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SYNTHESIS OF POLY(A) POLYMERASE FROM CONSERVED MESSENGER RNA IN GERMINATING EXCISED EMBRYOS OF WHEAT

SUJATA LAKHANI, A. NANDINI THIRU and R. C. SACHAR

Biochemistry and Molecular Biology Laboratory, Department of Botany, University of Delhi, Delhi 110007, India

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Key Word Index—*Triticum aestivum*; Gramineae; wheat embryos; poly(A) polymerase; conserved mRNA; *de novo* protein synthesis; cordycepin-mediated stimulation.

Abstract—A 3.5–6.0-fold stimulation of poly(A) polymerase activity was observed in excised wheat embryos germinated for 48 hr. Addition of primer RNA to the enzyme assay mixture was necessary for the incorporation of [^{3}H]AMP into the acid-precipitable polyadenylate product. Administration of six amino acid analogues (1 mM each) or cycloheximide (10 μg/ml) to the germinating embryos resulted in 77–82% inhibition of poly(A) polymerase activity. The inhibitory response, elicited by the analogues, was substantially counteracted by the simultaneous addition of the corresponding six amino acids (2 mM each). This indicated that *de novo* protein synthesis was necessary for the enhancement of poly(A) polymerase activity. Cordycepin, a potent inhibitor of transcription, failed to block poly(A) polymerase activity; instead, the drug invariably brought about a significant stimulation (*ca* 1.7–4.0-fold) of the enzyme activity. Cordycepin, however, inhibited acid phosphatase activity by 77% in germinating wheat embryos. Actinomycin D also failed to inhibit poly(A) polymerase activity in germinating wheat embryos. The lack of inhibition of poly(A) polymerase by transcriptional inhibitors during early germination suggested that the enzyme was translated from its conserved mRNA, already stored in the dry wheat embryos.

INTRODUCTION

Poly(A) polymerase has been reported in several plants, such as tobacco [1], maize [2], mung bean [Berry, M. and Sachar, R. C., unpublished results] and wheat [3,4]. Fractionation of wheat leaf extracts on DEAE-cellulose showed two isoenzymes of poly(A) polymerase. These were probably of nuclear and chloroplast origin [4]. Poly(A) polymerase from wheat germ was shown to possess both polymerizing and hydrolytic activity [5]. The preferential manifestation of the dual enzyme activity is dependent on the concentration of ATP. The hormonal regulation of poly(A) polymerase by GA_3 was reported in embryoless wheat half-seeds from this laboratory [6]. Subsequently, it was shown that the GA_3-mediated stimulation of poly(A) polymerase in wheat aleurone layers required *de novo* protein synthesis, but could occur under blocked conditions of transcription. Thus, the hormone seemed to regulate poly(A) polymerase activity at some post-transcriptional step [7].

Stimulation of poly(A)-containing mRNA has been reported in germinating embryos of wheat [8], cotton [9] and hazel [10]. The precise function of poly(A) tails is unknown, although it has been considered that polyadenylation may help in the transport of mRNA from the nucleus to the cytoplasm [11] and render translational stability to the mRNA [12, 13]. Poly(A) tails have also been assigned a protective role against nuclease digestion of mRNA from the 3′ end [14].

In this investigation, we report a 3.5–6-fold stimulation of poly(A) polymerase activity during early germination (48 hr) of excised wheat embryos. Inhibitor studies strongly indicated the presence of conserved message of poly(A) polymerase in quiescent wheat embryos. Paradoxically, treatment of embryos with cordycepin (3′-deoxyadenosine) effected significant enhancement of poly(A) polymerase activity over the controls.

RESULTS

Poly(A) polymerase was assayed in the G-25 fraction, isolated from germinated (48 hr) excised wheat embryos. The enzyme activity was determined as a measure of [^{3}H]AMP incorporation into the acid-precipitable polynucleotide product.* The enzyme activity showed a strict requirement for the exogenous addition of wheat embryo primer RNA. *Ca* 3.5–6-fold stimulation of poly(A) polymerase was observed in 48 hr germinated wheat embryos over that detected in the dry embryos.

