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MODULATION OF POLY(A)POLYMERASE BY cAMP IN *CICER ARIETINUM*

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Key Word Index—*Cicer arietinum*; Leguminosae; chick pea; cAMP; poly(A)polymerase; poly-(A$^+$)RNA.

Abstract—cAMP stimulated the activity of poly(A$^+$)polymerase in chick pea epicotyls with a parallel increase in poly(A$^+$)RNA levels. Experiments with inhibitors of RNA and protein synthesis and fractionation of partially purified enzyme on SDS–PAGE suggested a *de novo* synthesis of the enzyme.

INTRODUCTION

The regulatory role of cAMP in animal tissues and lower organisms is well documented [1, 2]. Its presence in bacteria, slime moulds, several fungi, algae and lower plants, in addition to animals, is irrefutable [3–6]. Attempts have been made to explore the possibility of similar roles of cAMP in higher plants [7–12]. Mass spectrometric and IR spectroscopic identification [13–15], the presence of cAMP binding proteins [15, 16], cAMP-dependent protein kinases [17, 18] and enzymes involved in cAMP metabolism [9] have suggested a regulatory role for this cyclic nucleotide in higher plants, where in a few cases it has been reported to mimic the action of hormones and act as a second messenger [19, 20].

Post-transcriptional addition of a poly(A$^+$) tail at the 3′ end of eukaryotic mRNA is an important step and is involved in mRNA stability and its translation [21]. Various plant hormones regulate poly(A$^+$)RNA levels in a number of plant species [22–27]. This important step is catalysed by poly(A$^+$)polymerase, which has been purified from a number of sources [28–31]. Hormonal control of this enzyme has also been reported [32–34].

Although cAMP has been implicated in the control of RNA synthesis in various plant tissues [22], no report exists of its role in post-transcriptional modification of mRNA. Bearing this in mind, we attempted to investigate a possible role of cAMP in the modulation of poly(A$^+$)polymerase activity and also the poly(A$^+$)RNA levels in *Cicer arietinum* epicotyls.

RESULTS AND DISCUSSION

In *Cicer arietinum*, maximum poly(A$^+$)polymerase activity was observed with poly(A$^+$) as primer, in the presence of Mn^{2+} at pH 8.0 and 30–32°. A time-course study revealed a linear increase in poly-(A$^+$)polymerase activity during the first 6 days of germination. This was accompanied by a parallel increase in poly(A$^+$)RNA levels.

There are only a few reports on the regulation of poly(A$^+$)polymerase activity in plants [32, 33, 35–37], and these indicate the regulation of this enzyme by hormones such as GA_3 and IAA. Because of the possible role of cAMP as a secondary messenger in the regulation of RNA synthesis in higher plants [12, 19], its involvement in the events leading to the synthesis of poly(A$^+$)RNA cannot be ruled out. Application of cAMP (10^{-5} M) resulted in an approximately two-fold increase in poly(A$^+$)RNA levels accompanied by a parallel 3.4-fold increase in poly(A$^+$)polymerase activity (Table 1). The results thus indicate that regulation of poly(A$^+$)polymerase by cAMP may lead to an increase in poly(A$^+$)RNA levels in *C. arietinum*.

The increased poly(A$^+$)polymerase activity in cAMP-treated seedlings could be due either to a conformational change in the pre-existing protein molecules or to its *de novo* synthesis. Administration of actinomycin D, cordycepin, cycloheximide and 5-fluorouracil to the germinating seedlings inhibited both cAMP-stimulated poly(A$^+$)polymerase activity as well as poly(A$^+$)RNA levels (Table 2). This suggested a fresh RNA and protein synthesis requirement for the observed cAMP stimulated response.

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Table 1. Effect of cAMP (10^{-5} M) on poly(A$^+$)polymerase activity and poly(A$^+$)RNA levels*

Treatment	Poly(A$^+$)polymerase [^{3}H]AMP incorporation (dpm mg^{-1} protein)	Relative activity	Poly(A$^+$)RNA [^{3}H]uracil incorporation (dpm mg^{-1} protein)	Relative activity
Control	50000	1.0	14200	1.0
cAMP (10^{-3} M)	36500	0.7	6700	0.5
cAMP (10^{-5} M)	172000	3.4	28900	2.0
cAMP (10^{-7} M)	90600	1.8	23500	1.7

* Seeds were germinated in the dark in the presence and absence of different concentrations of cAMP at 30±2°. Poly(A$^+$)polymerase was extracted from 6-day-old epicotyls. Enzyme activity was assayed in DEAE–sepharose fraction by incorporation of [^{3}H]ATP(sp. act. 3000 μCi mmol^{-1}). Labelled [^{3}H]uracil (sp. act. 360 GBq mmol^{-1}) was used for incorporation into RNA. RNA equal to 150000 dpm was loaded onto the affinity column, eluted and analysed for [^{3}H]uracil incorporation into poly(A$^+$)RNA.

