Ribonucleic Acid Synthesis Associated with a Developmental Change in the Gametophyte of Pteridium aquilinum

Received for publication October 4, 1971

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ABSTRACT

When bacteria are subjected to step-down conditions, there is an enhanced production of a messenger-type of RNA for a short time after the shift. A cultural shift, which appears to be similar to step-down, is described for gametophytes of Pteridium aquilinum. When the cultures are grown in white light, a population of rapidly dividing cells is produced, whereas in red light cell elongation predominates. If the cultures growing in white light are shifted to red light, a transition occurs which involves a rapid decrease in growth and nucleic acid synthesis. In particular, there is a marked decline in RNA synthesis for a short time following the shift and prior to the initiation of the new mode of growth. It appears that this observed change in RNA synthesis is related to the initiation of the new mode of growth.

When a bacterial culture is subjected to a nutritional shift, which results in an increased or decreased growth, associated temporal changes in macromolecular synthesis are observed. The synthesis of RNA is first observed to change, and this is followed by altered rates of protein and DNA synthesis (26). In particular, in step-down there is a transient period immediately following the shift to a poorer growth medium when there is a decrease in the accumulation of rRNA and an enhanced production of a messenger-type RNA (7, 9). Since the production of this RNA occurs prior to the establishment of the new growth rate, it has been suggested that the formation of this RNA is directly related to the initiation of the new phase of growth (14).

The present study describes a growth shift in a culture of plant cells which resembles step-down. By changing the light conditions, a culture of rapidly dividing cells is converted to a population with a low rate of cell division and a high potential for cell elongation. Prior to the initiation of the new mode of growth, there is, for a short period, an increased synthesis of a type of RNA which resembles D-RNA (DNA-like RNA) and rRNA precursor.

MATERIALS AND METHODS

Culture Conditions and Techniques. Methods of culture and growth measurements were as previously described (31, 32). Spores of P. aquilinum were collected in August, 1969 in Youngstown, Ohio. The spores were sterilized with a 1:8 dilution of commercially obtained Clorox for 8 min, and, after a wash with sterile water, the spores were inoculated into liquid medium at a concentration of 1 mg/10 ml of medium. The gametophytes were cultured on a modified Knudson's salt solution plus trace elements and 0.25% sucrose. The pH of the medium was adjusted to 5.5.

Cultures were maintained in a culture room at 25 ± 2° C with a light source of Sylvania “Gro-Lux” fluorescent lamps. The white light conditions consisted of continuous subillumination of white light at 9.5 × 10³ ergs/cm²·sec. Red light, at an energy level of 0.3 × 10³ ergs/cm²·sec with a light period of 12 hr light and 12 hr darkness, was obtained by using cinemoid filters Nos. 14, 16 (Kliegel Corporation, Long Island City, N.Y.). Light intensity was measured with a YSI Model 65 radiometer with a wavelength response of 0.25 to 3.30 µ. The development of the gametophytes was followed by determination of cell numbers microscopically. Estimates of total number of cells per culture vessel were made by estimating the number of spores in the original inoculum with a hemocytometer as previously described (32). Sample size for cell counts was 100 or more gametophytes.

Extraction and Determination of Total Nucleic Acid. Nucleic acids were extracted as described by Key and Shannon (15) with the modification that after hydrolysis of the RNA the contaminating carbohydrates were removed with Dowex-1 resin, as suggested by Smillie and Krotkov (30). The gametophytes from individual cultures were collected by filtration and then homogenized in 5 ml of ice-cold water with a mortar and pestle. Four-milliliter aliquots of this homogenate were precipitated in duplicate with perchloric acid (0.2 N final concentration) in ice for 30 min, washed two times with 0.2 N perchloric acid to 4 C, and then defatted for 30 min at 37 C with a 2:2:1 (v/v) mixture of ethanol:ether:chloroform. The pellet was then subjected to hydrolysis in 0.3 N KOH for 18 hr at 37 C, acidified to about 0.3 N with perchloric acid, and centrifuged in the cold. The supernatant, containing the RNA nucleotides, was removed, and the residue was washed with cold 0.2 N perchloric acid. The supernatant and washings were then adjusted to pH 8 with KOH, chilled, and centrifuged in the cold. The residue was then washed two times with cold water. The combined supernatants were then placed on a column of Dowex 1 × 8, chloride form, 200 to 400 mesh. The column was washed with 10 M NaCl, and the nucleotides were eluted with a HCl-NaCl solution (20 ml of 10 M HCl + 5.6 g of NaCl in 240 ml of water). RNA was then estimated with orcinol reagent according to the technique of Lin and Schjeide (17). The DNA was extracted from the KClO₄, precipitate by hydrolysis at 70 C for 40 min with 0.5 N perchloric acid. After hydrolysis the material was neutralized with KOH, centrifuged to remove KClO₄, and the DNA content of the supernatant was determined by the diphenylamine reaction (4).

