Synchronization of nuclear DNA synthesis in cultured *Daucus carota* L. cells by aphidicolin

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By inhibiting the α-like DNA polymerase, and therefore nuclear DNA synthesis, aphidicolin induces accumulation of suspension cultured carrot cells at the G_1/S boundary of the cell cycle. After a 24-h treatment with the drug the accumulation is complete, affecting all the cycling cells (95% of the population). Upon removal of the inhibitor, all cycling cells immediately resume nuclear DNA synthesis and move synchronously throughout the S-phase.

Aphidicolin *Synchronization* *Cultured plant cell* Daucus carota *Nuclear DNA synthesis*

1. INTRODUCTION

To study the events of the cell cycle or to obtain accumulation of cells at specific phases of the cycle, several attempts have been made to synchronize plant cell populations, either in culture or in plant explants. However, the published experimental methods are far from ideal because they are effective only on one specific cell type or are partially toxic or yield incomplete synchrony [1–4].

Thus, it is highly desirable to develop new synchronizing agents. Aphidicolin, a fungal toxin obtained from *Cephalosporium aphidicola* [5], synchronizes animal cells in culture [6] by inhibiting the replicative DNA polymerase α [7]. This drug specifically inhibits the plant α-like DNA polymerase [8]. The addition of aphidicolin to cultured plant cells causes a complete and reversible inhibition of nuclear DNA replication [9]. Nuclear DNA repair synthesis [10], organellar DNA replication [9], RNA and protein synthesis [8] do not appear to be disturbed.

These data suggest that aphidicolin is a possible candidate to synchronize plant cells without significant adverse effect on important cellular functions.

We report here that aphidicolin indeed induces accumulation of carrot cells at the G_1/S boundary of the cell cycle and that, upon its removal, all cycling cells immediately resume nuclear DNA replication and move synchronously through the S-phase.

Inactivation of aphidicolin occurs in the carrot cell culture. However, this is not an obstacle provided an inhibitory drug concentration is maintained throughout the synchronizing treatment.

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Abbreviations: DMSO, dimethyl-sulfoxide; *SCV*, ml sedimented cells/100 ml culture

2. MATERIALS AND METHODS

2.1. *Chemicals*

[*methyl*-1′,2′-^{3}H]Thymidine (25 Ci/mmol; 1 mCi/ml) was obtained from the Radiochemical Centre (Amersham). Aphidicolin was kindly supplied by Imperial Chemical Industries (England).

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2.2. *Plant cell growth*

Carrot (*Daucus carota* L.) cell suspension cultures were established and serially subcultured in Erlenmayer flasks as in [11]. Cell growth was followed by recording the *SCV*, i.e., the volume (ml) of cells sedimenting in 10 min in 100 ml culture, as in [12].

2.3. *Determination of aphidicolin concentration in the culture medium*

Culture samples (0.5 ml) were transferred to screw-cap test tubes and additioned with 2 ml acetone and 0.125 ml DMSO. After 24 h at room temperature, cell debris were discarded by low speed centrifugation. The concentration of aphidicolin in the supernatant was assayed by measuring the extent of inhibition of the DNA polymerizing activity of a purified mammalian DNA polymerase α, as in [13].

2.4. *Synchronization by treatment with aphidicolin*

Early exponential phase carrot cell cultures, obtained from the stock cultures by two successive transfers at 3-day intervals, were inoculated at low density (4–5 $\times$ 10^5 cells/ml, corresponding to *SCV* = 1.5) in fresh medium containing 20 μg aphidicolin/ml (DMSO solution) and incubated in standard growth conditions (100 ml culture in 500 ml Ehrlenmayer flasks, rotary shaker at 120 rev./min and 2.5 cm eccentricity, 26°C, dim light). After 12 h, 20 μg aphidicolin/ml were added again and incubation continued for an additional 12 h. At this time the drug was removed by washing the cells 4 times with culture medium and the incubation was continued at the same cell density and growth conditions.

