REQUIREMENT FOR THE SYNTHESIS OF DNA-LIKE RNA FOR GROWTH OF EXCISED PLANT TISSUE*

BY JOE L. KEY AND JOHN INGLE

DEPARTMENT OF BOTANY AND PLANT PATHOLOGY, PURDUE UNIVERSITY

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Although the mechanisms and control of cell elongation in plant tissue are still largely unknown, recent investigations have clarified certain aspects of this problem. Noodén and Thimann have presented evidence that the synthesis of some, if not total, protein is required for cell enlargement. They suggest that the role of auxin in the control of cell enlargement may be via nucleic acid metabolism, which in turn regulates synthesis of the essential protein. Key has reported that the synthesis of RNA, in addition to protein, is essential for the cell elongation of soybean hypocotyl tissue. The conclusions from these studies, and also those presented in this communication, assume the primary mechanism of inhibition by actinomycin D to be the specific blockage of DNA-dependent RNA synthesis. Subsequent studies with specific inhibitors of RNA synthesis have shown that only certain species of RNA need to be synthesized in order to support growth. Under the right conditions, actinomycin D and 5-fluorouracil inhibited 50 per cent of the RNA synthesis without affecting growth. The reported specificity of action of these inhibitors suggested to us that the synthesis of new ribosomal- and soluble-RNA was not necessary for the cell elongation.

We have examined the nature of the RNA synthesis that was required for growth. RNA, synthesized in control and inhibitor-treated tissue, has been fractionated by differential extraction, sucrose density gradient centrifugation, and methylated-albumin, kieselguhr column chromatography. By these techniques we have identified a fraction of RNA, the synthesis of which appears to be essential for growth of the tissue. The properties of this RNA fraction, its composition, heterogeneity of size, rate of labeling and turnover, are in many respects similar to those of the messenger-RNA described in bacterial systems. The requirement for the synthesis of this RNA for the process of cell elongation is discussed.

Materials and Methods.—Soybean: Seeds (Glycine max, var. Hawkeye) were germinated, and 1-cm sections of the hypocotyl, either from the elongating zone (0.25–1.25 cm below the cotyledons), or from the mature zone (1.5–3.5 cm below the cotyledons), were excised. Growth of the excised tissue was determined by the increase in fresh weight after incubation in sucrose-phosphate medium containing 2,4-dichlorphenoxyacetate at the concentration optimal for growth (10 μg/ml and 25 μg/ml for elongating and mature tissue, respectively). RNA synthesis was measured by the incorporation of adenosine diphosphate-8-C14 (0.5 μc per 1 gm tissue) into the RNA as previously described. Sections from elongating and mature regions of the hypocotyl showed qualitatively the same responses in terms of growth and RNA synthesis (see Table 1). Because of the greater absolute magnitude of the growth response with the elongating tissue, this was used in the majority of the experiments on growth and RNA synthesis. Mature tissue was used for most of the RNA characterization studies because of the greater incorporation of P32 orthophosphate into the RNA of this tissue.

Corn: Conditions for the growth of corn (Zea mays var. WF9 × M14), the preparation and incubation of mesocotyl (first internode) tissue, and the assay of ribonuclease activity have been described. Sections of mesocotyl tissue, consisting of the first 1 cm below the coleoptilar node, were incubated for 10 hr in a medium containing 10 μg/ml 2,4-dichlorphenoxyacetate. Growth and RNA synthesis were determined as with soybean.
Radish: Cotyledons were excised from 5-day-old seedlings (Raphanus sativus var. Cherrybelle) germinated in the light in a Hoagland minus-nitrate solution, and incubated in 0.05 M KNO₃ for 3 hr for the induction of nitrate reductase activity. RNA synthesis was measured as with soybean.

Growth of these excised tissues is primarily by cell enlargement. The inhibitors, actinomycin D and 5-fluorouracil, were used at 10 μg/ml (8 × 10⁻⁶ M) and 325 μg/ml (2.5 × 10⁻³ M), respectively, unless otherwise stated.