To determine the base composition of the RNA, cultures previously labeled with ³²P-orthophosphate were subjected to extraction and hydrolysis as described above. (Unlabeled RNA...
1–2 mg was added to each sample prior to the hydrolysis step to obtain sufficient absorbance for fractionation). After hydrolysis, the supernatant containing the RNA nucleotides was adjusted to pH 6 with KOH and centrifuged. The nucleotides were separated using a Dowex 1 × 8 formate column connected to an Instrumentation for Scientific Research ultraviolet optical unit, recorder, and fraction collector. A stepwise gradient of formic acid was used to elute the nucleotides (CMP 0.15 N, AMP 1.0 N, GMP 3.0 N, UMP 4.0 N containing 0.1 N ammonium formate [12]). The peak tubes for each nucleotide were combined, and an aliquot was removed for counting. Base composition is expressed as 32P distributed among the four nucleotide peaks.

**Extraction and Fractionation of RNA on Polyacrylamide Gels.** Undegraded nucleic acids were extracted by using the diethyl pyrocarbonate-sodium dodecyl sulfate method as described by Solymosy et al. (35). Gametophytes from individual cultures were collected by filtration and then homogenized at 4°C in 6 ml of 50 mM tris buffer at pH 7.6 containing 1% (w/v) sodium dodecyl sulfate, 5 mM MgCl₂, and 0.2 ml of diethyl pyrocarbonate. The homogenate was then incubated for 5 min at 37°C, centrifuged, and 0.6 g of NaCl was added to the supernatant. An additional incubation at 37°C for 5 min followed; the material was centrifuged, and the supernatant was added to three volumes of cold ethanol. The nucleic acids were collected by centrifugation after precipitation overnight at -16°C. In some cases DNA was removed from the precipitate by digestion with electrothoretically purified DNase at 10 μg/ml (19). The sample was then deproteinized with CHCl₃ and reprecipitated from ethanol.

The nucleic acids were fractionated by polyacrylamide gel electrophoresis according to the method of Loening (19) as previously described (33). Samples, dissolved in the running buffer and RNase-free sucrose, containing about 25 to 50 μg of nucleic acid, were layered on the 2.4% gels. The gels were electrophoresed for 3 hr at 5 mA/gel, at which time the 4S and 55 RNAs had run off the gel. At the completion of the run, the gels were soaked in distilled water for 30 min and then scanned at 265 nm by using a Joyce Loebel Polyfrac ultraviolet scanner. The gels were then frozen in Dry Ice and cut into 0.8-mm sections with a Joyce Loebel chopper for counting 32P-radioactivity.

**Culture Shift and Labeling Procedures.** Cultures, inoculated with a known quantity of spores, were placed in continuous high energy white light for a period of 4 days, at which time the gametophytes contained an average of two cells with about 80% of the gametophytes at the two-cell stage. At this time, the cultures were shifted to low energy red light with a light period of 12 hr light/24 hr. Samples were taken at different times by filtration of individual cultures after removal of 0.5 ml of a 50-ml culture for determination of developmental stage and cell number.

