Hormonal Control of Messenger Ribonucleic Acid Metabolism in Barley Aleurone Layers

[gibberellic acid/poly(A)-RNA/cordycepin/stable mRNA]

DAVID TUAN-HUA HO AND J. E. VARNER

MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Mich. 48824; and Department of Biology, Washington University, St. Louis, Missouri 63130

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ABSTRACT Ribonucleic acid containing poly(adenylic acid) [poly(A)-RNA] is present in barley aleurone layers. This poly(A)-RNA becomes labeled with radioactive precursors of RNA during the incubation of isolated aleurone layers with or without gibberellic acid. However, the rate of synthesis of poly(A)-RNA is enhanced by gibberellic acid. This enhancement begins within 3–4 hr of addition of the hormone and reaches a maximum, which is about 50–60% over the control, 10–12 hr after addition of the hormone. Cordycepin inhibits total RNA as well as poly(A)-RNA synthesis in barley aleurone layers. However, cordycepin inhibits the hormone-controlled synthesis of α-amylase (EC 3.2.1.1) only if it is added 12 hr or less after gibberellic acid. The insensitivity of α-amylase production to cordycepin after 12 hr of gibberellic acid treatment suggests that α-amylase is translated from stable messenger RNA.

Isolated barley aleurone layers respond to exogenous gibberellic acid (GA3) by synthesizing and secreting several hydrolyases, including α-amylase and protease, after an 8- to 10-hr lag period (1–3). During this lag period there is a proliferation of endoplasmic reticulum (4, 5) and a correlated formation of membrane-bound polysomes (6). Actinomycin D and 6-methylpurine block the hormone-controlled synthesis of hydrolyases (7, 8). Incorporation into salt-soluble RNA is enhanced by GA3 (9). Zwar and Jacobsen (10) demonstrated that the incorporation into polysomal RNA was increased in the presence of GA3, and it was recently reported that GA3 enhanced the synthesis of rapidly labeled RNA species in barley aleurone layer (11). These observations tend to support a previous suggestion that the synthesis of hydrolyases may depend on the GA3-mediated synthesis of their specific mRNAs (7). However, a possible post-transcriptional model, based on the necessity of membrane proliferation for the synthesis of secretory protein, has also been put forward (12).

Since almost all mRNAs in eukaryotic cells contain a covalently linked poly(adenylic acid) segment [poly(A)] (13, 14), mRNAs can be easily isolated by their affinity for immobilized oligo(deoxythymidylic acid) [oligo(dT)] or poly-uridylic acid [poly(U)]. Using this technique we now report that the synthesis of RNA containing a poly(A) segment [poly(A)-RNA] in barley aleurone layers is enhanced by GA3. Our results also suggest that α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) and probably other hydrolyases are translated from stable mRNAs, the stability of which may be related to the gibberellin effect in this system.

Abbreviation: GA3, gibberellic acid.

MATERIALS AND METHODS

Preparation and Treatment of Aleurone Layers. Aleurone layers were prepared by the methods of Chrispeels and Varner (1) from barley seeds (Hordeum vulgare L., cv. Himalaya) and incubated in 2 mM Na acetate buffer (pH 5.0) and 10 mM CaCl2, with or without 1 μM GA3 on a metabolic shaker at 25°. Radioactive adenosine, or uridine, or 32P was used to label RNA. To study the GA3 effect on poly(A)-RNA synthesis, we used the following labeling technique.

Double Labeling. Labeling was carried out by addition of 50 μCi of [3H]adenosine (specific activity: 30–50 Ci/mmol) to a sample containing GA3 and a sample without GA3, and 2 μCi of [14C]adenosine (specific activity: 310 mCi/mmol) to two other samples without GA3 at specific times. After 2 hr of further incubation, 3H-labeled layers were mixed with 14C-labeled layers (i.e., 3H-labeled sample containing GA3 with 14C-labeled sample without GA3; 3H-labeled sample without GA3 with 14C-labeled sample without GA3) and rinsed extensively with ice-cold adenosine solution (10 mM).

Total RNA Extraction. The aleurone layers were ground with 100 mM Tris·HCl buffer (pH 7.6), 1% Na dodecyl sulfate, and 0.1% diethyl pyrocarbonate in a chilled mortar. An equal volume of phenol-chloroform (1:1) mixture was added to the homogenate and the whole mixture was stirred vigorously for 10 min. After centrifugation at 10,000 rpm for 10 min, the aqueous phase was decanted and again extracted with an equal volume of phenol-chloroform mixture. The phenol-chloroform phase was extracted again with 100 mM Tris·HCl (pH 9.0). All aqueous phases were combined. After the addition of one-tenth volume of 1 M NaCl, RNA was precipitated by addition of 2.5 volumes of absolute ethanol and storing overnight at −20°.

