Vol. 102, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1981 Pages 617-623

THE RESPONSE OF BARLEY ALEURONE LAYERS TO GIBBERELLIC ACID INCLUDES THE TRANSCRIPTION OF NEW SEQUENCES

Irma Bernal-Lugo*, Roger N. Beachy and Joseph E. Varner

Plant Biology Program, Department of Biology,
Washington University, St. Louis, MO 63130.

Received July 31, 1981

Summary

In barley aleurone layers, gibberellic acid (GA_3) induces the synthesis of α-amylase while abscicic acid (ABA) inhibits this synthesis. The level of translatable mRNA for α-amylase is increased by GA_3 and by GA_3+ABA but it is not known if this increase is due to a direct enhancement of transcription of α-amylase gene by GA_3 (transcriptional control) or to the processing of a precursor RNA (post-transcriptional control). To answer this question we have measured the levels of α-amylase specific mRNA sequences in barley aleurone layers using [^{32}P]DNA complementary to α-amylase mRNA. The results show that exposure of barley aleurone layers to GA_3 or GA_3+ABA, leads to an increased accumulation of mature (∿1.6 Kb) α-amylase mRNA sequences.

INTRODUCTION

The synthesis of α-amylase in isolated barley aleurone layers is controlled by gibberelic acid (GA_3) and abscisic acid (ABA) (1,2,3). GA_3 brings about the de novo synthesis of the enzyme after a lag period of 6 to 10 hrs (4) and ABA prevents this response (1).

Inhibitor studies indicated that RNA synthesis is required for the GA_3 response and that the hormone increases the incorporation of labeled precursors into poly(A) containing RNA of aleurone cells (5,6). Using a protein synthesis cell-free system it was shown that the amount of translatable mRNA for α-amylase in aleurone layers begins to increase between 3-4 hrs. after GA_3 treatment (7).

---

*Present address:

Irma Bernal-Lugo, Departmento de Bioquimica, Division de Estudios de Post-Grads, Faculdad de Quimica, Universidad de Mexico, Mexico 20 DF

0006-291X/81/180617-07$01.00/0

The effect of ABA on the level of translatable α-amylase-mRNA (αAmRNA) has also been studied (2,3). The results showed that layers treated with GA_3 and ABA (GA_3+ABA) from the beginning of the incubation do not synthesize α-amylase although the αAmRNA starts to accumulate 10-12 hrs after hormone treatment (2,3). One interpretation of these data is that transcriptional events are directly required for the synthesis of α-amylase induced by GA_3, and that ABA blocks the translation of the mRNA *in vivo*. However, so far there is no direct evidence that the increase in translatable αAmRNA arises from an increase in cytoplasmic αAmRNA sequences or from some alteration in the structure of αAmRNA that increases its translational efficiency. To investigate these questions, we have used the gel blot hybridization technique with [^{32}P]labeled DNA complementary to α-amylase (αAcDNA) to detect and to quantitate the α-amylase specific RNA sequences. We report here that exposure of barley aleurone layers to GA_3 or to GA_3+ABA leads to an increased accumulation of mature (~1600 bases) α-amylase mRNA sequences.

Experimental Procedures

Materials. Barley seeds (*Hordeum vulgare* L. cv. Himalaya, 1974 crop) were supplied by the Department of Agronomy, Washington State University, Pullman, Washington and were stored at 5°C. Gibberellic acid, (±)cis-trans abscisic acid, sigma cell Type 50 cellulose, Sephadex G-150 were purchased from Sigma, oligo d(pT) cellulose T-1 was supplied by Boehringer-Manheim Corporation. L [^{35}S]methionine (1200 Ci/mmole) and [λ^{32}P]dATP (410 Ci/mmol) were from Amersham. Other chemicals were highest reagent quality available. An MV reverse transcriptase was obtained from Dr. J. Beard, Life Sciences, Inc., Gulfport, Florida.

Preparation and treatment of aleurone layers. Aleurone layers were isolated from 72 hr imbibed de-embryonated half seeds and incubated in solutions containing the various hormones, 20 mM sodium succinate buffer pH 5.2, and 10 mM $CaCl_2$, in a reciprocating metabolic shaker at 25°C. The hormone concentrations used in this work were 1.0 μM gibberellic acid and 20 μM of (±)cis-trans abscisic acid.