The mature region of soybean hypocotyl, labeled with P³² orthophosphate, was used for the characterization of newly synthesized RNA. The methods of preparation of Tris-RNA (mainly soluble- and ribosomal-RNA) and SLS-RNA (mainly DNA and DNA-like RNA), of fractionation of the RNA by sucrose density gradient centrifugation and by methylated albumin, kieselguhr (MAK) column chromatography, and of determination of base compositions of the RNA and DNA, have been described.²

Results.—(a) Inhibition of growth and RNA synthesis by actinomycin D and 5-fluorouracil: The inhibition of RNA synthesis by actinomycin D in excised soybean tissue, both elongating and mature, was accompanied by growth inhibition (Table 1). Quantitatively, however, growth was less sensitive to the inhibitor than was RNA synthesis. An examination of the inhibitory effects over a range of actinomycin D concentrations showed that up to 40 per cent of the RNA synthesis could be stopped without impairing growth (Fig. 1). A similar situation was observed when the inhibitory effects of the pyrimidine analogue, 5-fluorouracil, were studied. No inhibition of growth resulted from the highest concentration used (2.5 × 10⁻² M), whereas more than 50 per cent of the RNA synthesis was stopped (Fig. 1).

Additional parameters of growth in other tissues were measured to determine the generality of this 5-fluorouracil effect (Table 1). Growth of excised corn mesocotyl was unaffected while RNA synthesis was inhibited 50 per cent by the 5-fluorouracil. Furthermore, the increase in ribonuclease activity associated with growth of the mesocotyl¹⁰ was not inhibited; in fact, small stimulations were consistently observed. On the other hand, actinomycin D inhibited both growth and increase in enzyme activity, although these responses were again quantitatively smaller than that on RNA synthesis. Both RNA synthesis and the induction of nitrate reductase activity in radish cotyledons were inhibited 50 per cent by actinomycin D, whereas 5-fluorouracil, which reduced RNA synthesis by 50 per cent, had no effect on the induction of enzyme activity. Since it is thought that the increases in ribonuclease
and nitrate reductase activity are due to synthesis of new protein,\textsuperscript{10,11} the data show that growth of these tissues, as measured by either fresh weight or by protein synthesis, is insensitive to 5-fluorouracil, although RNA synthesis is inhibited by 50 per cent.

It has been reported that 5-fluorouracil\textsuperscript{6} and low concentrations of actinomycin D\textsuperscript{6} (0.1 \(\mu\)g/ml) differentially inhibit ribosome synthesis. It is, therefore, possible that the fraction of RNA which is not inhibited under these conditions represents a discrete species of RNA, the synthesis of which is essential for growth to proceed. The kinetics of growth inhibition which could be ascribed to the blockage of the synthesis of such a fraction of RNA is shown in Figure 2a. Tissue was pretreated with 5-fluorouracil so that the RNA being synthesized would be largely of this type. The growth rate of the tissue was identical to that of normal, auxin-stimulated growth.\textsuperscript{2} After the addition of actinomycin D, the growth rate remained the same as that of the control for 90 min, and then broke sharply, showing 70 per cent inhibition over the next 30-min period. Since, under the same conditions, actinomycin D inhibited the synthesis of RNA at a much earlier time (Fig. 2b), the lag before growth inhibition did not appear to result from slow penetration of the actinomycin D to its site of action. Thus, the data indicate that the tissue contains a supply of the 5-fluorouracil-resistant, actinomycin D-sensitive RNA sufficient to support growth of the tissue for 1–2 hr.

![Figure 2](image-url)

**Fig. 2.**—Inhibition by actinomycin D of growth and RNA synthesis of 5-fluorouracil-treated tissue. 1-gm samples of soybean hypocotyl (elongating region) were pretreated for 2 hr in medium containing 10 \(\mu\)g/ml 2,4-dichlorophenoxyacetate, 325 \(\mu\)g/ml 5-fluorouracil and 0.5 \(\mu\)c ADP-S-\(\text{C}^{14}\). At zero time actinomycin D (10 \(\mu\)g/ml) was added to one series of flasks. (a) Growth, measured by increase in fresh weight. (b) RNA synthesis, measured by incorporation of radioactivity into the RNA.

