RNA SYNTHESIS DURING THE DNA SYNTHESIS PERIOD OF
THE FIRST CELL CYCLE IN THE ROOT MERISTEM
OF GERMINATING VICIA FABA

K. M. JAKOB

Department of Plant Genetics, The Weizmann Institute of Science, Rehovot, Israel

SUMMARY

The synthesis of RNA during the first partially synchronous DNA synthesis period (S) in the primary root meristem of germinating Vicia faba seeds was studied.

Although there was a general temporal correlation between the synthesis of DNA and RNA during the first cell cycle, the bulk of RNA synthesis preceded the bulk of DNA synthesis during G1 and early S. Actinomycin D treatments during S showed that at least part of this RNA synthesis was necessary for the normal rate of DNA replication. It had been shown earlier that protein synthesis is required throughout the first S.

Fractionation of rapidly labeling RNA by polyacrylamide gel electrophoresis consistently revealed the synthesis of several distinct RNA species of high specific activity during first S. Actinomycin D reduced the height of all radioactive peaks. The estimated molecular weight of one of the rapidly labeling RNA species ($2.4 \times 10^8$) corresponds to the molecular weights reported for ribosomal precursor RNA in other species of higher plants. Ribosomal RNA was fully processed during first S.

The meristematic cells in the primary roots of germinating seeds of the broad bean, Vicia faba L. var. major begin their first cell division cycle from G1 (the period of interphase which precedes DNA synthesis). The cells subsequently pass through S (the DNA synthesis period) in partial synchrony. This can be concluded from the wave-like pattern of DNA synthesis with increasing time of seed soaking [8].

The primary root meristem represents therefore a favorable system for the in vivo study of the timing of metabolic events during the first cell cycle. The partial synchrony permits parallel analyses of biochemical and cytological events in the embryonic cells which participate in the division cycle.

Jakob & Bovey [8] showed that some protein synthesis throughout the first S period is essential for normal DNA replication. Cycloheximide severely reduced the rate of DNA synthesis by inhibiting protein synthesis, rather than by any direct effect of the antibiotic on DNA synthesis. The present paper deals with the question whether newly transcribed RNA is necessary for normal DNA synthesis. Such RNA presumably could mediate the protein synthesis required for DNA replication. Alternatively, the RNA essential for DNA synthesis could be entirely conserved during seed dormancy after having been transcribed during the previous cell cycle or during very early G1 on the mother plant. Conserved ribosomes and messenger RNA have been reported in embryos of germinating wheat [3, 15].
MATERIALS AND METHODS

Seeding culture

Seeds of *Vicia faba* L. var. *major* (Sutton's Exhibition Longpod or Aquadulce Claudia) were soaked for 14 h in tap water before removing the seed coats. Ten µg/ml chloramphenicol were added in order to prevent microbial growth to all culture and labeling solutions. The seedlings were then transferred to moist vermiculite and kept in the dark at 19°C for various periods. The primary roots were measured just before treatment or labeling and the seedlings were selected as previously described [8]. Treatments and labeling were performed on excised roots or on embryos from which the cotyledons had been removed at the time of seeding selection ("cotyledonless embryos").

Actinomycin D treatment and labeling

Excised roots were treated in the dark in aerated solutions of actinomycin D (300 µg/ml) and cotyledonless embryos on filter papers in Petri dishes. All controls were given identical conditions but without the antibiotic. Actinomycin D was a gift of Merck, Sharp & Dohme Research Laboratories, Rahway, N.J.

Radioactive nucleosides ([3H]-5-uridine, spec. act. 17-31 Ci/m mole; [3H]-methyl thymidine, spec. act. 18-19.9 Ci/m mole; and [14C]-2-thymidine, spec. act. 60 mCi/m mole) were administered to excised roots (about 4 mm long), or to cotyledonless embryos in aerated solutions. Pulse labels were terminated with either of two methods: (1) by fixation in absolute ethanol/glacial acetic acid (3:1) when radioactivity of the TCA-precipitable portions of homogenates was measured (tables 1, 2, fig. 1), or (2) by immersing 1 mm tips of roots in liquid air and storing them in a glass tissue grinder over dry ice for subsequent extraction and fractionation of RNA (figs 2-5).

Radioactivity of TCA-precipitable portions of homogenates

Fifteen 1 mm tips of fixed roots were defatted, homogenized and the radioactivity of the TCA-precipitable portion of the homogenate was measured as previously described [8]. Whatman GF/C glass fibre papers were used instead of membrane filters in the experiments in fig. 1 and table 2.