Effect of amino acid analogues and cycloheximide on poly(A) polymerase activity

In order to determine whether the increased poly(A) polymerase activity in wheat embryos was due to activation or *de novo* enzyme synthesis, the excised embryos were germinated in the presence of amino acid analogues and a protein synthesis inhibitor. Administration of six amino acid analogues (1 mM each) to the germinating wheat embryos effectively inhibited poly(A) polymerase activity (77% inhibition). This inhibitory response was substantially alleviated (*ca* 60%) by the simultaneous addition of the six corresponding amino acids (2 mM; Table 1). Embryos germinated in the continuous presence

*The polyadenylate nature of the product has been enzymatically and chemically characterized and is to be published elsewhere.

Table 1. Inhibition of poly(A) polymerase activity by amino acid analogues in germinated excised wheat embryos

Additions	Poly(A) polymerase activity, [^{3}H]AMP incorporation (dpm/mg protein)	Relative activity
Control	21 820	1.00
Six amino acid analogues (1 mM each)	5030	0.23
Six amino acid analogues (1 mM each) + six amino acids (2 mM each)	15 040	0.69

Excised wheat embryos were germinated for 48 hr on Nitsch's basal liquid medium containing six amino acid analogues (3,5-di-iodotyrosine, L-canavanine, DL-7-azatryptophan, L-thioproline, DL-ethionine and DL-*o*-fluorophenylalanine, (1 mM each) and on medium containing these analogues (1 mM each) together with their corresponding amino acids (L-tyrosine, L-arginine, L-tryptophan, L-proline, L-methionine and L-phenylalanine, 2 mM each). Poly(A) polymerase activity was assayed in the G-25 fraction as [^{3}H]AMP incorporation into the acid-precipitable polynucleotide product. Poly(A) polymerase activity in the G-25 fraction, prepared from dry excised embryos, was 6052 dpm/mg protein.

of cycloheximide (10 μg/ml) also showed a progressive inhibition of poly(A) polymerase at different stages of germination (Fig. 1). *Ca* 82% inhibition of enzyme activity was observed in cycloheximide-treated embryos at the 48 hr stage. These results strongly implied the requirement of *de novo* protein synthesis for the observed increase in poly(A) polymerase activity during early stages of embryo germination.

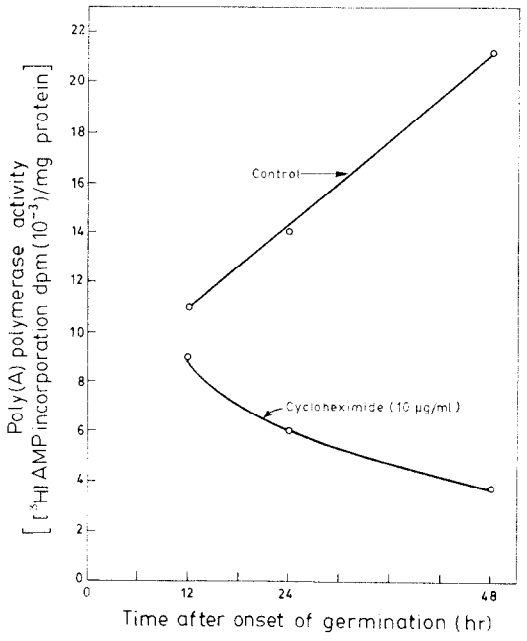

Fig. 1. Time course studies showing strong inhibition of poly(A) polymerase activity by cycloheximide in germinating excised wheat embryos. Excised wheat embryos were cultured in the presence and absence of cycloheximide (10 μg/ml). Poly(A) polymerase activity was assayed in the G-25 fraction at different stages of embryo germination.

Effect of cordycepin (3'-deoxyadenosine) and actinomycin D on poly(A) polymerase activity