2.5. *Assay of nuclear and of organellar DNA replication*

Two samples (1 ml) were withdrawn from cultures and incubated in the presence of 5 μCi of [^{3}H]thymidine at 26°C for 1 h on a rotary shaker. One of the samples contained 10 μg aphidicolin/ml. A further sample served as control for radioactivity incorporation at zero time incubation. Incubation was stopped by adding 1 vol. 10% trichloroacetic acid containing 0.1 mM thymidine. The suspension was then homogenized with a motor-driven teflon–glass Potter homogenizer (20 strokes) and the insoluble fraction collected on a Whatman GF/C glass filter and washed 6 times with 5% trichloroacetic acid containing 0.1 mM thymidine and 4 times with distilled water. The radioactivity on the filter was determined in an Insta-gel liquid scintillation cocktail with a Tri-Carb Packard liquid scintillation spectrometer (efficiency 31%). Incorporation due to replication of nuclear DNA was determined by subtracting the aphidicolin-insensitive incorporation from total incorporation (in the absence of aphidicolin). Incorporation due to organellar DNA synthesis was determined by subtracting the background value (i.e., the incorporation of radioactivity in a culture sample treated with trichloroacetic acid at zero time) from the aphidicolin-insensitive incorporation.

2.6. *Autoradiographic analysis of the cells after incorporation of [^{3}H]thymidine*

Culture samples (4 ml) were incubated at 26°C on a rotary shaker in the presence of [^{3}H]thymidine (5 μCi/0.4 μg/ml for the 1–3 h and 5 μCi/2 μg/ml for 24–48 h incubation times). Then, the cells were sedimented (3 min at 1000 $\times$ *g*) and washed 3 times with 5 ml culture medium containing 0.1 mM thymidine. Labelled DNA was revealed by means of autoradiography of 1 μm sections as in [9,14]. Results are expressed as % of nuclei showing extensive autoradiographic labelling.

3. RESULTS

Cultured plant cells grow asynchronously, with random distribution in the G_1, S, G_2 and M phases of the cell cycle and with a fraction of the cell population in a non-dividing state [1,3]. By inhibiting the α-like DNA polymerase and thus the replication of nuclear DNA [8,9], aphidicolin is expected to accumulate cells at the G_1/S boundary. To this purpose cells should remain in the presence of an inhibitory dose of the drug for a period of time corresponding at least to the cell cycle time minus the S phase duration.

The length of time required to obtain maximum accumulation of cells at the G_1/S boundary by aphidicolin was established by the experimental results reported in fig.1. Suspension cultures in the early experimental phase of growth, were sup-

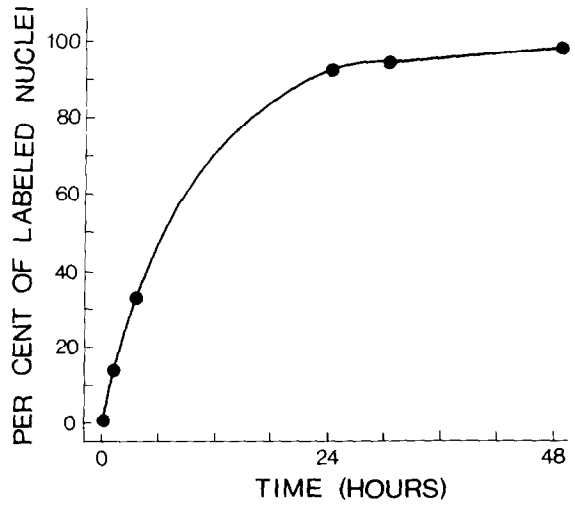

Fig.1. The increase in number of radioactive nuclei with increased time of incubation of *Daucus carota* cells with [^{3}H]thymidine. [^{3}H]Thymidine was added to a culture in the early exponential phase of growth and the incubation continued as in section 2.2. The percentage of labelled nuclei was determined by autoradiography as in section 2.6. Preparations appeared as in fig.2A and B, although smaller cell clusters were more frequent.

plemented with [^{3}H]thymidine. Culture samples were withdrawn at intervals and the percentage of labelled nuclei determined by autoradiography (fig.2A,B). The results show that, at 24 h, 95% of the nuclei were labelled. Thus, at this time 95% of cells had gone through at least a complete cell cycle (minus the S phase). The unlabelled 5% cell fraction appears to represent the non-cycling population, as suggested by the inability to obtain labelling of all nuclei even after 48 h incubation with the radioactive precursor. This phenomenon has already been observed in carrot as well as in other cell types [1,3,15,16].