RNA extraction and fractionation. Total RNA from aleurone layers was extracted by the method described by Hall et al. (8). Selection of poly(A)$^+$ RNA was done according to Mozer (9).

α-Amylase mRNA. Barley α-amylase mRNA was prepared as described by Mozer (9).

Cell-free protein synthesis. Poly(A)$^+$RNA was translated for 90' at 25°C in wheat germ translation system (10). The reaction mixture (25 µl) contained 5 µl wheat extract, 37 mM Hepes (pH 7.6), 155 mM potassium acetate, 10 mM potassium chloride, 4.0 mM dithiothreitol, 3.0 mM magnesium acetate, 0.33 mM spermidine, 40 µM aminoacids minus methionine, 4 µM methionine, 2 µCi L[^{35}S]methionine, and an energy source consisting of 1 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 3 mM phosphocreatine and 20 µg/ml phosphocreatine kinase and 0.5 µg of poly(A)$^+$RNA. The incorporation of [^{35}S]methionine into protein was determined as described by Mans & Novelli (11). Aliquots of the translation mixture (5-10 µl) were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (12). After electrophoresis, the gels were prepared for fluorography and exposed to Kodak XAR-Omat film (13).

Preparation of DNA complementary (cDNA) to α-amylase mRNA. [^{32}P]-DNA complementary to α-amylase mRNA was synthesized in a reverse transcriptase (RNA-dependent DNA polyermase) reaction with the conditions described by Meyers and Spiegelman (14), using as template 50 µg/ml mRNA enriched in α-AmRNA. Following the reaction, samples were treated with base to hydrolyze the RNA, neutralized and extracted with phenol-chloroform and desalted by passage through a Sephadex G-150 column. Void volume fractions were collected and ethanol precipitated. After centrifugation the pellet was dissolved in water and used as the probe.

RNA blotting and hybridization. Poly(A)$^+$RNA was separated by electrophoresis under denaturing conditions on 1.1% agarose gels by procedures similar to those of Thomas (15). After electrophoresis the RNA was transferred (blotted) to a sheet of nitrocellulose (15). The nitrocellulose was dried under a lamp for 2 hrs. and baked overnight at 60°C. The blots were prehybridized at 42°C for 12 hrs in a buffer containing 50% (v/v) formamide, 0.76 M NaCl, 0.076 M Na citrate (pH 7.0), 50 mM sodium phosphate (pH 6.5), sonicated denatured salmon sperm DNA at 250g/ml and 0.02% each of bovine serum albumin, ficoll, and polyvinyl pyrrolidone. RNA/cDNA hybridization was carried out in 4 vol. of prehybridization buffer and 1 vol. of 50% (wt/v) dextran sulfate at 42°C for 24 hr. with gentle shaking.

After hybridization, the paper was washed 4 times for 5 min each at room temperature in 0.30 M NaCl/30 mM sodium citrate/0.1% NaDodSO$_4$ and then for 15 to 30 min at 50°C in 15 mM NaCl/1.5 mM sodium citrate/0.1% NaDodSO$_4$ until the radioactivity in the final wash was close to background (usually three or four washes). The paper was then exposed to X-ray film at -70°C with a Dupont Cronex Lightning-plus intensifying screen. The intensities of the resulting α-amylase mRNA bands were quantitated by exposing the blot for different lengths of time until the intensities of each sample looked similar. This was checked by scanning the autoradiographs with a Gilford spectrophotometer.