For extraction of total RNA and DNA, the cells were labeled by adding sterile, carrier-free 32P-orthophosphate to the cultures, 50 μc/culture for 3 hr for RNA and 10 μc/culture for 24 hr for DNA. Cultures to be used for extraction of undegraded nucleic acids were first concentrated 10-fold in a sterile Millipore filter apparatus. To the 5 ml of culture suspension remaining on the filter was added sterile, carrier-free 32P-orthophosphate for a period of either 2 hr (150 μc) or 7 hr (50 μc). Following the labeling period the cells were collected by filtration, washed two times with 50 mM Na₂PO₄, and once with distilled water. The cells were then either dried directly on the filter for measurement of total uptake or washed off the filter into a mortar and extracted as described above.

**Measurement of Radioactivity.** After extraction and hydrolysis of labeled cultures, aliquots of RNA and DNA were absorbed onto 2.1-cm glass filters. The filters were then dried and placed in scintillation vials with 10 ml of scintillation fluid containing per liter of toluene 5.0 g of PPO and 0.3 g of dimethyl POPPO. The vials were then counted in a Beckman LS-133 liquid scintillation spectrometer. RNA nucleotides recovered from Dowex 1 × 8 columns and cell samples dried on filters were counted in the same manner. The 32P-radioactivity of individual slices of the gel was determined by drying the slices on filter paper, which was then placed in scintillation vials containing 10 ml of scintillation fluid. All samples were counted to 5% error.

**RESULTS**

**Growth Pattern.** In continuous, high intensity white light, spores of *P. aquilinum* germinate and produce a cluster of densely cytoplasmic isodiametric cells having an average diameter of about 21 μ. Up to a period of about 7 days when a meristem becomes evident, all of these cells undergo cell division. Cultures grown in red light produce filaments of highly elongated cells (about 18 × 147 μ) with cell division confined to the apical cell of the filament (34). During these early stages of development, a rapid increase in cell number occurs in white light, whereas cultures developing in red light grow much more slowly (Fig. 1).

If cultures are permitted to develop in white light to about the two-cell stage (4 days) and shifted to red light, a biphasic growth curve is obtained (Fig. 1). Within the first 12 hr after transfer to red light, cell division stops. During the next 12 to 24 hr, the cells start to enlarge, and then usually one of the cells initiates filamentous growth, which is observed as an outpocketing. Continued development in red light leads to formation of a filament.

**Nucleic Acid Synthesis.** The accumulation of DNA and RNA was examined for cultures grown in white light and red light and for cultures subjected to shift conditions (white to red light). When cultures were maintained solely in either white or red light, the greatest increase in both DNA and RNA occurred in white light (Table I). When cultures were shifted from white to red light, there was a decrease in accumulation.
Table I. A Comparison of the Accumulation of DNA and RNA in Cultures Grown Under Different Light Conditions

Cultures inoculated with equal numbers of spores were permitted to develop in white light (W), red light (R) or under shift conditions, white to red light (W-R). The cultures were shifted to red light after 4 days in white light. At 4 days and daily intervals thereafter individual cultures were sacrificed for determination of total DNA and RNA.

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>Accumulation of Nucleic Acids</th>
<th>µg/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA W</td>
<td>R</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>44</td>
<td>17</td>
</tr>
</tbody>
</table>

**Fig. 2.** Change in RNA synthesis, as measured by uptake of 32P-orthophosphate, in cultures grown in white light (○) and red light (○). Arrow indicates when the cultures were shifted to red light.

Table II. Base Composition of 32P-RNA in the Shift Experiments

Cultures growing in white light, and after shift to red light, were labeled for 3 hr with 32P-orthophosphate (50 µg/culture).

<table>
<thead>
<tr>
<th>Culture Period</th>
<th>Composition of Radioactive RNA</th>
<th>32P-Mole Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMP</td>
<td>AMP</td>
</tr>
<tr>
<td>W-4, 4 days white light</td>
<td>23.6</td>
<td>24.1</td>
</tr>
<tr>
<td>R-12, 4 days white light and 12 hr red light</td>
<td>20.5</td>
<td>28.9</td>
</tr>
<tr>
<td>R-4, 4 days white light and 4 days red light</td>
<td>23.5</td>
<td>24.9</td>
</tr>
</tbody>
</table>

of DNA and RNA relative to cultures retained in white light (Table I).