We also checked the presence of an aphidicolin-inactivating activity in carrot cell cultures since conversion of the drug into inactive derivatives has been reported in rat liver cells [17]. The experimental results (to be published elsewhere) have shown that carrot cells are indeed endowed with an intracellular aphidicolin-inactivating activity, the rate of inactivation being dependent on the cell concentration. It was decided to overcome the problem by using step-wise addition of an appropriately high drug concentration.

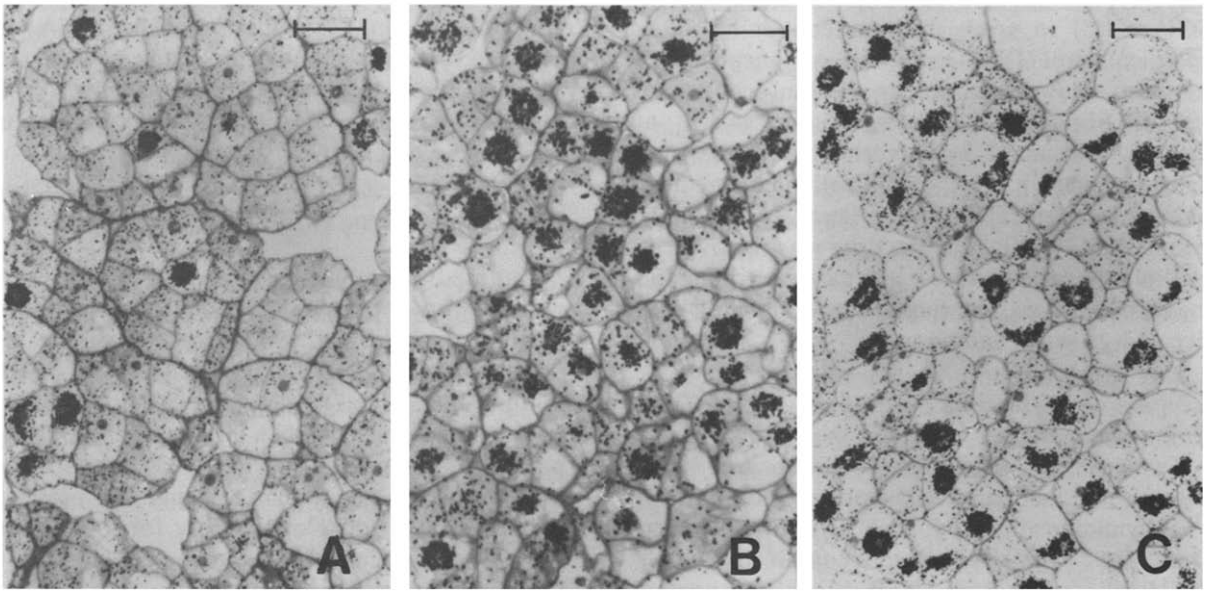

Fig.2. Autoradiographs, under the light microscope, of sectioned *Daucus carota* cells: (A,B) asynchronous cell population labelled for 3 h and for 24 h, respectively; (C) aphidicolin synchronized cells labelled for 3 h immediately after the drug removal. [^{3}H]Thymidine was added to culture samples (4 ml), the samples were incubated, fixed and labelled DNA revealed by autoradiography as in section 2.6. Photographs show exceptionally large cell clusters. The pattern of labelling showed no variation in clusters of smaller size (4–20 cells); bars, 30 μm.

Thus, in order to obtain accumulation of carrot cells at the G_1/S boundary and to induce synchronous release into the S-phase, aphidicolin (20 μg/ml) was added to a carrot cell suspension culture in the early exponential phase of growth at 4×10^5 cells/ml. Incubation was continued for 24 h with a second addition of aphidicolin (20 μg/ml) at 12 h. Aphidicolin concentration was in this way kept above the minimum inhibitory level (4 μg/ml) throughout the synchronizing period (fig.3). Replication of nuclear DNA was completely inhibited during the 24 h period (fig.3). The aphidicolin-insensitive organellar DNA synthesis, which accounted for 22% of total [^{3}H]thymidine incorporation at the moment of drug addition and proceeded at an unaltered rate