Poly(A)-RNA Isolation. Preparation of the Fiberglas filter with immobilized poly(U) and the filtration procedure were according to Sheldon et al. (15). Oligo(dT)-cellulose was purchased from Collaborative Research (Waltham, Mass.). The cellulose column (1 × 5 cm) was equilibrated with 10 mM Tris·HCl buffer (pH 7.6), and 0.5 M KCl (binding buffer). RNA sample was dissolved in binding buffer and applied to the column. After thorough washing with binding buffer and Tris buffer containing 0.1 M KCl, the bound RNA was then eluted with buffer alone.

Chemical Labeling of the 3'-End of the RNA Molecule. To a solution of about 50 μg of RNA in 100 μl of H2O was added 20
μl of an aqueous solution containing 2 nmol of NaIO₄. The oxidation was allowed to proceed for 2 hr in the dark at room temperature. Then 5 μl of 0.1 M NaB₄H₄ (50 μCi) in 0.1 M KOH was added and the reaction mixture was kept in the dark at room temperature for 2 hr. A drop of 1 M acetic acid was added at the end of 2 hr to convert excess NaB₄H₄ to boric acid and tritium gas. The labeled RNA was collected by ethanol precipitation.

**Poly(A) Segment Isolation.** Bound RNA eluted from the oligo(dT)-cellulose column was made to 10 mM Tris-HCl, 0.1 M NaCl, 1 mM MgCl₂. After digestion with pancreatic ribonuclease A (2 μg/ml) and T1 RNase (10 units/ml) for 30 min, diethyl pyrocarbonate or Na dodecyl sulfate was added to inactivate the enzymes. The RNase-resistant fragment was precipitated by ethanol.

**Base Composition Analysis.** RNA samples labeled with ³²P were hydrolyzed with 0.3 M KOH at 37°C for 18-20 hr. After neutralization with perchloric acid, the hydrolysate was subjected to paper electrophoresis as described (16). The ultraviolet light-absorbing region on the paper was cut off and radioactivity was determined directly.

**Enzyme Assay.** The activity of α-amylase was assayed as described by Varner and Mense (17).

### RESULTS

**Characterization of Poly(A) Segment and Poly(A)-RNA of Barley Aleurone Layers.** Total RNA extracted from barley aleurone layers can be separated into unbound and bound fractions by oligo(dT)-cellulose chromatography (Fig. 1). The unbound RNA includes nonspecifically bound RNA washed out by 0.1 M KCl. As shown in Table 1, the bound RNA is rich in AMP and polydispersed in size, and this RNA species contains ribonuclease-resistant poly(A). The length of the poly(A) segments ranges from 80 to 200 nucleotides.

### TABLE 2. Localization of poly(A) segment on RNA molecule

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cpm</th>
<th>Cpm</th>
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<tbody>
<tr>
<td>Poly(A) from Sigma</td>
<td>Before RNase treatment</td>
<td>After RNase treatment</td>
</tr>
<tr>
<td></td>
<td>4,996.5</td>
<td>4,493.5</td>
</tr>
<tr>
<td>E. coli tRNA</td>
<td>65,095.0</td>
<td>4,911.5</td>
</tr>
<tr>
<td>Barley poly(A)-RNA</td>
<td>73,956.0</td>
<td>70,340.5</td>
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</table>

RNA samples were chemically labeled as described in Materials and Methods before they were subjected to ribonuclease treatment.
(Fig. 2). The 3'-OH end of the bound RNA was chemically labeled by NaIO₄ oxidation followed by NaB₃H₄ reduction (18). After the ribonuclease treatment, all the label went to the poly(A) segment (Table 2), indicating the poly(A) segment is located adjacent to the 3'-OH end of the RNA molecule.

**Effect of GA₃ on Poly(A)-RNA Metabolism.** Poly(A)-RNA binds to the poly(U) filter, which can be subjected to scintillation counting directly; this provides us with an efficient way to analyze the effect of GA₃ on poly(A)-RNA synthesis of barley aleurone layers.

The double labeling technique described in Materials and Methods maximizes the sensitivity of the experiment; this design also eliminates possible artifacts generated by differential degradation during the isolation of poly(A)-RNA. From the ³H/¹⁴C ratio, one can calculate the hormonal effect as the percent enhancement of poly(A)-RNA synthesis over the control. For example, at 12 hr after addition of GA₃, the ³H/¹⁴C ratio of the mixture of the sample containing GA₃ and the sample without GA₃ is 38.05 and that of the mixtures of samples both without GA₃ is 24.22; therefore, the GA₃ enhancement is 57%.

We observed that GA₃ enhanced the synthesis of poly(A)-RNA with a lag period 3–4 hr. This enhancement reached a maximum, which was about 50–60% over control, at 10–12 hr, then decreased afterward (Fig. 3). This GA₃ effect can still be observed if uridine is used as the labeled precursor, indicating that the synthesis of the portion of the poly(A)-RNA molecules not containing poly(A) is also enhanced by GA₃.

Abscisic acid, a naturally occurring plant hormone that antagonizes GA₃-mediated hydrolase synthesis in barley aleurone layers, prevents the GA₃ effect on poly(A)-RNA synthesis.