The rate of RNA and DNA synthesis in cultures subjected to shift conditions was measured by incorporation of carrier-free 32P-orthophosphate. There was a severe drop in the incorporation of isotope into RNA when the cultures were shifted to red light (Fig. 2). This was followed by a slight increase in RNA synthesis, which then stabilized to a new rate considerably lower than that occurring in white light. In addition to the labeling of the cultures at 24-hr intervals in both white and red light, the cultures were labeled every 4 hr after transfer to red light. The severest depression in RNA synthesis occurred about 12 hr after transfer. In the figure, the specific radioactivity of the RNA is expressed per culture. The same drop in activity is observed if the specific radioactivity is expressed per cell or per unit RNA, since these values change very little after the shift.

The incorporation of label into DNA showed a pattern similar to that observed for RNA. For DNA, however, the drop in synthesis immediately after the shift was not so severe and appeared to occur at a somewhat later time, about 18 to 20 hr after shift. Total uptake of label for cultures growing in red light was lower than for cultures growing in white light. When white-light cultures were shifted to red light, a gradual decrease in uptake was observed. After about 30 to 36 hr, this decrease stabilized to a rate of uptake comparable to material cultured only in red light. No sharp decrease in uptake was observed coinciding with the sharp drop in RNA synthesis at about 12 hr after the shift to red light.

**The 32P-Base Composition of Shift RNA.** Since there was a marked drop in RNA synthesis in the shift cultures, the 32P-base composition of cultures at different stages of growth was examined. Cultures were pulsed for 3 hr with 50 µg of sterile 32P-orthophosphate at three different periods of development: (a) after 4 days growth in white light (two-cell stage), (b) 12 hr after shift to red light (stage at which cell division has stopped), (c) 4 days after the shift to red light (filamentous growth). The DNA was extracted and fractionated as described in “Materials and Methods.”

Coinciding with the drop in specific radioactivity, a change in the 32P-base composition of the RNA was observed after the shift to red light (Table II). Twelve hours after transfer to red light the cultures had a base composition notably high in AMP and low in GMP. The base compositions of cultures grown in white light and those that had been in red light several days did not show the high content of AMP.

**Characterization of Shift RNA by Gel Electrophoresis.** The nucleic acids were characterized by fractionation on polyacrylamide gels. Cultures growing in white light for 4 days were labeled for 7 hr with 50 µg of sterile 32P-orthophosphate, and the nucleic acids were extracted by the pyrocyanate method. The nucleic acids were electrophoresed on 2.4% polyacrylamide gels for 3 hr at 5 ma/gel. On the basis of cochromatography with nucleic acids extracted from Escherichia coli (21) and susceptibility to degradation with DNase, the three absorbance peaks were identified as (a) DNA, (b) 1.3 × 10⁵ mol wt cytoplasmic rRNA, and (c) 0.7 × 10⁴ mol wt cytoplasmic rRNA (Fig. 3A). It can be observed there is good coincidence between the absorbance and the 32P profiles.

To further characterize the shift, RNA cultures were pulsed with 150 µg of sterile 32P-orthophosphate for 2 hr at three different times: (a) after 4 days growth in white light, (b) 12 hr after transfer to red light, and (c) 4 days after transfer to red light. The nucleic acids were then extracted, the DNA was removed with DNase, and the RNA was electrophoresed on 2.4% polyacrylamide gels for 3 hr at 5 ma/gel. Prior to the shift, most of the newly synthesized 32P-RNA was associated with the two RNA absorbance peaks, with a small radioactive peak following the rRNA (Fig. 3B). Immediately following shift to red light, the major portion of the 32P-RNA appears as a broad peak following the rRNA and trailing into the rRNA region of the gel (Fig. 3C). After 4 days in red light, the radioactive pattern was similar to that observed prior to the shift.
DISCUSSION

Cultures of gametophytes, in the early stages of development, show two distinct patterns of development when grown under different light conditions. A rapidly dividing population of isodiametric cells is produced in high intensity white light, whereas a population of highly elongate cells with a low potential for division is produced in red light (34). If cultures are allowed to develop in white light to the two-celled stage and then shifted to red light, the following sequence is observed: (a) cell division stops, (b) the cells enlarge, (c) one or both of the cells starts to elongate, (d) a filamentous type of growth then continues. Thus in the shift experiments there is a transition from a cell with a high potential for cell division to a cell with a low potential for cell division and a high potential for elongation. Of particular interest to this investigation are two aspects of this transition: (a) there is a lag period which precedes the initiation of the new mode of growth, and (b) the transition occurs without cell division, i.e., the same cell which had a high potential for division in white light elongates in red light.

