Polyamine-induced DNA Synthesis and Mitosis in Oat Leaf Protoplasts

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ABSTRACT

Freshly isolated protoplasts from leaves of oat seedlings (var. Victory) which do not divide when cultured on a wide range of media are capable of incorporating tritiated leucine, uridine, and thymidine into trichloroacetic acid-insoluble macromolecules. Over 70% of the leucine and uridine incorporated over an 18-hour period are found in protein and RNA, respectively, as shown by hydrolysis of the macromolecular products with a specific protease or RNase. In contrast, little or none of the tritiated thymidine is incorporated into macromolecules hydrolyzable by DNase over an 18- to 96-hour period. Incorporation of thymidine into trichloroacetic acid-insoluble material declines sharply with increasing time of culture after 18 hours. However, addition of diamines or polyamines to the medium not only prevents the decline, but actually increases net thymidine incorporation, including a fraction going into DNA. A significant increase in mitoses and binucleate protoplasts is also observed in 72- to 180-hour cultures.

The inability of oat leaf protoplasts to synthesize significant quantities of DNA suggests that they are arrested at the G1 phase of the cell cycle. Treatment with polyamines appears to promote both DNA synthesis and the inception of mitotic activity in oat protoplasts, as in numerous animal and microbial cells.

Protoplasts obtained from leaves of certain plant species, especially members of the Solanaceae like tobacco and potato, can synthesize new walls, undergo nuclear and cellular division, and ultimately regenerate entire plants (4, 22). Protoplasts from leaves of cereals do not undergo sustained mitotic division when cultured under similar conditions on a wide range of media (6, 17). These nondividing protoplasts show a time-dependent decrease of incorporation of precursors into presumptive protein and nucleic acids (8) and an increase in potentially detrimental hydrolyses such as RNase and Protease (10). This decrease in net synthetic activity and lack of mitosis in cereal protoplasts may result from a complex of senescence-induced changes (1) or injury caused by plasmolysis (19) that must precede protoplast isolation. These stresses can produce severe metabolic alterations, contributing to a blockage of the cell cycle and a consequent lack of mitosis.

Much evidence indicates that the naturally occurring diamines and polyamines are involved in regulating nucleic acid synthesis and cell division in microorganisms and animals (2); similar evidence is far less extensive for plants (3, 23). Our previous experiments established that polyamines can: (a) stabilize oat leaf protoplasts against spontaneous or induced lysis; (b) increase the incorporation of amino acids and nucleosides into trichloroacetic acid-insoluble materials in such protoplasts (1, 15); (c) retard Chl breakdown; and (d) decrease or prevent the rapid postexision rise of RNase and protease activity in oat leaves (16).

Recently, substantial evidence has accumulated suggesting an important role of polyamines in regulating DNA synthesis (5, 7) and an orderly progression through the animal cell cycle (9, 13, 24). For example, specific inhibitors of polyamine synthesis have been shown to prevent certain mammalian cells from entering mitosis (12, 24), while addition of spermidine or spermine to such cells results in rapid resumption of cell division (7, 21). Because of these facts, we considered it desirable to test the possibility that polyamine treatment might enhance cell division in plant protoplasts. We report here the characterization by enzymic digestion of macromolecular products resulting from incorporation of [3H]leucine, [3H]uridine, and [3H]thymidine into oat protoplasts, as well as effects of polyamines on DNA synthesis and on mitotic activity in protoplasts from oat leaves.

MATERIALS AND METHODS

The first leaf of 7-day-old seedlings of Avena sativa L. (var. Victory) was used for isolation of protoplasts. The seeds were grown in Vermiculite in controlled growth rooms with a 16-h photoperiod of 12,000 lux as detailed in an earlier report (8).

Isolation and Culture of Protoplasts. The leaves were sterilized by immersion in 70% ethanol for 2 min, two washes with sterile-distilled H2O, further dipping in 10% Clorox for 5 min containing Tween 20 (1 drop/10 ml), and finally five to six rinses with sterile-distilled H2O. All manipulations were performed aseptically in a laminar flow hood.