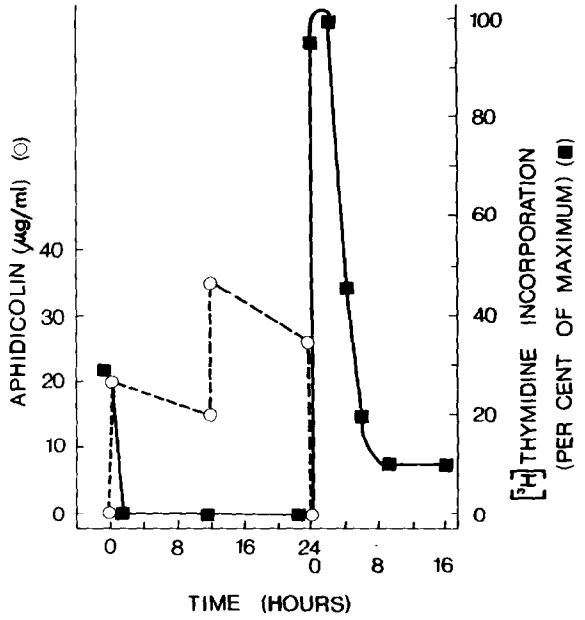

Fig.3. Nuclear DNA synthesis (□) in cultured cells of *Daucus carota* exposed for 24 h to aphidicolin. A cell culture was incubated as in the text and 20 μg/ml aphidicolin added at time zero and 12 h. Aphidicolin concentration (○) was measured by the biochemical test as in section 2.3. At 24 h aphidicolin was removed by 3 washes with fresh culture medium and incubation continued in the same experimental conditions. Nuclear DNA synthesis was assayed as in section 2.5 by subtracting the aphidicolin-insensitive [^{3}H]thymidine incorporation from total incorporation; maximum [^{3}H]thymidine incorporation was 74137 cpm/ml culture.

during the incubation in aphidicolin, was subtracted from the values shown in the figure. The mitotic index, which was ~3% in the initial cell cultures, was down to zero at time 24 h (not shown). Upon removal of aphidicolin, nuclear DNA synthesis, as assessed by 1 h pulse-labellings with [^{3}H]thymidine, resumed immediately, reached a maximum at 1–3 h and dropped at time 5–6 h.

An autoradiographic analysis of radioactively labelled cells with the [^{3}H]thymidine labelling performed for 3 h starting at the moment of drug removal, showed that 95% of the nuclei were labelled (fig.2C).

Thus, the immediate resumption of nuclear DNA synthesis and the observation that all cycling cells have radioactively labelled nuclei demonstrate that aphidicolin is highly effective and specific in synchronizing nuclear DNA replicative synthesis.

4. DISCUSSION

The experimental data have shown that treatment with appropriate doses of aphidicolin causes accumulation of cultured carrot cells at the G_1/S boundary of the cell cycle. The blockage is complete, affecting all the cycling cells (95% of the population) and reversible, resulting in immediate and synchronous resumption of nuclear DNA synthesis following removal of the drug. However, organellar DNA synthesis is apparently not affected by the treatment. The non-synchronized cells (~5%) most likely represent the fraction of the population that does not multiply, as described in carrot [16] and in other cell cultures [15,18].

Research is now under way to establish how long synchronization is maintained throughout the phases of the cell cycle and through subsequent cycles. Preliminary results show synchronous mitoses 9–10 h after the removal of aphidicolin from the carrot cell culture.

Furthermore, the sensitivity of all tested plant DNA polymerases α to aphidicolin [7,8,19,20] suggests that this drug is a universal synchronizing agent for plant cells. In fact, Nishinari et al. and Nagata et al. are now providing evidence for a highly effective aphidicolin induced accumulation of mitotic cells in tobacco suspension cultures (personal communication). Synchronous mitoses were also observed by one of us (in preparation) in radi-

cle tip cells 6 h after removal of aphidicolin from germinating seedlings of *Haplopappus gracilis*.

Obviously, a single treatment with aphidicolin as used in the above experiments, allows the accumulation at the G_1/S boundary of those cells which were out of the S-phase at the moment of drug addition. A small fraction of the cells is blocked in the process of DNA synthesis; if necessary, these cells, which are estimated to represent <10% of total population, might be forced to accompany more precisely the main stream by an appropriate second exposure to aphidicolin.

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