Cultures of these two different types of cells show large differences with respect to the accumulation of DNA and RNA, with the greatest increase occurring in the rapidly growing cultures. Thus the two different patterns of growth are easily distinguishable in terms of their nucleic acid synthesis. Similar differences, especially with reference to RNA, have been reported for other fern gametophytes (3, 10, 25, 27, 28).

In terms of the observed differences in nucleic acid accumulation, the drop in the rate of synthesis observed in the shift experiment was predictable. In addition, however, a severe drop in RNA synthesis in particular was observed during the transitory period prior to the establishment of the new mode of growth. On the basis of \(^3^P\)-base composition and migration on gels, it appears that the pattern of RNA synthesis during this period is distinctly different from that occurring prior to or about 24 hr or more after the shift.

The RNA produced during this period had a base composition high in adenine and low in guanine, and on the gels most of the counts occurred in the region normally assigned to the rRNA precursor (5, 8, 16, 29) and trailing into the rRNA region of the gel. The base composition of this RNA is suggestive of the D-RNA which has been described in other plant tissues (6, 11, 12, 20). It has been suggested that D-RNA probably represents both mRNA and nuclear RNA (20). Ingle and Key (13) have demonstrated that purified D-RNA migrates to a position of rRNA precursor on gels and trails into the rRNA region, similarly to the pattern observed here. Thus it appears the shift RNA described here probably represents a mixture of D-RNA and rRNA precursor.

The decrease in growth rate and RNA synthesis, coupled with the transient production of RNA resembling D-RNA in the shift experiments, suggests a pattern of development similar to step-down in bacterial cultures. Classically, the term step-down has been applied to the observed decrease in growth that occurs when bacteria are shifted from a rich to a poor energy source (9, 26). Immediately following such a shift there is a marked drop in RNA synthesis, followed by the establishment of a new rate of RNA synthesis characteristic of the new growth rate. During the transitory period, there is the formation of a heterodisperse RNA having a base composition distinct from the bulk RNA (7, 9, 14). The most recent evidence on RNA synthesis during step-down suggests that during the interim there is a decrease in the accumulation of rRNA (6, 24, 25) and an accumulation of a messenger-type RNA either due to preferential synthesis (14) or increased stability (7).

In addition to bacterial cultures, nutritional step-down has been observed in yeast (24), cultured plant cells (1), and cultured mammalian cells (2, 22, 36). It has, in fact, been suggested that step-down may not necessarily involve a nutritional shift, but may generally involve any situation which induces a sharp decrease in growth (23). Loening (18) has suggested that the sharp decrease in growth and net macromolecular synthesis observed in excised tissue is representative of a step-down situation. Also, in the plant systems which have been subjected to step-down conditions there has been an increased synthesis of D-RNA (1, 20, 29). It thus appears that the pattern of growth and RNA synthesis observed here is comparable to step-down described in other cultured material. As an additional proof that developmental change observed here represents step-down, it has been previously observed that filament formation will result from any condition which inhibits growth, including: various inhibitors and changes in nutrition, light, and availability of oxygen (31, 32).

In summary, a developmental change is described which is comparable to step-down observed in bacteria and other cultured material. Prior to the initiation of the new mode of growth, there is an increased synthesis of a type of RNA which resembles D-RNA and probably represents a combination of rRNA precursor, rRNA, and mRNA. It appears that the formation of this distinct type of RNA is related to the initiation of the new mode of growth in a manner similar to that observed during the "diauxic lag" in E. coli (14).

Acknowledgments—I would like to acknowledge the technical assistance of Richard Dix and Carol Redmond. This research was supported in part by a grant from the University Research Council and by National Science Foundation Grant GF 7608.
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**LITERATURE CITED**


