HORMONAL REGULATION OF POLY(A) POLYMERASE ACTIVITY BY GIBBERELLIC ACID IN EMBRYO-LESS HALF-SEEDS OF WHEAT (*TRITICUM AESTIVUM*)

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1. Introduction

Poly(A) polymerase enzyme has been reported in a variety of animal and plant tissues [1]. The enzyme catalyzes the addition of AMP residues at the 3′-terminus hydroxyl-end of mRNA. ATP is the true substrate of this enzyme. The enzyme has been purified to homogeneity and its reaction product characterized in calf thymus tissue [2,3]. Treatment of resting lymphocytes with phytohemaglutins (lectins) stimulated (3–6-fold) poly(A) polymerase activity [4]. Among plants, poly(A) polymerase activity is reported in tobacco [5], maize [6,7], wheat [8,9] and cotton [10] but so far, the enzyme has not been purified. Polyadenylation plays a crucial role in processing of hnRNA to mRNA [1]. It is thus logical to consider that processing of mRNA is regulated either by the de novo synthesis or by the activation of poly(A) polymerase. At present, however, practically nothing is known about the regulation of poly(A) polymerase activity in animal and plant cells. We now report the hormonal control of poly(A) polymerase activity in embryo-less wheat half-seeds by the exogenous application of gibberellic acid. Inhibitor studies indicated the requirement of de novo protein synthesis for GA_3-stimulated poly(A) polymerase activity.

2. Materials and methods

2.1. *Enzyme preparation*

Embryo-less half-seeds of wheat (*Triticum aestivum*, var. *sonalika*) were pre-washed for 5 h in distilled water at 20°C. Half-seeds, surface sterilized with $HgCl_2$ soln. (0.02%), were imbibed (48 h) under aseptic conditions (25°C) in presence and absence of GA_3 (10^{-5} M) soln. Chloramphenicol (50 μg/ml) was routinely added during the imbibing of half-seeds to prevent bacterial contamination. The effect of cycloheximide (20 μg/ml) and various amino acid analogues (D,L-ethionine, D,L-7-azatryptophan, D,L-*o*-fluorophenylalanine, L-thioproline, 1 mM each) was tested on poly(A) polymerase activity in GA_3-treated half-seeds. After imbibing, the half-seeds (10 g) were homogenized in Tris–HCl buffer (100 ml, 50 mM, pH 8.0) containing β-mercaptoethanol (5 mM), Triton X-100 (0.2%) and polyvinyl polypyrrolidone (2%). Acid washed sand was used as an abrasive for grinding the half-seeds. The homogenate was centrifuged at 20 000 × *g* for 15 min. The supernatant was fractionated by solid ammonium sulphate (30–50% saturation). The ammonium sulphate fraction precipitate was completely desalted on Sephadex G-25 column (1.8 cm × 17 cm). This partially purified fraction was designated as 'G-25 fraction' and was used for the assay of poly(A) polymerase activity.

2.2. *Assay of poly(A) polymerase*

The assay mixture comprised of [^{3}H]ATP (4 μCi, spec. act. 15.00 mCi/mmol), unlabelled ATP (0.4 μmol), Tris–HCl buffer (100 μmol, pH 8.0), β-mercaptoethanol (2 μmol), $MnCl_2$ (2 μmol), wheat embryo primer RNA (1 mg) and G-25 fraction (500 μg protein) in a final volume of 500 μl. The incubation mixture was incubated at 37°C for 30 min and the reaction was terminated by adding equal volume of trichloroacetic acid (10%) containing sodium pyrophosphate (2 mM). Bovine serum albumin (500 μg) was added as a carrier protein at the time of ter-

Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; GA_3, gibberellic acid; hnRNA, heterogeneous nuclear RNA; mRNA, messenger RNA, PAGE, polyacrylamide gel electrophoresis; poly(A), polyadenylic acid; POPOP, 1,4-bis[2-(5-phenyloxazole)]-benzene; phenyl-oxazolylphenyl-oxazolylphenyl; PPO, 2,5-diphenyloxazole

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minating the reaction. The trichloroacetic acid-precipitable fraction was collected on Whatman 3 MM circular discs (24 mm). The precipitate was washed (×6) with a 7 ml aliquot of chilled trichloroacetic acid (5%) and finally rinsed with a mixture of alcohol: ether (1:1, 10 ml) and ether (15 ml). Radioactivity was determined in the dried filter discs by using toluene–PPO–POPOP scintillation fluid (1 litre:5.0 g: 0.3 g). Radioactive counts of samples were corrected by subtracting the values of boiled enzyme controls. Primer RNA was isolated from wheat embryos, excised from 2-day-old seedlings, by the slightly modified procedure in [11]. This RNA preparation was relatively rich in messenger fraction. Protein was estimated by the method in [12].