Frequency of mitoses

Mitotic indices were obtained from Feulgen squashes of root tips fixed after various periods of seed soaking as described earlier [9]. Each mitotic index was based on the mean of eight 1 mm tips; 500 cells were analysed in each tip.

Extraction of RNA

Sixty 1 mm tips were glass homogenized in 2 ml pH 8.4 TNEP buffer (0.05 M Tris-HCl, 0.01 M NaCl, 0.001 M EDTA, 100 µg/ml polyvinyl sulfate) to which 0.025 ml diethyl pyrocarbonate and 0.4 ml of 10% sodium lauryl sulfate were added; (modified from [22]). Three ml of water-saturated phenol were added, followed by further homogenization and high speed centrifugation (34 800 g) at 5°C. The interphase, phenol layer, and cellular debris were extracted at 52°C with 2 ml TNEP (pH 7.4) and high speed centrifugation at 5°C. The first phenol supernatant was hot phenol chloroform extracted as described by Penman [18]. The supernatant and interphase of this extraction were combined with those of the extracted cellular debris. This mixture was then re-extracted with hot phenol chloroform and chloroform [18] until disappearance of the interphase and the nucleic acid was precipitated in 2 vol of absolute ethanol at -20°C and centrifuged. The precipitate was dissolved in sterile glass-distilled water to dissolve remaining DNase inhibitors and then reprecipitated in 2 vol of absolute ethanol after the addition of NaCl to make a 0.03 M solution. The precipitate was dissolved in water and DNase solution was added for a final solution containing 0.05 M Tris-HCl, pH 7.4, 0.01 M MgCl and 100 µg/ml Sigma ribonuclease-free pancreatic DNase (modified from [18]). After 1 h incubation at 37°C, the RNA solution was chilled and precipitated with ethanol.

Polyacrylamide gel electrophoresis

The RNA was fractionated according to Loening [11, 13, 14]. RNA was redissolved in water; concentrated electrophoresis buffer (E) with sucrose were added for the final E buffer concentration described by Loening & Ingle [14]. Samples containing 50 µg RNA were layered over 2.4% polyacrylamide gels followed by electrophoresis at 60 mA per 6 mm diameter gel tube [11, 14]. Gels were scanned and then sliced using devices described by Gressel & Wolowelsky [6, 7]. Two 1 mm gel slices per scintillation vial were digested at 37°C in 0.4 ml NCS solubilizer (Amersham/Searle). Radioactivity was determined in toluene scintillation fluid (4 g PPO, 0.1 g POPOP in 1 l toluene) with a scintillation spectrometer.

RESULTS

Pattern of whole RNA synthesis during first cell cycle

The temporal relationship between the rates of RNA synthesis and DNA synthesis was determined by double labeling of DNA and RNA at various times after the beginning of imbibition (fig. 1). Preliminary findings [8] of a general parallelism between the patterns of synthesis of the two nucleic acids during the first cell cycle were confirmed. There was

Exptl Cell Res 72
also a striking similarity between the pattern of synchronous mitoses and that of DNA synthesis (fig. 1).

The curves of RNA and DNA synthesis in fig. 1 suggest that much of the RNA synthesis precedes DNA synthesis during early S (h 30–34). There is also some RNA synthesis during G1 (h 16–27) in these experiments.

**Dependence of the first S period on RNA synthesis**

The RNA synthesis inhibitor actinomycin D was used to determine whether any of the RNA synthesized during the first S period is necessary for normal DNA synthesis. Table 1 shows a 60–70% inhibition of $^3$H-5-uridine incorporation when excised roots were treated with 300 μg/ml actinomycin D for 1–4 h during first S. Removal from actinomycin D treatment did not reduce the inhibitory effect throughout S (table 1).

Actinomycin D had little or no effect on DNA synthesis when given 3 h or less prior to a $^3$H-thymidine pulse (table 2). At the same time it was known (table 1) that actinomycin was able to inhibit RNA synthesis within 1 h of treatment. Slow penetration of the antibiotic therefore could not explain the lack of effect on DNA synthesis. Thus actinomycin D apparently had no effect on DNA synthesis per se in *Vicia*, yet some RNA, produced more than 3 h before the $^3$H-thymidine pulse, was necessary for S. Actinomycin treatment 5 or more hours before $^3$H-thymidine labeling which led to a 60–70% reduction in the rate of $^3$H-5-uridine incorporation, reduced the $^3$H-thymidine incorporation up to 53% (table 2). It seems therefore that the RNA synthesis preceding DNA

---

**Table 1. Inhibition of $^3$H-5-uridine incorporation by actinomycin D during S of the first cell cycle (homogenates)**

<table>
<thead>
<tr>
<th>Periods of actinomycin D treatment (hours after beginning of imbibition)</th>
<th>$^3$H-5-uridine from hour 36–37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Expt 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control (cpm incorporated)</td>
<td>3735</td>
</tr>
<tr>
<td>Percentage of control</td>
<td>30.6</td>
</tr>
<tr>
<td>36–37</td>
<td>35–37</td>
</tr>
<tr>
<td>34–37</td>
<td>33.4</td>
</tr>
<tr>
<td>33–37</td>
<td>23.07</td>
</tr>
<tr>
<td>25–28</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> X of 2 replications; all actinomycin D treatments were significantly different from the control at the 1% level.