To ascertain whether the stimulation of poly(A) polymerase also required fresh transcriptional activity during the early stages of embryo germination, cordycepin (500 μM) was administered at the inception of germination. The drug proved effective in arresting embryo germination, but failed to inhibit poly(A) polymerase activity. Surprisingly, a 2.25-fold increase in poly(A) polymerase activity was observed in cordycepin-treated embryos (77 900 dpm/mg protein) over that of the controls (34 600 dpm/mg protein) after 48 hr of germination. Embryos germinated (48 hr) in the presence of actinomycin D (100 μg/ml) also showed no inhibition of poly(A) polymerase activity; instead there was an increase of nearly 30% in enzyme activity in drug treated embryos over that of controls (data not presented). In the other experiments, cordycepin was administered to the embryos 12 hr after the onset of germination. This would necessarily permit the initiation of all metabolic activities in the embryo before the drug could prevent transcriptional activity. In this case too, the drug stimulated poly(A) polymerase activity by *ca* 1.7–4.0-fold in 48 hr germinated embryos (Table 2). Cordycepin (500 μM), however, brought about significant inhibition (76.6% inhibition) of acid phosphatase activity in 48 hr germinated wheat embryos, thus confirming the effective penetration of the drug in the embryonic tissue [15].

Since the ammonium sulphate fraction precipitate (0–50%) from wheat embryos is likely to have a number of enzyme activities which could effect the incorporation of label from [^{3}H]ATP into the acid-precipitable fraction, it became necessary to check whether the enzyme activity in cordycepin-treated embryos is also dependent on the presence of primer RNA. The data on the incorporation of

Table 2. Stimulation of poly(A) polymerase activity by the mid-course addition of cordycepin to the germinating excised wheat embryos

Additions	Experiment 1		Experiment 2		Experiment 3	
	Poly(A) polymerase activity, [^{3}H]AMP incorporation (dpm/mg protein)	Relative activity	Poly(A) polymerase activity, [^{3}H]AMP incorporation (dpm/mg protein)	Relative activity	Poly(A) polymerase activity, [^{3}H]AMP incorporation (dpm/mg protein)	Relative activity
Control	30810	1.00	37300	1.00	22900	1.00
Cordycepin (500 μM)	51200	1.66	75800	2.03	91000	3.97

Excised wheat embryos were initially germinated on Nitsch's medium for 12 hr. Thereafter, one set of embryos was kept as control, while the other was administered cordycepin (500 μM). Poly(A) polymerase activity was assayed in the G-25 fraction prepared from 48 hr germinated embryos.

Table 3. Poly(A) polymerase activity in the presence and absence of exogenous primer RNA in cordycepin-treated embryos

Additions	Poly(A) polymerase activity, [^{3}H]AMP incorporation (dpm/mg protein)	% increase of label incorporation over the (−)-primer
Control		
1. (−)-Primer	5100	—
2. (+)-Primer	22900	77.5
Cordycepin		
3. (−)-Primer	32900	—
4. (+)-Primer	91000	63.8

Excised wheat embryos were germinated for 48 hr in basal medium to which cordycepin (500 μM) was added 12 hr after the onset of germination. Poly(A) polymerase activity was assayed in the ammonium sulphate fraction precipitate (0–50%) in the presence and absence of exogenous primer RNA (1 mg).

label into the acid-precipitable fraction with and without the primer RNA is presented in Table 3. It became evident that the bulk of label incorporation in controls (78%) and cordycepin-treated embryos (64%) is strictly dependent on the exogenous addition of primer RNA to the assay reaction mixture.

Briefly, the inhibition of poly(A) polymerase by translational inhibitors, but not by transcriptional inhibitors, strongly favours the presence of conserved message for poly(A) polymerase in wheat embryos.

DISCUSSION

The presence of conserved mRNA has been reported in quiescent embryos of many flowering plants, like bean [16], pea [17], rice [18], cotton [19]; and in the classical example of unfertilized sea urchin eggs [20, 21]. In wheat embryos, the presence of conserved mRNA was shown by DNA–RNA competition hybridization techniques [22]. However, the validity of this study was questioned by Purdom *et al.* [23] who demonstrated that the RNA from dry embryos used by Chen and coworkers for hybridization with wheat embryo DNA was actually ribosomal and not messenger RNA. Subsequently, the presence of conserved messengers in ungerminated wheat embryos was proved by translating the isolated messenger fraction in a cell-free system [24, 25]. Conserved messengers have also been reported for specific enzymes, like *o*-diphenolase in wheat embryos [26], carboxypeptidase [27], isocitrate lyase [28] and protease [29, 30], in cotton; and acid phosphatase in wheat half-seeds [31]. In isolated wheat aleurone layers, gibberellic acid-mediated stimulation, of poly(A) polymerase is dependent upon translation of a conserved mRNA. This is based on the observation that the hormone-triggered stimulation of poly(A) polymerase is effectively blocked by cycloheximide and amino acid analogues, clearly implicating the requirement of *de novo* protein synthesis. However, inhibition of transcription by cordycepin (500 μM) failed to inhibit the GA_3-stimulated poly(A) polymerase. This suggested that the expression of conserved message of poly(A) polymerase in wheat aleurone layers is regulated by a phytohormone [7]. The significance of long-lived mRNA in seeds of flowering plants and the role they play during early germination has been extensively discussed by Payne [32].

