Nucleic Acid Metabolism During Cytokinin Induced Cellular Differentiation

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Abstract. Edstrom's microphoresis technique has been employed to determine the quantitative alterations in nucleic acid content and base composition of individual cells associated with the initiation of bud primordia in Funaria hygrometrica. The filamentous protonemata of this moss initiates bud cells which through repeated divisions form the leafy gametophores. The cytokinin, 6-furfurylamino purine (kinetin), was used to induce the differentiation of bud cells from protonematal cells. The total RNA content of kinetin-induced bud cells (22.0 \( \mu g/\text{cell} \)) was nearly 15 times that of protonematal cells (1.6 \( \mu g/\text{cell} \)). The same dramatic increase in total RNA was apparent in bud cells which developed spontaneously in older cultures. As would be predicted, the adenine (A) to guanine (G) ratio for DNA from bud and protonematal cells was identical (0.7). The A:G ratio for RNA from bud cells (1.0) was much lower than that from protonematal cells (1.7). Thus, kinetin-induced differentiation in this system involves a dramatic increase in total RNA, the base composition of which approaches that of DNA. The base composition (A:G ratio) of DNA remains constant.

Changes in nucleic acid metabolism are particularly relevant to an understanding of cellular differentiation. In multicellular organisms the problem of measuring nucleic acids has been approached either with direct photometric measurements on histological sections, or by microdissection of individual cells and organelles followed by microextraction and microelectrophoresis (microphoresis) of nucleic acids by special methods developed by Edstrom (7, 8, 9). These latter methods permit the nucleic acid content and composition of individual cells and cellular units to be measured quantitatively. We have used these methods to determine changes in nucleic acid metabolism associated with spontaneously and hormonally-induced cellular differentiation. Spores of the moss Funaria hygrometrica germinated on an agar medium form protonemata, a mass of filamentous cells. The protonemata branch and certain ones eventually divide to form bud primordia. These bud cells continue to divide and differentiate into the leafy gametophore or moss plant. The cell division which gives rise to the bud cell is thus of great consequence, for from a non-meristematic protonematal cell there arises a cell which remains meristematic. The question is how do the nucleic acid contents and composition differ in the bud cell and in the protonematal cell from which it originated? Bud cells develop spontaneously in older cultures but can be induced to form in younger cultures by an exogenous supply of cytokinins. Is the cytokinin treatment reflected in the nucleic acid metabolism of protonematal and bud cells?

Materials and Methods

Spores of Funaria hygrometrica were grown aseptically on Koller medium minus \( \text{NH}_4\text{NO}_3 \) as recommended by Szewykowski (17). Micronutrients were supplied by Heller's solution (14). The medium plus 1 % agar was contained in plastic Petri dishes. Spore capsules were surface sterilized by using 95 % ethanol for 1 min followed by a wash in a 0.2 % \( \text{HgCl}_2 \) solution for 5 min and then a sterile water wash. The capsules were transferred to a
Petri dish and opened with sterile needles. Dispersion of the spores across the agar surface was facilitated by adding 1 ml of sterile distilled water to each dish. The cultures were maintained at 22.5 ± 1.0° and received continuous illumination of 200 ft·c from cool-white fluorescent lamps.

Fourteen days after inoculation the protonemata were harvested and fixed in freshly prepared acetic acid:absolute ethanol, 1:3 v/v, for 90 min at room temperature followed by absolute ethanol and benzene, 90 min each. Next, the tissues were infiltrated with soft paraffin for 4 hr, transferred to additional soft paraffin for 10 hr and finally hard paraffin for 4 hr. All infiltrations were performed at 56°. The resulting paraffin blocks were stored at 0°.

Prior to the enzymatic extraction of RNA and DNA nucleotides, the tissues were deparaffinized in chloroform at 56° for 7 min. Successive transfers at room temperature into chloroform, chloroform, absolute ethanol and 0.01 N acetic acid were made after intervals of 5 min each. Once deparaffinized and hydrated, the tissues were placed on a cover slip and then into a de Fonbrune oil chamber. Dissection and isolation of cells was accomplished by means of a de Fonbrune micromanipulator with glass needles (6). The ultramicrochemical methods for determinations of DNA and RNA have been described by Edstrom (7, 8, 9).

The RNA of the cells was hydrolyzed with pancreatic ribonuclease (Worthington Biochemical Corporation) buffered at pH 7.6, and the resulting oligoribonucleotides extracted during three 1 hr incubation periods at 38°. The dried extracts were redissolved in a glycerol containing buffer and photographed together with a reference system. Photomicroscopy was performed with a microscope equipped with quartz optics and actinic UV light (265 nm) obtained from a Zeiss monochromator used in conjunction with a Zeiss power supply and a Xenon 450 w lamp. UV absorption was detectable on the negatives as areas of low grain density. The absolute amount of RNA was determined by scanning the negatives on a Joyce Loebl recording microdensitometer. From calculations based on these curves, quantities as low as 200 μg RNA could be analyzed.

