. M. STALZMANN. 1969. Studies on *in vitro* lymphocyte proliferation in cultures synchronized by the inhibition of DNA synthesis. I. Variability of S plus G_2 periods of first generation cells. *Exp. Cell Res.* **56**:453.
10. VAN'T HOF, J. 1965. Cell population kinetics of excised roots of *Pisum sativum*. *J. Cell Biol.* **27**:179.
11. VAN'T HOF, J. 1966. Experimental control of DNA synthesizing and dividing cells in excised root tips of *Pisum*. *Amer. J. Bot.* **53**:970.
12. VAN'T HOF, J. 1968. Control of cell progression through the mitotic cycle by carbohydrate provision. I. Regulation of cell division in excised plant tissue. *J. Cell Biol.* **37**:773.
13. WEBSTER, P. L., and J. VAN'T HOF. 1970. Initiation of DNA synthesis and mitosis in stationary, transitional and proliferative phase meristems: requirements for RNA and protein synthesis. *Amer. J. Bot.* **57**:130.
14. WHITE, P. R. 1943. A Handbook of Plant Tissue Culture. The Jaques Cattell Press, Lancaster, Pa.
15. WHITSON, G. L., G. M. PADILLA, and W. D. FISHER. 1966. Morphogenetic and macromolecular aspects of synchronized *Tetrahymena*. *In* Cell Synchrony. I. L. Cameron and G. M. Padilla, editors. Academic Press Inc. New York. 289.
16. WILSON, G. B., and J. H. MORRISON. 1959. The mitotic cycle and the ontogeny of neoplastic growth. *Cytologia* (*Tokyo*). **24**:43.