2.3. *Acrylamide gel electrophoresis*

The G-25 fraction was fractionated on 10% polyacrylamide gels [13]. Primer RNA (1 mg/gel column) was added during polymerization of the acrylamide gels. The gels were pre-washed electrophoretically (2 mA/gel column) with Tris–glycine buffer (pH 8.3) containing β-mercaptoethanol (5 mM) for 1 h before loading the sample. The G-25 fraction (1 mg sample protein) containing tracking dye (bromophenol blue, 0.005%) and sucrose (5%) soln. was loaded on each gel column and electrophoresed for 1 h at 4°C. Thereafter, the incubation mixture (0.16 ml) containing [^{3}H]ATP (4 μCi) + unlabelled ATP (0.4 μmol) + $MnCl_2$ (2 μmol) + β-mercaptoethanol (2 μmol) + Tris–HCl buffer (100 μmol, pH 8.0) were loaded on each gel column and electrophoresed for additional 1 h at 37°C. The gels were frozen in liquid nitrogen and sliced into 1 mm thick sections with a gel slicer. The slices were placed on discs of Whatman 1 MM and dried in oven. Radioactivity was scanned in dried gel slices in toluene-based scintillation fluid.

3. Results

Partially purified fraction (G-25 fraction), isolated from GA_3-treated wheat half-seeds, showed 2–3-fold stimulation of poly(A) polymerase activity over the untreated controls (table 1, fig.1). The physico-chemical properties of poly(A) polymerase in controls and GA_3-treated half-seeds were identical in several respects, such as pH optimum, response to ionic strength, enzyme stability and electrophoretic mobility on acrylamide gels. In both fractions, the pH activity

Table 1
Stimulatory effect of GA_3 on poly(A) polymerase activity in wheat half-seeds

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)			
	Expt 1		Expt 2	
	dpm/mg protein	Relative activity	dpm/mg protein	Relative activity
Control	3316	1.00	4388	1.00
GA_3 (10^{-5} M)	6604	1.99	12 820	2.92

Embryo-less half-seeds, pre-washed for 5 h, were imbibed in GA_3 (10^{-5} M) soln. for 48 h. The enzyme activity was assayed in G-25 fraction by the incorporation of labelled [^{3}H]ATP into acid-precipitable fraction

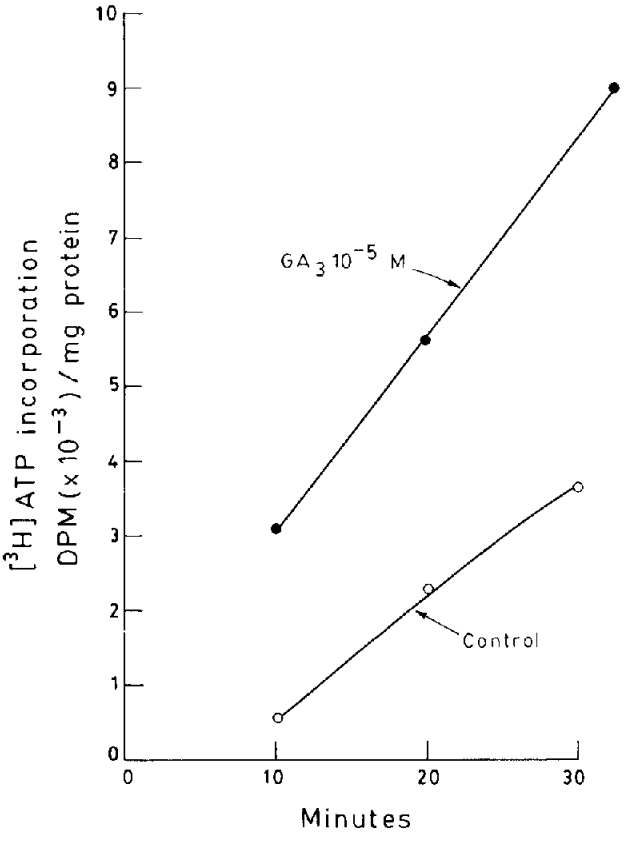

Fig.1. [^{3}H]ATP incorporation into poly(A) product as a function of time in control and GA_3-treated half-seeds of wheat. Poly(A) polymerase activity was assayed in G-25 fractions.