For analysis of RNA base composition, the RNA extracts were hydrolyzed in 4 N HCl contained in a microsyringe at 100° for 30 min. The hydrolysates were applied to a single cellulose fiber mounted on a quartz coverslip. Electrophoretic separation of the 4 major bases along the fiber was achieved with ca. 4000 V in ca. 4 min and the fiber was immediately photographed. The negatives were scanned on the microdensitometer and the ratios and percentage composition of RNA bases were calculated from the absorption curves.

Subsequent to the extraction of RNA outlined above, cells were extracted with electrophoretically purified deoxyribonuclease (Worthington Biochemical Corporation). The enzyme was buffered at pH 5.2 and used for four 1 hr extractions at 38°. The dried extracts were dissolved in droplets of 1 N HCl and incubated in an oil chamber for 18 hr at 38°. The extracts were applied to a cellulose fiber and the DNA bases separated by microphoresis. Using the same photographic-photometric procedure outlined for RNA, the adenine:guanine ratio for DNA was determined. In complementary DNA, this ratio defines the other base quotients. The absolute amount of DNA can be estimated by including a measured amount of cytidine in the extract prior to microphoresis.

Results and Discussion

Plants of Funaria hygrometrica grown on the modified Koller nutrient medium plus 4.65 μM kinetin initiated bud-like cells 14 days after inoculation. On control medium lacking kinetin, bud cells were observable 21 days after inoculation. Induction of bud formation in mosses by cytokinins was first recognized by Gorton et al. (10, 11). The effect of growth regulators and culture methods on the development of Funaria hygrometrica are reported by Szweykowski (15, 16). Further references are provided by Brandes and Kende (5), Bopp and Brandes (3), and Jahn (12).

Table I shows that the RNA content of bud cells was nearly 15 times greater than that of the protonemal cells from which they originated. The exact significance of this increase, however, can not be fully appreciated until the cytoplasmic volumes of protonemal and bud cells are accurately determined and compared. The increase reported here was obtained in bud cells which developed spontaneously (lot A) as well as in bud cells whose initiation was induced by kinetin (lot B).

Table 1. Total RNA in Cells From Protonemata and Buds of Funaria hygrometrica

<table>
<thead>
<tr>
<th>Lot</th>
<th>Cell</th>
<th>RNA</th>
<th>Cells/ estimation</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg/cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Protonema</td>
<td>1.8 ± 0.29</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>bud</td>
<td>17.4 ± 0.98</td>
<td>50</td>
<td>9</td>
</tr>
<tr>
<td>B</td>
<td>Protonema</td>
<td>1.6 ± 0.11</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>bud</td>
<td>22.0 ± 1.74</td>
<td>50</td>
<td>15</td>
</tr>
</tbody>
</table>
development of buds in older cultures lacking an exogenous supply of cytokinins, presumably occurs in conjunction with an increase in endogenous cytokinin action. Evidence for a naturally occurring cytokinin, "bryokinin," has been reported by Bauer (2) and Klein (13).

Using deoxyribonuclease, DNA oligonucleotides were extracted from cells which had been previously isolated by microdissection and extracted with ribonuclease. The adenine:guanine (A:G) ratio for DNA from protonemal and bud cells was identical (Table II) as would be expected. The A:G ratio for DNA is known to vary among taxonomically diverse organisms, but at the same time does reflect taxonomic relationships. Guanine-rich DNA noted here in a moss, is also common to green algae. Saccodesmus quadricauda is reported (18) to have an A:G ratio near 0.6. Finally, using the same photographic-photometric procedures, the A:G ratios of RNA from protonemal and bud cells were compared. Table II shows that the A:G ratio for RNA from bud cells is much lower than the ratio determined for protonemal RNA and thus resembles more closely the A:G ratio determined for DNA. Inhibitor studies with Actinomycin D have provided circumstantial evidence for DNA dependent RNA synthesis during bud initiation (4). Using Edstrom methodology, RNA from various cellular fractions of the alga, Acetabularia, was also found to be adenine-rich (1).

In summary, the differentiation of a meristematic bud cell from a non-meristematic protonemal cell involves ca. 15 fold increase in RNA. The newly synthesized RNA approaches DNA in its base composition. These trends are apparent in bud cells induced by an exogenously supplied cytokinin as well as in bud cells of spontaneous origin. The base composition of DNA in protonemal and bud cells was found to be identical.

**Table II. Adenine/Guanine Ratios for RNA and DNA From Cells of Funaria hygrometrica**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Nucleic acid</th>
<th>Adenine</th>
<th>Guanine</th>
<th>Cells/ estimation</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protonema</td>
<td>RNA</td>
<td>1.7 ± 0.12</td>
<td>120</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DNA</td>
<td>0.7 ± 0.03</td>
<td>120</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Bud</td>
<td>RNA</td>
<td>1.0 ± 0.05</td>
<td>100</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DNA</td>
<td>0.7 ± 0.02</td>
<td>50</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

Plants were grown for 14 days on nutrient agar containing 4.65 µM kinetin.

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**Literature Cited**