Protoplasts were isolated by stripping off the lower epidermis and floating the leaves, stripped side down, on 0.5% (w/v) Cellulysin (B grade, Calbiochem) in B5 medium (11) or in 1 mM phosphate buffer (pH 5.8) with 0.4 M sorbitol or 0.6 M mannitol for 2 h at 30 ± 1°C. The released protoplasts were collected by centrifugation at 50g for 5 min, then washed three times with the above medium by centrifugation and resuspension. The final protoplast pellet was resuspended at a concentration of about 3-107 protoplasts/ml and cultured in B5 medium with the following additives: 100 g/l mannitol, 15 g/l sucrose, 2 mg/l a-naphthylacetic acid, 1 mg/l BA, 6,000 mg/l CaCl2, 100 mg/l l-ascorbate, 100 mg/l inositol, 250 mg/l xylose, 250 mg/l arabinose, and 1 mM of any of the three polyamines (cadaverine, spermine, spermidine) as their HCl salts (Sigma). This concentration was chosen on the basis of previously reported favorable results (1), and because higher concentrations seemed to produce aberrant appearance in treated leaf segments (16). The culture medium was filter-sterilized and the pH was adjusted to 5.8. Control treatments included no polyamines. The protoplast suspensions were cultured as 20-μl drops hanging from covers of Petri dishes; 5 ml of the same medium was included in the bottom of each Petri dish to establish vapor phase equilibrium with the drops. The dishes were placed in moist chambers in the dark and the protoplasts were cultured for varying times at room temperature (about 23°C).

Incorporation of Labeled Precursors into Macromolecules. Al-

1 This work was supported by National Science Foundation Grant DAR 7813294 to A. W. G.
igots of 0.5-ml suspensions of protoplasts containing 2-5 x 10^6 protoplasts/ml were incubated usually for 4 h with 20 μl of 10 μCi/ml L-[4,5-3H(N)]leucine (60 Ci/mmol); 100 μCi/ml [5-3H]-uridine (27.6 Ci/mmol); or 100 μCi/ml [methyl-3H]thymidine (56.4 Ci/mmol). All radioactive precursors were purchased from New England Nuclear Co. The incubation was performed in triplicate in covered disposable microbeakers in a Dubnoff metabolic shaking incubator (40 reciprocal strokes/min) at 23 ± 1 C. At the end of each incubation period 20-50-μl aliquots were pipetted onto discs of Whatman No. 3MM filter paper and the incorporation of label into trichloroacetic acid-insoluble materials was measured as described in an earlier paper (8). The washed and air-dried discs were placed in 2 ml Aquasol (New England Nuclear) in minivials, and radioactivity was determined in an Autonon scintillation counter.

The specificity of incorporation of each precursor was assessed by determination of the degree to which the incorporation product could be hydrolyzed by appropriate specific enzymes. Leucine incorporation into protein was tested by Pronase (Calbiochem) incubated at 37 C for 1 h prior to use to inactive residual DNase and RNase activity. Filter paper discs containing the labeled material were incubated at 37 C for 1 h in 50 μg/ml Pronase in 100 mM Tris buffer (pH 8.0). Uridine incorporation into RNA was tested by pancreatic RNase ( Worthington), heated 5 min at 100 C prior to use to eliminate DNase and protease activity. Discs were incubated in 50 μg/ml RNase in 100 mM acetic acid (pH 5.5). Thymidine incorporation into DNA was tested by DNase (Worthington) stipulated as being RNase-free. Discs were incubated in 50 μg/ml DNase in 25 mM Tris buffer (pH 7) with 5 mM MgCl2. The activity of this DNase was tested against 3H-labeled λ-phage DNA. Over 90% of the counts from this material was solubilized by the enzyme. Preliminary experiments revealed that no counts from labeled macromolecules were lost from the filter paper discs during incubation in buffer alone; only active enzymes appeared to solubilize, and thus remove the counts from each of the labeled precursors. In each case, after appropriate enzymic treatment, the residual undigested macromolecule on the filter paper was precipitated with trichloroacetic acid, processed as described above, and counted.

Staining Procedure. Mitotic activity was determined by counting nuclei of protoplasts stained with 1% aceto-carmine or modified carbolfuchsin, according to the method of Kao (14). Briefly, aliquots of the protoplast suspension were mixed with the fixative acetic acid-ethanol (1:9, v/v) in 0.2 M mannitol and kept at 4 C for 24 h. The fixed protoplasts were stained and then gently spread to visualize the chromosomes without excessive crushing of the cell. The data presented are from single experiments, which generally were representative of a number of experiments (two to four) in each treatment.