### TABLE 1

<table>
<thead>
<tr>
<th>Soybean hypocotyl</th>
<th>RNA synthesis</th>
<th>Increase in fresh weight</th>
<th>RNA synthesis</th>
<th>Increase in fresh weight</th>
<th>RNA synthesis</th>
<th>Increase in ribonuclease activity</th>
<th>Induction of nitrate reductase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elongating tissue*</td>
<td>12</td>
<td>33</td>
<td>6</td>
<td>42</td>
<td>10</td>
<td>56</td>
<td>52</td>
</tr>
<tr>
<td>Mature tissue†</td>
<td>33</td>
<td>100</td>
<td>35</td>
<td>100</td>
<td>98</td>
<td>118</td>
<td>44</td>
</tr>
<tr>
<td>Corn mesocotyl‡</td>
<td>34</td>
<td>50</td>
<td>32</td>
<td>50</td>
<td>50</td>
<td>158</td>
<td>104</td>
</tr>
<tr>
<td>Radish cotyledon‡</td>
<td>44</td>
<td>100</td>
<td>44</td>
<td>100</td>
<td>44</td>
<td>104</td>
<td>44</td>
</tr>
</tbody>
</table>

Actinomycin D and 5-fluorouracil were used at 8 \(\times\) \(10^{-4}\) \(M\) and 2.5 \(\times\) \(10^{-4}\) \(M\), respectively.

* 8 hr incubation.
† 12 hr incubation.
‡ See Materials and Methods.
5-fluorouracil, and that this was the only RNA synthesis required for growth of the excised plant tissue, prompted an investigation of the nature of this RNA fraction. Tissue was incubated with orthophosphate-P$^{32}$ (carrier free) for 7 hr, and the RNA was differentially extracted to give Tris-RNA, which contained the bulk of the soluble- and ribosomal-RNA, and SLS-RNA, which included DNA and a DNA-like fraction of RNA, referred to as D-RNA. These RNA preparations were further fractionated on MAK columns (Fig. 3). RNA prepared from control tissue showed the typical resolution into soluble-RNA, DNA, and light and heavy ribosomal-RNA. The newly synthesized P$^{32}$ RNA was present in each of these regions, and an additional fraction of radioactivity, D-RNA, eluted at a higher salt concentration than the heavy ribosomal-RNA. Fractionation of RNA prepared from 5-fluorouracil-treated tissue showed that synthesis of ribosomal-RNA was inhibited by 90 per cent. Soluble-RNA synthesis was inhibited by 75 per cent, but much of the radioactivity present in this fraction could result from end-group addition on to the soluble-RNA. The synthesis of D-RNA, however, was inhibited by only 10 per cent (after correction for the contaminating heavy ribosomal-RNA). These results suggest that the synthesis of D-RNA is essential for the growth of the tissue, while synthesis of new ribosomal- and soluble-RNA is not.

Some of the properties of D-RNA have been determined. The composition of this fraction of RNA differed from that of the total RNA and the other P$^{32}$ RNA fractions, but showed some similarity (in relative GMP and AMP contents) to

![Figure 3](image1.png)

**Fig. 3.**—MAK separations of RNA prepared from control and 5-fluorouracil-treated tissue. 15-gm samples of soybean hypocotyl (mature region) were pretreated for 2 hr in control and 5-fluorouracil (325 $\mu$g/ml) media. 500 $\mu$C P$^{32}$ orthophosphate were added for 7 hr incubation. Tris- and SLS-RNA's were prepared and chromatographed on MAK columns. Samples were run on to the column in 0.4 M NaCl, sol-RNA was eluted with 0.6 M, and then a linear gradient from 0.7 M (300 ml) to 1.1 M (300 ml) was started.