Presently, we observed that the stimulation of poly(A) polymerase activity in germinating excised wheat embryos is not inhibited by cordycepin. However, strong inhibition of enzyme activity by amino acid analogues and cycloheximide in germinating embryos implicated the necessity of *de novo* protein synthesis. Thus, the inhibitor studies indicated that poly(A) polymerase is translated from a conserved message already stored in the dry wheat embryos. The effective penetration of cordycepin in wheat embryos is based on the following facts: the drug strongly inhibited [8-^{14}C]adenine incorporation (80–82% inhibition; [33, 34]) into the RNA fraction of germinating excised wheat embryos; and it also caused severe inhibition of acid phosphatase activity in 48 hr germinated excised wheat embryos (76.6% inhibition; [15]).

In addition, we observed that the administration of cordycepin to excised embryos resulted in an enhancement of poly(A) polymerase activity (1.7–4.0-fold stimulation). This paradoxical stimulatory effect of cordycepin could be explained by postulating that the drug is responsible for blocking the transcriptional activity of some inhibitory factor of poly(A) polymerase. Other workers have reported that actinomycin D causes the unusual stimulation of tyrosine aminotransferase in hepatoma cells [35, 36], tryptophan pyrrolase in rat liver cells [37], protease in cotton embryos [30] and ribonuclease in *Bacillus subtilis* [38]. This paradoxical effect of actinomycin D was first termed 'super-induction' by Tomkins *et al.* [35].

In germinating cotton embryos, protease activity was stimulated by actinomycin D, while cordycepin inhibited the enzyme activity. Since actinomycin D failed to inhibit protease activity, Ihle and Dure [30] suggested that protease was translated from its conserved messenger. Cordycepin inhibits polyadenylation of the protease conserved mRNA and this resulted in the inhibition of protease activity. The stimulatory effect of actinomycin D on protease activity was attributed to the inhibition of a protease inhibitor mRNA. However, these experiments cannot be accepted as conclusive for the following reasons. (a) Actinomycin D blocked only 70% of trans-

criptional activity in cotton embryos. In the absence of total inhibition of RNA synthesis, the possibility of fresh synthesis of mRNA for protease cannot be ruled out during early germination of cotton embryos. (b) Cordycepin blocks both transcription and polyadenylation in germinating cotton embryos. Therefore, the inhibition of protease activity by this drug could as well be due to inhibition of its messenger fraction (see Payne [32]).

The stimulation of poly(A) polymerase in wheat embryos by cordycepin necessarily reflects the elimination of some inhibitory factor at the transcriptional step. This inhibitor probably plays a significant regulatory role in the expression of poly(A) polymerase during early germination. The activation of a preformed poly(A) polymerase by cordycepin is ruled out in wheat embryos in view of the absolute requirement for de novo protein synthesis for the increased enzyme activity.

We consider that all poly(A) polymerase activity in the first 48 hr is coded by conserved mRNA when transcription is blocked by administration of cordycepin. However, it is quite possible that the induction of poly(A) polymerase (48 hr) is contributed by the combination of newly synthesized and conserved mRNA in the absence of a transcriptional inhibitor. Dry wheat embryos are known to possess conserved mRNA which can support general protein synthesis in a cell-free system [25]. At the same time, it has also been reported that the synthesis of new mRNA in wheat embryos begins after 2 hr of imbibition [39]. In view of these observations, we are inclined to believe that the synthesis of mRNA specific for poly(A) polymerase could occur during the first 48 hr of germination. However, it is not easy to discern the contribution of newly synthesized and conserved mRNA towards the synthesis of poly(A) polymerase. The situation becomes somewhat complex because there is stimulation of poly(A) polymerase in presence of a transcription inhibitor. The lack of inhibition of enzyme activity would prove the presence of conserved mRNA, while the stimulation of poly(A) polymerase would indicate that some sort of protection is offered to the stored mRNA or to the processing enzyme itself against some inhibitor which is not synthesized in presence of cordycepin.