RESULTS AND DISCUSSION

Characterization of Macromolecular Products. Freshly isolated protoplasts from oat leaves, despite the injury and osmotic stress experienced during isolation, are able to incorporate [3H]leucine, [3H]uridine, and [3H]thymidine into trichloroacetic acid-insoluble macromolecules (Table I; see also ref. 8). The net incorporation increased with time in culture for 18 h. Separate digestion of the trichloroacetic acid-insoluble components with Pronase and RNase showed that in both fresh and 18-h-old protoplasts, over 70% of the leucine and uridine were incorporated into authentic protein and RNA, respectively. In contrast, none of the thymidine was incorporated into DNA of freshly isolated protoplasts, and only about 13% was incorporated into DNA of the cultures aged for 18 h before exposure to [3H]thymidine (Table I). Even when the protoplasts were cultured for longer periods (4-8 days), DNA synthesis did not occur significantly and the protoplasts did not show any mitotic division. In tobacco protoplasts, on the other hand, both incorporation of thymidine into DNA and cell division have been observed (25). Oat protoplasts can synthesize abundant protein and RNA, but not significant quantities of DNA, and hence appear to be arrested at the G1 phase of the cell cycle.

Effect of Polyamines on Incorporation of Thymidine. The ability of oat protoplasts to incorporate [3H]thymidine into trichloroacetic acid-insoluble material decreases rapidly with time of culture of protoplasts after 18 h (Table II). Treatments with 1 mM cadaverine, spermidine, or spermine not only prevent this decline, but increase the extent of incorporation of thymidine into trichloroacetic acid-insoluble materials. Spermine treatments were most effective in enhancing incorporation and stabilizing the protoplasts during culture.

The rapid decline in the ability of the control protoplasts to synthesize DNA during culture suggests that these protoplasts are subject to postisolation senescence. Reduction of such senescence, as shown by polyamine-induced net increase in thymidine incorporation, is supported by earlier observations of polyamines as senescence inhibitors in oat leaves (16) and oat protoplasts (1, 10, 15). The effect of polyamines on DNA synthesis and Mitotic Activity. Measurements of the amount of incorporated [3H]thymidine removed by digestion with DNase (Table III) show that only about 12% of such thymidine was present in DNA in control protoplasts. Addition of cadaverine or spermine to the culture medium in-

<p>| Table I. Characterization of Macromolecular Products Resulting from Incorporation of Tritium-labeled Leucine, Uridine, and Thymidine into Oat Leaf Protoplasts |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Age of Protoplasts</th>
<th>[3H]Leucine</th>
<th>[3H]Uridine</th>
<th>[3H]Thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronase</td>
<td>Incorporation into Protein</td>
<td>RNase</td>
<td>Incorporation into RNA</td>
</tr>
<tr>
<td>0</td>
<td>514*</td>
<td>120</td>
<td>77</td>
</tr>
<tr>
<td>18</td>
<td>4852</td>
<td>836</td>
<td>83</td>
</tr>
</tbody>
</table>

* Incorporation data given as cpm/5 × 10^6 protoplasts.
Table III. Effect of Polymamines on Incorporation of \[^{3}H\]Thymidine into DNA of Oat Leaf Protoplasts Cultured as Hanging Drops for 96 h

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Incorporation of [^{3}H]Thymidine into DNA</th>
<th>Incorporation into DNA</th>
<th>cpm/6 × 10^6 protoplasts</th>
<th>Δ</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[[^{3}H]Thymidine +DNase +DNase] Difference</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, B5</td>
<td>284</td>
<td>251</td>
<td>33</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>+1 mm cadaverine</td>
<td>326</td>
<td>267</td>
<td>59</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>+1 mm spermidine</td>
<td>412</td>
<td>328</td>
<td>84</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

Increased this value to about 18 and 21%, respectively. Although these polyamine-mediated increases in DNA synthesis are relatively small, they are consistent and significant not only in the 4-day old cultures, but also in cultures continued for longer periods.

The increased DNA synthesis led us to investigate the effect of polymamines on mitotic activity. Whereas control protoplasts showed only sporadic nuclear divisions, treatments with 1 mm spermidine or spermine significantly increased their frequency (Table IV). Polymamine treatments produced numerous binucleate protoplasts as well as typical mitotic figures (Fig. 1). These observations demonstrate that (a) oat leaf protoplasts cultured on usual defined media are able to synthesize abundant proteins and RNA, but not substantial quantities of DNA; and (b) treatments with polymamines measurably increase DNA synthesis as well as mitotic activity in these cells.

Several recent studies (9, 13) with mammalian cells indicate that polyamine biosynthesis and accumulation are temporal and quantitative prerequisites for progression through the cell cycle. Recently, in plant systems also, the endogenous levels of polymamines have been correlated with activities of embryogenic cells and pea seedlings (18, 20). It is possible that the level of polymamines and their precursor amino acids, as well as the activities of key enzymes involved in polyamine biosynthesis and degradation, should show some relation to metabolic vigor and mitotic activity of oat protoplasts. We are now conducting such investigations, as well as studies of the general significance of polymamines in plant physiology.

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