![Figure 4](image2.png)

**Fig. 4.**—Sedimentation properties of Tris-RNA and SLS-RNA. 15 gm of soybean hypocotyl (mature region) were labeled with 500 $\mu$C P$^{32}$ for 1 hr, and Tris- and SLS-RNA's were prepared. One third of each was centrifuged on a sucrose density gradient (5–20%) for 16 hr at 23,000 rpm (-70°) in an SW 25 rotor.
soybean DNA—hence its name (Table 2). Sucrose density gradient centrifugation showed that the newly synthesized RNA in the SLS-RNA fraction (composed mainly of D-RNA after 1 hr incubation in P\textsuperscript{32}) was heterogeneous in size over the ribosomal region of the gradient (Fig. 4). The rate of accumulation of radioactivity into D-RNA and ribosomal RNA is shown in Figure 5a. During the first hour, the rate of accumulation into D-RNA was twice that into ribosomal RNA. The accumulation rate continued to increase for the ribosomal-RNA, but after 1 hr leveled off and then decreased with the D-RNA, suggesting either a breakdown of this fraction, or its incorporation into some other, stable RNA. The difference in composition between the D-RNA and the other RNA fractions, however, argues against this latter possibility. The accumulation of radioactivity in Tris-RNA and SLS-RNA during a 30-min P\textsuperscript{32} incubation, at which time the SLS-RNA is largely D-RNA,\textsuperscript{13} and then a subsequent chase in P\textsuperscript{32} medium is shown in Figure 5b. There was a loss of radioactivity from the SLS-RNA during the latter part of the chase, whereas the radioactivity of the Tris-RNA continued to increase. Such chase experiments indicate a mean life of around 2 hr for the D-RNA.

Discussion.—The growth of excised plant tissues, as measured by increase in weight and protein synthesis, is strongly inhibited by high concentrations of actinomycin D (10 \(\mu\)g/ml). This is suggestive of a requirement for continual DNA-dependent RNA synthesis\textsuperscript{3} in the growth process.\textsuperscript{2} However, low concentrations

![Figure 5](image-url)

**TABLE 2**

Compositions of Nucleic Acid Fractions from Soybean Tissue

<table>
<thead>
<tr>
<th>Composition</th>
<th>CMP</th>
<th>AMP</th>
<th>GMP</th>
<th>UMP or TMP</th>
<th>GMP/AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>23.9</td>
<td>23.2</td>
<td>31.9</td>
<td>21.1</td>
<td>1.37</td>
</tr>
<tr>
<td>Total DNA</td>
<td>19.5</td>
<td>30.5</td>
<td>19.5</td>
<td>30.5</td>
<td>0.64</td>
</tr>
<tr>
<td>sol-RNA</td>
<td>26.4</td>
<td>20.1</td>
<td>33.4</td>
<td>20.4</td>
<td>1.66</td>
</tr>
<tr>
<td>l. rib-RNA</td>
<td>20.7</td>
<td>24.5</td>
<td>29.8</td>
<td>25.0</td>
<td>1.22</td>
</tr>
<tr>
<td>b. rib-RNA</td>
<td>22.4</td>
<td>24.4</td>
<td>33.0</td>
<td>20.3</td>
<td>1.36</td>
</tr>
<tr>
<td>D-RNA</td>
<td>22.1</td>
<td>34.2</td>
<td>20.8</td>
<td>22.9</td>
<td>0.81</td>
</tr>
</tbody>
</table>

* \(\mu\)moles per 100 \(\mu\)moles.
of actinomycin D (0.1 µg/ml) and 5-fluorouracil, which do not affect growth of the excised soybean hypocotyl, inhibit total RNA synthesis by 50 per cent. The synthesis of soluble- and ribosomal-RNA is strongly and selectively inhibited by 5-fluorouracil in the hypocotyl tissue, whereas D-RNA synthesis is only slightly impaired. Collectively, these observations indicate a requirement for the continual synthesis of a particular species of RNA, namely, the D-RNA, for the growth of the excised plant tissue. No additional synthesis of soluble- and ribosomal-RNA appears necessary. Since auxin (2,4-dichlorophenoxycetate) was used to promote maximum growth of the excised tissue, the synthesis of D-RNA appears essential for the auxin response.