RESULTS

Estimation of the purity of α-amylase mRNA. In order to determine the purity of the α-amylase mRNA isolated by DMSO-sucrose density gradient *in vitro* translation products directed by α-amylase mRNA were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. A fluorograph

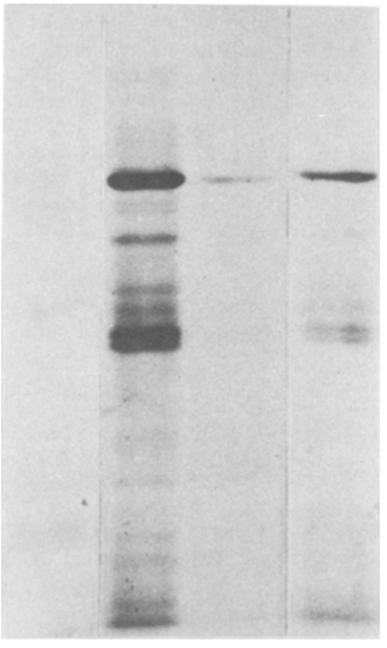

Fig. 1. Fluorogram of SDS-polyacrylamide gel analysis of protein synthesized in wheat germ extract under the direction of total poly(A)$^+$RNA and purified α-amylase mRNA. Polypeptides synthesized in the assay when no RNA is added, A; translation products using total poly(A)$^+$RNA isolated from aleurone layers treated with 1 mM GA_3 for 16 hrs, B; translation products using a DMSO-sucrose gradient fraction rich in α-amylase mRNA, C; the same as C with longer exposure, D.

of the total translation products obtained showed a major band of radioactivity in the position of pre-α-amylase (fig 1c) (9). However, if the same gel is exposed for a longer time, several minor bands migrating faster than α-amylase were also observed (Fig 1D); however, greater than 75% of the polypeptides produced in these reactions were present in the major band. In contrast, when the products obtained from translation of total poly(A)$^+$RNA were analyzed the pattern shown is more complex, although the major band was pre-α-amylase (Fig 1B). From this experiment we concluded that this fraction enriched in α-amylase mRNA was an appropriate template for preparing α-amylase cDNA. The preparation does, however, contain some other RNA sequences.

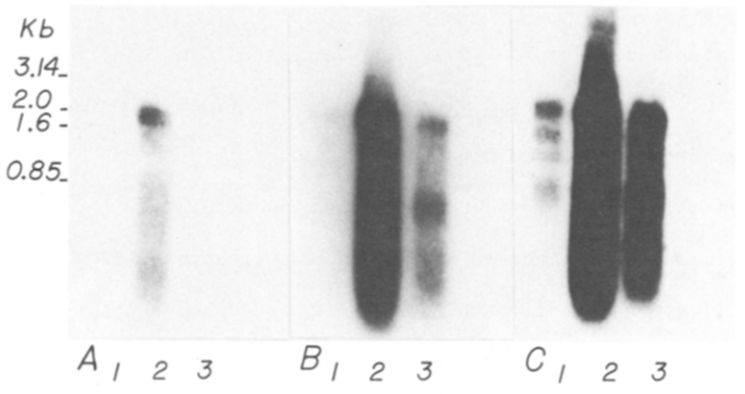

Fig. 2. Analysis of total poly(A)$^+$RNA from control and hormone treated aleurone layers. Poly(A)$^+$RNA was isolated from aleurone layers, and analyzed by RNA gel blot hybridization with [^{32}P]αAcDNA probe. The size markers were coat protein gene of C_c-TMV, 16S rRNA, 18S rRNA, and 23S rRNA and were visualized by staining with ethidium bromide. The lanes contained 5 µg of poly(A)$^+$RNA from control layers (lane 1), from aleurone layers treated with 1 µM GA for 16 hrs (lane 2), and from aleurone layers treated with 1 µM GA and 20 µM ABA for 20 hrs (lane 3). A, B, and C indicate film exposure times of 1, 12, 36 hrs, respectively. The arrow indicates the position of native α-amylase.

Quantification of α-amylase sequences. The mRNA which sediments at approximately 17S and includes the peak of pre-α-amylase translational activity was used as a primer for cDNA synthesis. The cDNA synthesized in these reactions was about 400 nucleotides. We have used this α-amylase cDNA (αAcDNA) to quantify the levels of α-amylase mRNA (αAmRNA) sequences.

