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# 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 ($GA_3$) by synthesizing and secreting several hydrolases, 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 hydrolases (7, 8). Incorporation into salt-soluble RNA is enhanced by $GA_3$ (9). Zwar and Jacobsen (10) demonstrated that the incorporation into polydispersed RNA was increased in the presence of $GA_3$, and it was recently reported that $GA_3$ 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 hydrolases may depend on the $GA_3$-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 $GA_3$. Our results also suggest that α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) and probably other hydrolases are translated from stable mRNAs, the stability of which may be related to the gibberellin effect in this system.

Abbreviation: $GA_3$, 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 $CaCl_2$, with or without 1 μM $GA_3$ on a metabolic shaker at 25°. Radioactive adenosine, or uridine, or $^{32}P$ was used to label RNA. To study the $GA_3$ effect on poly(A)-RNA synthesis, we used the following labeling technique.

*Double Labeling.* Labeling was carried out by addition of 50 μCi of [$^3$H]adenosine (specific activity: 30–50 Ci/mmol) to a sample containing $GA_3$ and a sample without $GA_3$, and 2 μCi of [$^{14}$C]adenosine (specific activity: 310 mCi/mmol) to two other samples without $GA_3$ at specific times. After 2 hr of further incubation, $^3$H-labeled layers were mixed with $^{14}$C-labeled layers (i.e., $^3$H-labeled sample containing $GA_3$ with $^{14}$C-labeled sample without $GA_3$; $^3$H-labeled sample without $GA_3$ with $^{14}$C-labeled sample without $GA_3$) 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 $H_2O$ was added 20

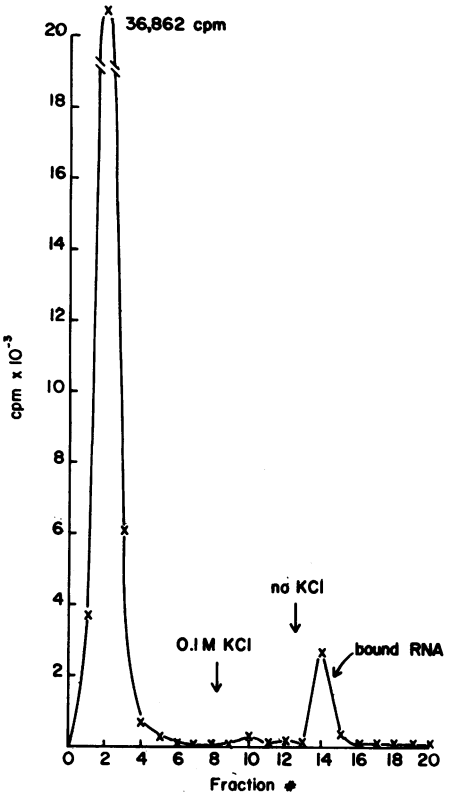


FIG. 1. Chromatography of total barley RNA on oligo(dT)-cellulose. Fractions of 4.5 ml were collected.

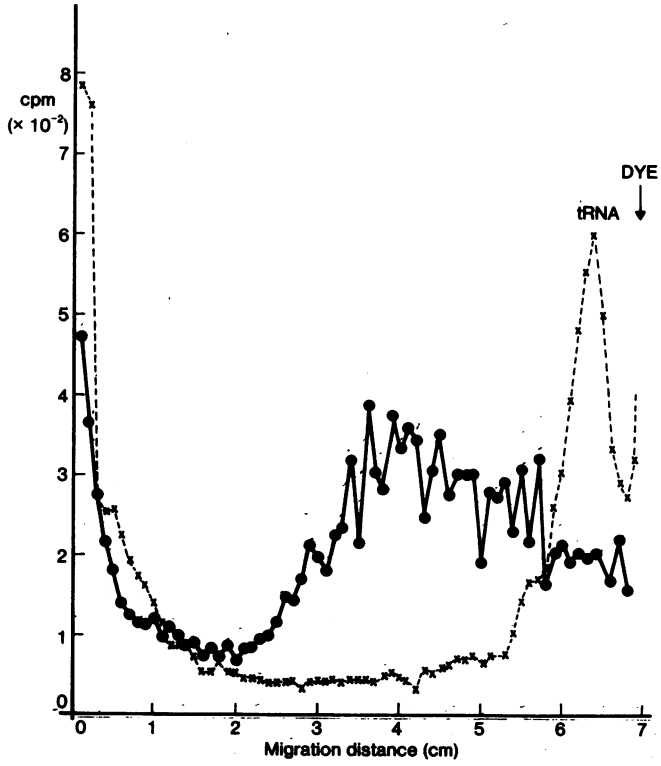


FIG. 2. Polyacrylamide gel electrophoresis of poly(A) from barley aleurone layers. Poly(A) segment labeled with [$^3$H]adenosine was isolated as described in *Materials and Methods* and then analyzed on 10% polyacrylamide gel. Tritiated tRNA, analyzed on a separate gel, was used as a marker.