The resistance of the D-RNA to 5-fluorouracil, under conditions where synthesis of ribosomal- and soluble-RNA is largely inhibited, cannot be readily explained by the mechanisms proposed for the inhibitory action of this pyrimidine analogue. The lack of inhibition by 5-fluorouracil of the increase in ribonuclease or nitrate reductase activity is similar to that found for constitutive enzymes in bacteria by Horowitz and Chargaff. They found, however, that the induction of β-galactosidase was inhibited. Nemeth has also reported that 5-fluorouracil completely blocked the normal developmental increase in tryptophan pyrrolase activity in the newborn pig, and inhibited by 70 per cent the adaptive increase in activity in response to tryptophan. Studies using synthetic messenger-RNA containing this analogue in cell-free systems have shown that although the homopolymer of polyfluorouridyl acid was inactive as a template for the synthesis of polyphenylalanine, the inclusion of small quantities of 5-fluorouracil in polyuridylic acid did not significantly reduce its template activity. Consequently, the apparent sensitivity of messenger-RNA, and hence protein synthesis, to this inhibitor may depend on the amount and the position of incorporation of the analogue in the molecule.

The properties of the D-RNA, its heterogeneity of size, its different base composition, its rate of synthesis, and its subsequent degradation are similar to those of messenger-RNA in bacterial systems. Although there is not complete agreement between the compositions of the D-RNA and DNA, the differences could result from the copying of only one strand of the DNA or from the transcription of only part of the genome. The demonstration of nucleotide sequence complementarity between the D-RNA and DNA is of major importance, and is under investigation by means of hybridization techniques. The stability of the D-RNA, with a mean life of around 2 hr, is in excellent agreement with the prediction of stability of the essential RNA from the kinetics of growth inhibition by actinomycin D (Fig. 2). These studies indicate that the process of cell enlargement is under gene regulation, with information release occurring through the DNA-directed formation of template- or messenger-RNA, as described for bacteria.

Summary.—Studies on the growth of excised soybean hypocotyl, corn mesocotyl, and radish cotyledon, as measured by increase in weight or enzyme activity, indicate a requirement for the synthesis of a particular species of RNA. The pyrimidine analogue, 5-fluorouracil, which has no inhibitory effect on growth, strongly inhibits the synthesis of ribosomal- and soluble-RNA without affecting the synthesis of one fraction of RNA. Subsequent inhibition of synthesis of this fraction of RNA with actinomycin D results in growth inhibition. This fraction of RNA has been identified with the DNA-like RNA by methylated albumin, kieselguhr chromatography.
The properties of the DNA-like RNA, its composition, heterogeneity of size, rate of synthesis, and turnover are similar to those described for messenger-RNA of bacterial systems. Cell enlargement therefore appears to be under the control of the genome through the synthesis of intermediate, messenger-RNA molecules.

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STRAND SELECTIVE TRANSCRIPTION OF T4 DNA IN VITRO*

BY MELVIN H. GREEN

DEPARTMENT OF BIOLOGY, UNIVERSITY OF CALIFORNIA (SAN DIEGO AT LA JOLLA)

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Studies on the transcription of viral DNA by the DNA-dependent RNA polymerase led to the paradox that whereas one strand of DNA acts as a template for the synthesis of RNA in vivo,1–5 both strands are transcribed in vitro.6–8 Recently, Hayashi et al.9 demonstrated that only one strand of the double-stranded circular form of bacteriophage φX174 DNA is copied in vitro, and more precisely, that it is the same strand as the one utilized in vivo. However, breakage of the circular φX174 DNA molecule by sonication resulted in the transcription of both strands of the primer. It was therefore concluded that the mechanism of strand selection by the RNA polymerase depended upon the physical integrity of the DNA primer, a condition not satisfied in the earlier studies of this reaction.6–8

The first indication that the E. coli RNA polymerase might show strand prefer-