<sup>b</sup> One replication only.
Table 2. Inhibition of $^3$H-thymidine incorporation by a pretreatment with actinomycin D (homogenates)

<table>
<thead>
<tr>
<th>Time of actinomycin D pretreatment (hours before labeling)</th>
<th>Time of $^3$H-thymidine treatment (h)</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>34</td>
<td>36</td>
<td>39</td>
</tr>
</tbody>
</table>

Control (cpm $^3$H-thymidine incorporated)

<table>
<thead>
<tr>
<th></th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7 755</td>
<td>7 158</td>
<td>8 381</td>
</tr>
</tbody>
</table>

Percentage of control

<table>
<thead>
<tr>
<th></th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>82.8</td>
<td>91.0</td>
<td>73.3*</td>
</tr>
<tr>
<td>5</td>
<td>63.9</td>
<td>66.6b</td>
<td>46.8a</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>84.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>57.1a</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>55.8a</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>52.1b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>54.5b</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>47.1b</td>
<td></td>
</tr>
</tbody>
</table>

Cotyledonless embryos were pretreated for 3 h with 300 μg/ml actinomycin D; $^3$H-thymidine (20 μCi/ml; 1 h) incorporation was then measured on excised roots at various times after beginning of imbibition. Expts 1, 3: $\bar{X}$ of 3 replications; expt 2: $\bar{X}$ of 2 replications.

$^a$ Significant difference at the 5% level.

$^b$ Significant difference at the 1% level.

RNA synthesis during S period

The foregoing leads to the question: which species of RNA are synthesized prior to and during S? Nucleic acids were extracted from root tips of cotyledonless embryos after labeling at various times during first S. The nucleic acids were then fractionated by polyacrylamide gel electrophoresis.

Three UV absorbing peaks are seen in a scan following 180 min electrophoresis (fig. 2): DNA (left), heavy ribosomal RNA (rRNA) (middle) and light rRNA (right). The absorbancy peak of the tRNA is lost during long electrophoresis runs but can be seen on the right side of 80 min runs (fig. 5 A).

Absorbancy scans of the sort shown in figs 2–5 have also been obtained earlier during G1 (18 h of soaking; see fig. 1). At that time there is very little, if any, $^3$H-5-uridine incorporation into rRNA and the processing of rRNA is slow even at later times (see figs 2–5). This suggests that most, if not all, of these 260 nm absorbancy peaks represent nucleic acids which had been synthesized during embryo and seed maturation on the mother plant before seed dormancy, and which had been conserved from the previous cell cycle or from very early G1.

A number of distinct and consistent radioactive peaks of high specific activity, interspersed with the polydispersed radioactivity, were obtained during 1–2 h labeling periods (figs 2, 3 B). One of these $^3$H-5-uridine labeled peaks had a slower mobility than DNA (fig. 2). Greater amounts of this radioactive RNA appeared with longer labeling periods, or with relatively short post labeling incubation periods (fig. 3 A, B). It is probable that this

RNA species synthesized during the first S

...
RNA from root tips of cotyledonless embryos labeled from h 29-30. (A, B) 80 μCi/ml ³H-5-uridine; DNase treated; 180 min electrophoresis. (B) Incubated for 30 min after labeling in 1 000 fold concentration of uridine.

The above estimate for the putative precursor of *Vicia* is similar to the molecular weights reported for ribosomal precursor RNA in other higher plants [4, 5, 10, 19].

Radioactivity in rRNA appeared first following 3–4 h of post-labeling incubation. The amount of $2.4 \times 10^6$ ³H-5-uridine labeled material decreased in size during 6 h post-labeling incubation in 1 000 fold non-radioactive uridine, while the counts in the rRNA peaks increased during this period (fig. 4). A straightforward precursor-product relationship between the presumptive precursor and the rRNA has however not been established so far, because of the continuous incorporation of ³H-5-uridine in spite of the massive dilution during the ‘chase’ period. The combined radioactivity of the two rRNA peaks after 6 h ‘chase’ (fig. 4B) was 6.5 times that of the precursor peak after a 1 h pulse (fig. 4A).