The conserved mRNA of poly(A) polymerase seems fairly long-lived as the increase in poly(A) polymerase is not inhibited by cordycepin in embryos germinated for 72 hr (unpublished data). Perhaps it would be necessary to grow embryos for a much longer duration for determining the stage at which the degradation of conserved mRNA begins.

Thus, the presence of conserved mRNA for poly(A) polymerase in quiescent wheat embryos and its active translation during early stages of embryo germination could be advantageous to the system for rapid polyadenylation of the newly formed mRNAs.

EXPERIMENTAL

Isolation and culture of excised wheat embryos. Dry wheat embryos (*Triticum aestivum var. Sonalika*) were isolated by a slightly modified procedure of ref. [40]. The excised embryos were surface-sterilized with $HgCl_2$ soln (0.02%), thoroughly rinsed with sterile H_2O and cultured on an aseptic liquid basal medium [41] in the presence of chloramphenicol (50 μg/ml). The embryos were germinated in the dark at 25°.

Enzyme extraction. The germinated embryos were homogenized in Tris–HCl buffer (50 mM, pH 8) containing β-mercaptoethanol (5 mM) and polyvinyl polypyrrolidone (PVP, 4% w/v). Acid-washed sand was used as an abrasive. The homogenate was spun at 10000 *g* for 10 min and the supernatant was mixed with an equal vol. of satd $(NH_4)_2SO_4$ soln. The $(NH_4)_2SO_4$ fraction ppt (0–50% satn) was desalted on a Sephadex G-25 column (30 × 3.8 cm). This was designated the G-25 fraction and used for the assay of poly(A) polymerase activity. Proteins were determined by the method of ref. [42]. All operations were carried out at 4°.

Assay for poly(A) polymerase activity. The assay reaction mixture for poly(A) polymerase contained Tris–HCl buffer (100 μmol, pH 8), β-mercaptoethanol (2 μmol), $MnCl_2$ (2 μmol), wheat embryo primer RNA (1.0 mg), unlabelled ATP (0.4 μmol), [G-^{3}H]ATP (4 μCi, sp. act. 1500 mCi/mmol) and G-25 fraction (1 mg protein), in a final vol. of 500 μl. The reaction mixture was incubated at 37° for 30 min, and terminated with an equal vol. of TCA (10%) containing $Na_2P_2O_7$ (2 mM). The contents were chilled and the acid-insoluble product was washed on 3 MM Whatman filter discs with TCA (5%, 40 ml), Et_2O–EtOH (1:1, 10 ml) and Et_2O (15 ml). The filter discs were dried at 60° and the radioactivity determined using scintillation fluid containing PPO (5 g) and POPOP (0.3 g) in 1 l. of distilled toluene. The radioactivity of the boiled enzyme control was subtracted from the values of each sample.

Primer RNA. Prepared from the embryonic tissue of 48 hr germinated seedlings according to the procedure of ref. [43].

Treatment of embryos with amino acid analogues and inhibitors of protein and RNA syntheses. Excised wheat embryos were germinated (48 hr) under aseptic conditions on Nitsch's liquid basal medium containing six amino acid analogues (1 mM each of 3,5-di-iodotyrosine, L-canavanine, DL-7-azatryptophan, L-thioproline, DL-ethionine and DL-*o*-fluorophenylalanine), six amino acid analogues (1 mM each) together with six corresponding amino acids (2 mM each of L-tyrosine, L-arginine, L-tryptophan, L-proline, L-methionine and L-phenylalanine), cycloheximide (10 μg/ml), cordycepin (500 μM) and actinomycin D (100 μg/ml). Cordycepin and actinomycin D were administered to the dry wheat embryos at the start of germination, and the effect of the drug was tested on poly(A) polymerase activity after 48 hr of embryo germination. The effect of cordycepin was also tested by its mid-course addition to 12 hr germinated embryos. In this case too, enzyme activity was assayed after culturing embryos for 48 hr.

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