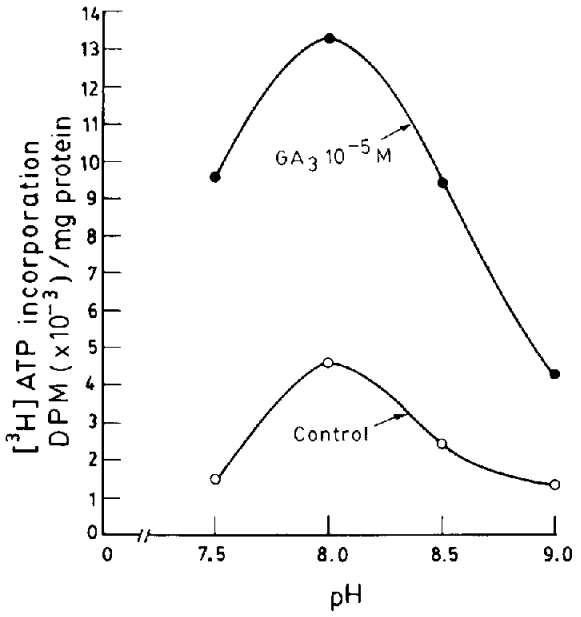

Fig.2. [^{3}H]ATP incorporation into poly(A) product as a function of pH in control and GA_3-treated half-seeds of wheat. Poly(A) polymerase activity was assayed in G-25 fractions.

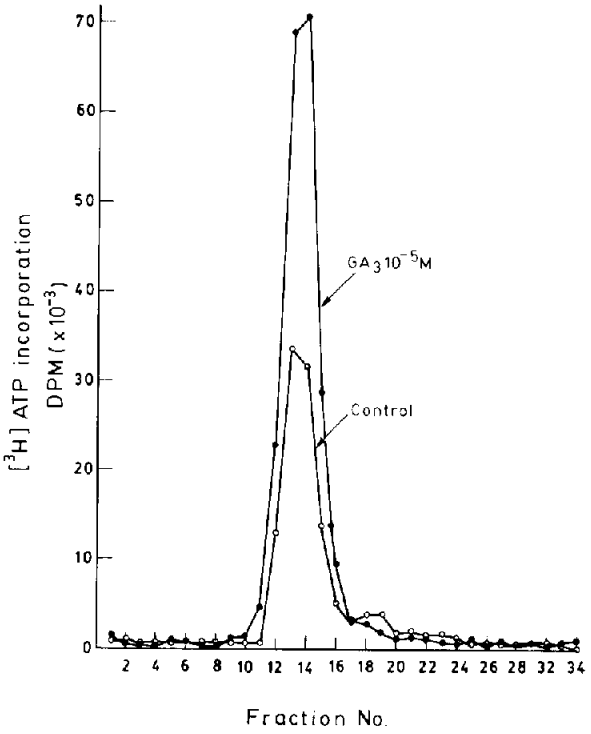

Fig.3. Activity peaks of poly(A) polymerase, in control and GA_3-treated half-seeds, obtained by fractionating G-25 fractions on polyacrylamide gel electrophoresis.

profile was similar, with a pH optimum at 8.0 (fig.2). The enzyme activity was inhibited by ammonium sulphate (20–40 mM) to 50–60%. The G-25 fraction retained full enzyme activity for 4–5 days when stored at −10°C after freezing in liquid nitrogen. Poly(A) polymerase activity was completely abolished when the G-25 fraction was heated at 55°C for 2 min. Fractionation of G-25 fraction on PAGE showed no multiple forms of poly(A) polymerase. Both control and GA_3-treated half-seeds showed a single activity peak of poly(A) polymerase (fig.3). The position of the activity peak in control coincided with that of the GA_3-treated half-seeds. This was also confirmed by fractionating mixed enzyme preparations on acrylamide gels. Thus, the electrophoretic mobility of GA_3-stimulated poly(A) polymerase activity peak was identical to that of the control enzyme. A 2-fold stimulation of poly(A) polymerase activity by GA_3 was also witnessed on acrylamide gels (fig.3). No radioactive peak was observed when [^{3}H]ATP was passed through the gels devoid of G-25 fraction. It may be stated that the assay of poly(A) polymerase activity on acrylamide gels invariably showed higher enzyme activity (in terms of radioactive incorporation) in contrast to that observed with routine filter

Table 2
Inhibition of GA_3-stimulated poly(A) polymerase activity by cycloheximide in wheat half-seeds

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)	
	dpm/mg protein	Relative activity
Control	1816	1.00
GA_3 (10^{-5} M)	4800	2.64
GA_3 (10^{-5} M) + cycloheximide (20 μg/ml)	2252	1.24

The pre-washed half-seeds were imbibed (48 h) in the continuous presence of GA_3 and cycloheximide. The enzyme activity was assayed in G-25 fraction by the incorporation of labelled [^{3}H]ATP into acid-precipitable fraction

Table 3
Inhibition of GA_3-stimulated poly(A) polymerase activity by amino acid analogues in wheat half-seeds

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)	
	dpm/mg protein	Relative activity
GA_3 (10^{-5} M)	11 000	1.00
GA_3 (10^{-5} M) + four amino acid analogues (1 mM each)	5640	0.51