Equal amounts of poly(A)$^+$RNA isolated from aleurone layers treated with different hormones were electrophoresed under denaturing conditions on agarose gels. The RNA was transferred to nitrocellulose filter and hybridized with αAcDNA probe (Fig 2). A major band of hybridization of about 1600 nucleotides was observed with poly(A)$^+$RNA from layers treated with GA_3 for 16 hrs. (Fig. 2A lane 2). Longer exposures of the same blots were necessary to detect the αAmRNA sequences in poly(A)$^+$RNA isolated from layers treated with GA_3+ABA (Fig 2B lane 3) and without GA_3 (Fig 3B, lane 1). One of the striking features of the hybridization

pattern apparent only after long exposures, is the apparent complexity of the extracted RNA. This suggests the presence of many more RNA species than detected by in vitro translation (Fig. 1). Densitometric analyses of these autoradiograms indicated that the αAmRNA sequence is approximately 40 fold more abundant in layers treated with GA_3 for 16 hrs. (Fig 2A lane 2) than in layers without treatment (Fig 2C lane 1). The relative concentrations of αAmRNA sequence show a strong correlation with the amount of α-amylase being synthesized by these layers after GA_3 treatment. These results therefore show that increased synthesis of α-amylase in aleurone layers treated with GA_3 is due to corresponding increases in αAmRNA sequences.

Analysis of poly(A)$^+$RNA from layers treated with GA_3+ABA show that in the presence of ABA, there is a smaller increase in the GA_3-induced α-AmRNA (Fig 2B, lane 3). In this experiment αAmRNA increased 3 fold.

DISCUSSION

Using the blot hybridization technique, we have shown that incubation of barley aleurone layers with GA_3 or GA_3+ABA causes an increase in the concentration of the α-amylase mRNA (Fig 2). The size of the α-amylase mRNA sequences detected in the present work in control or hormone-treated tissue (Fig 2), 1.6 Kb, agrees well with previous measurements of the size of this mRNA (9). This result obtained with a partially purified αAcDNA probe, confirms an earlier suggestion, based upon measurements using labeled RNA precursors (5,6), and in vitro translation assays (7), that incubation with GA_3 increases αAmRNA sequences in barley aleurone layers. It is interesting to note that the levels of α-amylase mRNA in cells treated with GA_3+ABA is lower than in cells treated with GA_3 only. Whether this difference is due to a difference in transcriptional rate (synthesis) or difference in degradation is currently unknown.

ACKNOWLEDGEMENTS: This work was supported by a grant from the Monsanto Agricultural Products Co. and by an International Research Fellowship (NIH) awarded to IB-L. We thank Michael Mitas for technical help.

REFERENCES

1. Chrispeels, M.J. and Varner, J.E. (1967) Plant Physiol. 42: 398-406.

2. Higgins, T.J.V., Zwar, J.A. and Jacobsen, J.V. (1977) in J.H. Weil and L. Bogorad, ed., Acides Nucleiques et synthese des proteines chez les vegeteux p. 481-486 C.N.R.S.

3. Mozer, T.J. (1980) Cell 20: 479-485.

4. Filner, P. and Varner, J.E. (1967) Proc. Natl. Acad. Sci. U.S.A. 58: 1520-1526.

5. Jacobsen, J.V. and Zwar, J.A. (1975) Proc. Natl. Acad. Sci. U.S.A. 71: 3290-3293.

6. Ho, D.T.H. and Varner, J.E. (1975) Proc. Natl. Acad. Sci. U.S.A. 71: 4783-4786.

7. Higgins, T.J.V., Zwar, J.A. and Jacobsen, J.V. (1976) Nature 260: 166-169.

8. Hall, T.C., Ma, Y., Buchbinder, B.U., Pyne, J.W., Sun, S.M., and Bliss, T.A. (1978) Proc. Natl. Acad. Sci. U.S.A. 75: 3196-3200.

9. Mozer, T.J. (1980) Plant Physiol. 65: 834-837.

10. Bruening, G., Beachy, R.N., Scalla, R., and Zaitlin, M. (1976) Virology 71: 498-517.

11. Mans, R.J. and Novelli, G.D. Biochim. Biophys. Res. Comm. 3: 540-543.

12. Laemmli, U.K. (1970) nature 227: 680-685.

13. Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46: 83-88.

14. Meyers, J. and Spiegelman, S. (1978) Proc. Natl. Acad. Sci. U.S.A. 75: 5329-5333.

15. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77: 5201-5205.