**Effect of Cordycepin on the Production of α-Amylase.** Cordycepin, 2'-deoxyadenosine, is generally believed to work as a chain terminator during RNA synthesis. In animal tissue, cordycepin preferentially inhibits poly(A) synthesis (19). However, we observed that cordycepin inhibited the synthesis of both poly(A)-RNA and RNA not containing poly(A) in barley aleurone layers.

Cordycepin, but not 2'-deoxyadenosine, at a concentration of 0.1 mM, effectively inhibits α-amylase production if applied at the same time as GA₃. This inhibitory effect decreases if the analog is added after GA₃ (Fig. 4). Cordycepin added 12 hr or more after GA₃ has no inhibitory effect on α-amylase production (Fig. 4). A similar effect of cordycepin on GA₃-enhanced protease production was also observed (data not shown).

Cordycepin inhibits uridine incorporation into total RNA to the same extent whether it is added at zero time or 12 hr after GA₃ (data not shown). It also inhibits about 50% of total protein synthesis, as checked by the incorporation of radioactive leucine (Table 3), indicating that the mRNAs for nonhydrolase proteins are not stable.

**DISCUSSION**

We have demonstrated that poly(A)-RNA is present in barley aleurone layers and that the synthesis of poly(A)-RNA is enhanced by GA₃. This enhancement begins after a lag period of 3–4 hr and reaches a maximum at 10–12 hr after the addition of GA₃. The barley aleurone layers is a nondenying tissue, and neither its respiration rate nor its energy charge is changed significantly by the application of hormone (Keates and Varner, unpublished observation). Therefore, the GA₃-enhanced poly(A)-RNA synthesis cannot be a result of hormone-enhanced growth or hormone-enhanced energy metabolism. α-Amylase has been shown to be synthesized after the addition of the hormone in GA₃-treated aleurone layers (2). However, the production of α-amylase is no longer sensitive to cordycepin if cordycepin is added 12 hr or more after GA₃, suggesting that α-amylase may be encoded from a stable mRNA.

In principle, the control of hormone-enhanced synthesis of specific proteins could be either on the transcriptional or the post-transcriptional level. It has been shown that hormone-controlled synthesis of egg white proteins in chick oviduct is

<table>
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<tr>
<th>Table 3. Effect of cordycepin on α-amylase and total protein synthesis</th>
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<tr>
<td>+ GA₃ only</td>
</tr>
<tr>
<td>α-Amylase (μg/10 layers)</td>
</tr>
<tr>
<td>[3H]Leucine incorporation (cpm/10 layers)</td>
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* Numbers in parentheses indicate the percentage of inhibition.
due to the accumulation of specific mRNAs (20, 21). Palmiter (22) has found that ovalbumin has a fairly stable mRNA whose synthesis is enhanced by estrogen. It was recently concluded (23) that withdrawal of estrogen will decrease the half life of ovalbumin mRNA, indicating that this mRNA is somehow stabilized in the hormone-treated tissue.

In the GA$_4$-treated barley aleurone layers, about 40% of the newly synthesized protein is estimated to be $\alpha$-amylase (Flint and Varner, unpublished observation). The percentage of the sum of all kinds of hydrolases in terms of total protein is even higher. Thus, if GA$_4$ mediates the transcriptional machinery, there should be a detectable enhancement of the synthesis of total mRNA. The timing of the GA$_4$ effect on poly(A)-RNA synthesis correlates with the time of polysome formation, indicating that RNA synthesis may be a prerequisite of polysome formation. Gibberellic acid causes an increase in the activity of two enzymes, phosphorylcholine cytidyl and phosphorylcholine-glyceride transferases, required for the biosynthesis of lecithin, which is a major component of membrane phospholipids (12). However, GA$_4$ appears to enhance the activity of these two enzymes by some activation process not involving protein synthesis or RNA synthesis (24). Therefore, it is unlikely that the enhanced synthesis of poly(A)-RNA is necessary for the observed membrane proliferation.

Zwar and Jacobsen (10) were able to show a GA$_4$ effect on the incorporation of labeled uridine and adenosine into polydisperse RNA in the period 8–16 hr after addition of GA$_4$. Recently, they have verified the previous results and shown that the polydisperse RNA is poly(A)-RNA (25). However, because they used long labeling periods, their results may reflect the accumulation instead of the rate of the synthesis of poly(A)-RNA. Johnson and Kende (12) suggested that the synthesis of GA$_4$-enhanced hydrolases may be dependent on the availability of proper membranes, i.e., endoplasmic reticulum, for the attachment of polysomes that carry the hydrolase-specific mRNAs. It has been shown in rat liver that the average half-life of mRNA associated with free polysomes appears to be relatively short compared to mRNA associated with membrane-bound polysomes (26). Therefore, the accumulation of polydisperse RNA may be a consequence of stabilization of mRNA for $\alpha$-amylase, and possibly other hydrolases as well, by its association with the polysomes bound to endoplasmic reticulum.

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