In addition to the presumptive precursor peak is the ribosomal precursor of *Vicia faba* and it accumulates because of its slow processing to ribosomal RNA during the first cell division cycle. It has an approximate molecular weight of $2.4 \times 10^6$ as estimated by the method of Loening [13], using $0.7 \times 10^6$ and $1.3 \times 10^6$ as standard values for the present rRNA [12, 19].

Experimental Cell Research 72
RNA synthesis during S period

RNA, other radioactive peaks also recurred consistently in most experiments (figs 2, 3B). Some preliminary statements can be made about these RNA species. One peak of radioactivity usually appeared under the absorbancy peak of light rRNA after 1½–2 h labeling (fig. 3B). This suggested that some light rRNA may have been processed during this relatively short labeling period, while the heavy rRNA component appeared to be processed much more slowly. This has been reported also by Leaver & Key [10] in carrot discs. The remaining radioactive peaks (figs 2, 3B) could represent intermediates in the processing of larger ribosomal precursor RNA molecules to rRNA. Alternatively, all or some of them could represent non-ribosomal RNA species such as heterogeneous and/or messenger RNA.

RNA was fractionated from actinomycin D treated meristems on polyacrylamide gels in order to check for possible differences in actinomycin sensitivity among the species of rapidly labeling RNA. All radioactive peaks including tRNA were reduced in height (fig. 5A). Fig. 5B suggests that there were some differences in the extent of reduction among the various RNA species.

DISCUSSION

The requirements of RNA and protein synthesis for DNA replication have been described for mammalian cells (see reviews [2, 17 and 16, 23]) and for cultured amphibian lenses [1, 20]. In the broad bean, Vicia, it has been possible to study these phenomena in a naturally synchronous tissue. The result of this and of an earlier study on Vicia [8] indicate that the availability of certain RNA and protein molecules is a prerequisite for normal DNA synthesis in the meristematic cells of primary roots. Moreover, the embryonic tissue apparently maintains a relatively larger ‘store’ of the RNA molecules than of the protein molecules required for the continuation of DNA synthesis. This is inferred from experiments in which either protein or RNA synthesis were suppressed. This ‘store’ is maintained through synthesis of these molecules during S. Protein synthesis required for DNA synthesis becomes limiting within 1 h of cycloheximide treatment [8] while RNA becomes limiting to DNA syn-

Fig. 5. Abscissa: slice number; ordinate: (left top): —, A540; (right top): cpn × 10−3; ——, control; ——Δ—, 300 μg/ml actinomycin D from h 29-32; (right bottom): ——, actinomycin D/contrcl × 100.

Inhibition of RNA synthesis by actinomycin D treatment. (A) RNA from root tips of cotyledonless embryos labeled from h 29-32 with 80 μCi/ml 3H-5-uridine; DNase treated; 90 min electrophoresis; (B) effect of actinomycin D on the synthesis of various RNA fractions.
thesis only later; i.e. about 5 h after actinomycin D treatment. This result is consistent with the hypothesis that protein synthesis during S is mediated by RNA synthesized earlier during S or during G1.

The present results are in agreement with the recent findings of Webster & Van't Hof [21]. These authors used similar inhibitors in order to demonstrate the requirements for RNA and protein synthesis necessary for the sucrose-induced resumption of DNA synthesis in stationary phase meristems of cultured pea roots.

The most important question which arises from these experiments is, which of the rapidly labeling RNA species represent intermediates in the processing of precursor RNA to rRNA and what happens to those that do not? It is of course possible that most of the RNA which is synthesized during the first S represents exclusively ribosomal precursors, rRNA and tRNA. This would imply that the protein synthesis which has been shown to be essential for DNA synthesis [8], is mediated entirely by conserved messenger RNA [3].

The prerequisite of RNA synthesis for DNA replication could be due to a shortage of ribosomes and/or tRNA for the maintenance of the normal rate of protein synthesis during S. However it is possible that some of the radioactive species of RNA which occur during S will not fit into the above-mentioned categories. These would then be of special interest for their possible involvement in the first cell cycle. Studies which attempt to resolve some of these open questions about the RNA synthesized during the first cell cycle in Vicia primary roots are now in progress.

The advice of Dr Robert A. Weinberg on extraction procedures and the technical assistance of Mr Israel Tal and Miss Leah Schwartz are gratefully acknowledged.

The author is a Michael and Audrey Sacher Research Fellow.

REFERENCES


Note added in proof: The following reference dealing with RNA synthesis during seed germination became available after submission of the present paper: Melera, P W, Plant physiol 48 (1971) 73.

Received September 7, 1971
Revised version received November 10, 1971