The pre-washed half-seeds were imbibed for 48 h in the continuous presence of GA_3 and four amino acid analogues (D,L-ethionine, D,L-7-azatryptophan, D,L-o-fluorophenylalanine, L-thioproline, 1 mM each). The enzyme activity was assayed in G-25 fraction by the incorporation of labelled [^{3}H]ATP into acid-precipitable fraction

assay procedure (fig.1,3). This unusual high rate of labelled [^{3}H]ATP incorporation observed on acrylamide gels could be due to the physical separation of some inhibitory factor of poly(A) polymerase activity. In [14] ATP levels were reported to regulate poly(A) polymerase and poly(A) hydrolytic activities in wheat germ extracts. They have further suggested that the two reactions in vivo may be performed by a single enzyme [14,15]. Therefore, another possibility is that poly(A) hydrolytic activity was low on acrylamide gels and this could account for the increased labelled [^{3}H]ATP incorporation.

Treatment of half-seeds with cycloheximide (20 μg/ml) strongly inhibited (86% inhibition) the GA_3-stimulated poly(A) polymerase activity (table 2). Also the addition of amino acid analogues (1 mM each) to the GA_3-treated half-seeds inhibited (49% inhibition) poly(A) polymerase activity, though to a lesser extent (table 3). In other sets of experiments, the analogues showed only 30% decrease in GA_3-stimulated poly(A) polymerase activity. However, this inhibitory effect of analogues was completely counteracted by the simultaneous addition of normal amino acids at high concentration (2 mM each). It, therefore, appears that de novo protein synthesis was necessary for the GA_3-regulated poly(A) polymerase activity. Besides, judging from the physico-chemical properties of this enzyme, it seems unlikely that the stimulation of poly(A) polymerase activity, in response to GA_3, is due to any structural modification of the preformed enzyme molecules.

The G-25 fraction showed a negligible phosphatase activity at pH 8.0, so that the assay mixture is not capable of building up a large pool of [^{3}H]ADP. This then excluded the possible synthesis of poly(A) through the activity of polynucleotide phosphorylase. Actinomycin D (10 μg/ml), which is an usual inhibitor of RNA polymerase activity, failed to inhibit poly(A) polymerase activity when added to the assay mixture.

4. Discussion

Poly(A) polymerase plays a pivotal role in the processing of hnRNA to mRNA by the addition of poly(A) tails to the messenger fraction [1]. Thus, one of the points of post-transcriptional regulation of mRNA could involve the de novo synthesis or activation of poly(A) polymerase. So far, there is no evidence to indicate the hormonal control of poly(A) polymerase activity in animal and plant cells. In animal systems, a solitary effort was made in which estrogen-treated oviduct tissue of quail showed enhanced transcriptional activity. However, no increase in poly(A) polymerase activity was observed, although the hormone did stimulate the specific activity of RNA polymerase I and II [16]. Increased polyadenylation of conserved messenger is reported in sea urchin eggs soon after fertilization, but nothing is stated about the regulation of poly(A) polymerase activity [17]. In barley aleurone layers also, GA_3 is reported to increase the level of RNA that contain poly(A) tails (poly(A) RNA) [18]. It was further shown that GA_3 increases the specific synthesis of both poly(A) and presumptive mRNA fractions [18]. However, it was not known whether the increased poly(A) synthesis was on account of more availability of messenger fraction, or was achieved by the enhancement of poly(A) polymerase activity. This investigation has revealed that GA_3 is responsible for 2–3-fold stimulation of poly(A) polymerase activity in wheat half-seeds and this could account for the increased polyadenylation of messenger fraction in cereals. Administration of cycloheximide and amino acid analogues significantly blocked GA_3-stimulated poly(A) polymerase activity in wheat half-seeds. This indicated the requirement of de novo protein synthesis for the hormonal control of enzyme activity. The stimulation of poly(A) polymerase activity (8–10-fold) was also recorded

in germinating excised wheat embryos (48 h). This enzyme activity was strongly inhibited by cycloheximide and amino acid analogues. This then also suggested the requirement of de novo protein synthesis for increased poly(A) polymerase activity during early germination of wheat embryos (Lakhani and Sachar, unpublished). It is pertinent to mention that GA_3 enhances the level of translatable α-amylase mRNA, containing poly(A) tails, in barley aleurone layers [19]. Both cycloheximide and amino acid analogues decreased the level of translatable α-amylase mRNA in GA_3-treated aleurone tissue, indicating the requirement of de novo protein synthesis [20]. In view of these observations, it is now visualized that one of the crucial roles of GA_3 during early seed germination in cereals is the regulation of poly(A) polymerase activity which in turn controls the polyadenylation of messenger fraction at the post-transcriptional step.

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