$\mu$l of an aqueous solution containing 2 nmol of $NaIO_4$. The oxidation was allowed to proceed for 2 hr in the dark at room temperature. Then 5 $\mu$l of 0.1 M $NaB^3H_4$ (50 $\mu$Ci) 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 $NaB^3H_4$ 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 $MgCl_2$. After digestion with pancreatic ribonuclease A (2 $\mu$g/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 $^{32}P$ were hydrolyzed with 0.3 M KOH at 37° for 18–20 hr. After neutralization with perchloric acid, the hydrolyzate 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 $\alpha$-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 1. *Base composition (%) of RNA species isolated from barley aleurone layers*

| Sample | CMP | AMP | GMP | UMP |
|---|---|---|---|---|
| Total RNA | 25.1 | 22.9 | 33.5 | 18.5 |
| Bound RNA [poly(A)-RNA] | 23.7 | 30.2 | 25.1 | 21.0 |
| RNase-resistant RNA [poly(A) segment] | 0.8 | 94.1 | 3.6 | 1.5 |

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

| Sample | Cpm: Before RNase treatment | Cpm: After RNase treatment | % Remaining |
|---|---|---|---|
| Poly(A) from Sigma | 4,996.5 | 4,493.5 | 89.9 |
| *Escherichia coli* tRNA | 65,025.0 | 4,911.5 | 7.6 |
| Barley poly(A)-RNA | 73,656.0 | 70,340.5 | 95.5 |

RNA samples were chemically labeled as described in *Materials and Methods* before they were subjected to ribonuclease treatment.

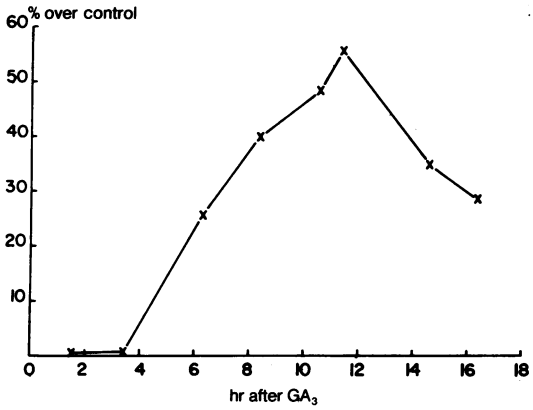


FIG. 3. Effect of $GA_3$ on poly(A)-RNA synthesis in barley aleurone layers.

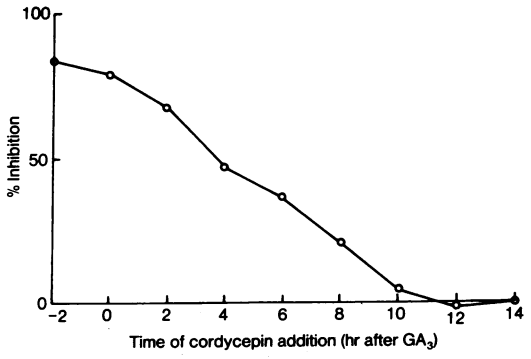


FIG. 4. Effect of cordycepin on $\alpha$-amylase activity. Cordycepin was added at different times after $GA_3$ as indicated, and aleurone layers were further incubated until 24 hr after addition of $GA_3$, when the activity of $\alpha$-amylase was assayed.

(Fig. 2). The 3′-OH end of the bound RNA was chemically labeled by $NaIO_4$ oxidation followed by $NaB^3H_4$ 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 $GA_3$ 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 $GA_3$ 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 $^3H/^{14}C$ 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 $GA_3$, the $^3H/^{14}C$ ratio of the mixture of the sample containing $GA_3$ and the sample without $GA_3$ is 38.05 and that of the mixtures of samples both without $GA_3$ 24.22; therefore, the $GA_3$ enhancement is 57%.

We observed that $GA_3$ 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 $GA_3$ 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 $GA_3$.

Abscisic acid, a naturally occurring plant hormone that antagonizes $GA_3$-mediated hydrolase synthesis in barley aleurone layers, prevents the $GA_3$ effect on poly(A)-RNA synthesis.

*Effect of Cordycepin on the Production of $\alpha$-Amylase.* Cordycepin, 3′-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 $\alpha$-amylase production if applied at the same time as $GA_3$. This inhibitory effect decreases if the analog is added after $GA_3$ (Fig. 4). Cordycepin added 12 hr or more after $GA_3$ has no inhibitory effect on $\alpha$-amylase production (Fig. 4). A similar effect of cordycepin on $GA_3$-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 $GA_3$ (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 $GA_3$. This enhancement begins after a lag period of 3–4 hr and reaches a maximum at 10–12 hr after the addition of $GA_3$. The barley aleurone layers is a nondividing 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 $GA_3$-enhanced poly(A)-RNA synthesis cannot be a result of hormone-enhanced growth or hormone-enhanced energy metabolism. $\alpha$-Amylase has been shown to be synthesized after the addition of the hormone in $GA_3$-treated aleurone layers (2). However, the production of $\alpha$-amylase is no longer sensitive to cordycepin if cordycepin is added 12 hr or more after $GA_3$, suggesting that $\alpha$-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 3. *Effect of cordycepin on $\alpha$-amylase and total protein synthesis*

| | + $GA_3$ only | + $GA_3$ with with cordycepin |
|---|---|---|
| $\alpha$-Amylase ($\mu$g/10 layers) | 426.7 | 426.2 (0.1)* |
| [$^3$H]Leucine incorporation (cpm/10 layers) | 247,390 | 134,174 (45.8) |

* 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 $GA_3$-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 $GA_3$ mediates the transcriptional machinery, there should be a detectable enhancement of the synthesis of total mRNA. The timing of the $GA_3$ 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, $GA_3$ 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 $GA_3$ effect on the incorporation of labeled uridine and adenosine into polydisperse RNA in the period 8–16 hr after addition of $GA_3$. 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 $GA_3$-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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