Table 2. Effect of actinomycin D (Act D), cordycepin, cycloheximide (CHI) and 5-fluorouracil (5-FU) on poly(A$^+$)polymerase activity and poly(A$^+$)RNA levels*

Treatment	Poly(A$^+$)RNA [^{3}H]uracil incorporation (dpm mg^{-1} protein)	Relative activity	Poly(A$^+$)polymerase activity, [^{3}H]uracil incorporation (dpm mg^{-1} protein)	Relative activity
Control	15300	1.0	52600	1.0
cAMP (10^{-5} M)	31900	2.1	137100	2.6
Act D (30 μg ml^{-1})	7800	0.5	28400	0.5
Cordycepin (5×10^{-4} M)	8900	0.5	29100	0.6
cAMP (10^{-5} M)+Act D (30 μg ml^{-1})	22500	1.5	77400	1.5
cAMP (10^{-5} M)+cordycepin (5×10^{-4} M)	19900	1.3	85000	1.6
CHI (10 μg ml^{-1})	4800	0.3	15800	0.3
5-FU (10^{-3} m)	6200	0.4	21600	0.4
cAMP (10^{-5} M)+CHI (10 μg ml^{-1})	15800	1.1	64800	1.2
cAMP (10^{-5} M)+5-FU (10^{-3} M)	20600	1.3	74100	1.4

* Seeds were germinated in the dark in the presence of specific concentrations of different inhibitors. The activity of poly(A$^+$)polymerase was measured in the DEAE–sepharose fraction. The rest of the procedure was as described in the footnote to Table 1.

Studies on the effect of pH and thermal inactivation curves (data not given) suggested that both cAMP stimulated and control poly(A$^+$)polymerase possessed similar properties, indicating no conformational change on cAMP treatment. These results were confirmed further when partially purified DEAE–sepharose eluted fractions of both control and cAMP-treated seedlings were fractionated on polyacrylamide gel electrophoresis. Both cAMP-treated and control poly(A$^+$)polymerase showed a single activity peak at the same elution volume. However, a 2.3-fold higher activity was observed in cAMP-treated seedlings (Fig. 1). This clearly suggests that the observed stimulation in poly(A$^+$)polymerase as a result of cAMP application could be due to *de novo* synthesis of the enzyme.

The action of cAMP in stimulating the enzyme poly(A$^+$)polymerase was highly specific, since related adenine derivatives failed to mimic the action (Table 4). Moreover, the observed stimulation by cAMP could not be attributed to the cAMP acting as an additional source of nitrogen, as the seedlings grown in the presence of a few common amino acids did not show any enhanced stimulation. Dibutyryl-cAMP, a less polar structural analogue of cAMP, caused nearly the same stimulation as that of cAMP, indicating that non-permeability to cAMP in the plant cells did not arise.

It has been shown previously that the application of IAA (10^{-7} M) to *C. arietinum* seedlings results in an approximately two- to three-fold increase in poly(A$^+$)polymerase activity [34], and it is suggested that cAMP may act as a secondary messenger to the hormone.

EXPERIMENTAL

Seeds of chick pea (*Cicer arietinum*) var. Pusa 209 were surface sterilized and germinated in the dark at 30° in Petri plates containing acid-washed quartz

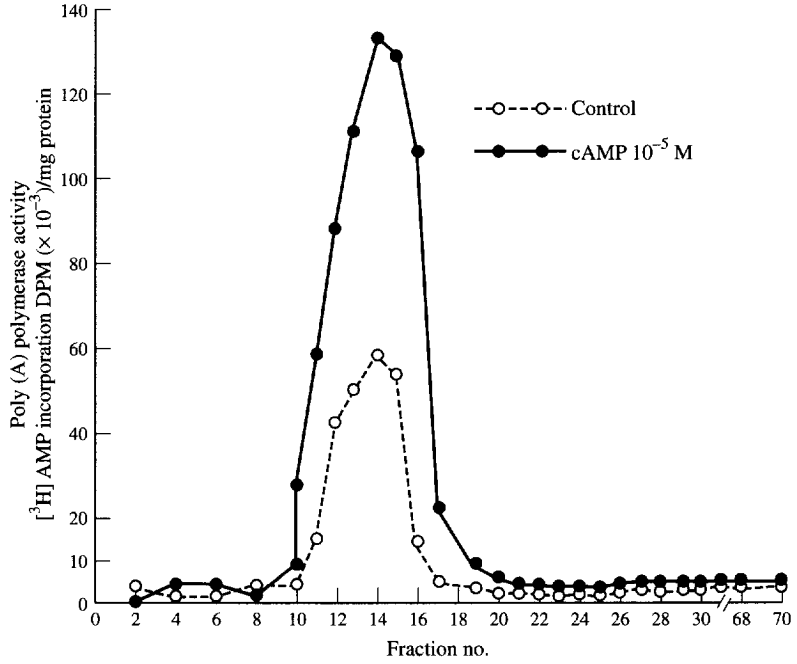

Fig. 1.

Table 3. Effect of a mixture of amino acid analogues (AAAs) on cAMP-stimulated poly(A⁺)polymerase activity*

Treatment	Poly(A⁺)polymerase activity, [³H]AMP incorporation (dpm mg⁻¹ protein)	Relative activity
Control	51 900	1.0
cAMP (10^{-5} M)	132 700	2.6
AAAs (1 mM)	29 000	0.6
AAAs (2 mM)	42 900	0.8
AAAs (1 mM) + amino acid (2 mM)	47 900	0.9
cAMP (10^{-5} M) + AAAs (1 mM)	80 000	1.5
cAMP (10^{-5} M) + AAAs (1 mM) + amino acid (2 mM)	128 600	2.5

*Seeds were germinated in the presence and absence of cAMP (10^{-5} M) at $30 \pm 2°$ in the dark for 6 days. Treatment of a mixture of different amino acid analogues, namely canavanine, ethionine, *p*-fluorophenylalanine, thioproline, azatryptophane and dinitrotyrosine, and the corresponding amino acids was done over a period of 48 hr. Epicotyls were harvested. Enzyme was assayed in the DEAE–sepharose fraction. The rest of the procedure was as described in the footnote to Table 1.

Table 4. Effect of various adenine derivatives of cAMP on poly(A⁺)polymerase activity in *C. arietinum* epicotyls*

Treatment	Poly(A⁺)polymerase activity, [³H]AMP incorporation (dpm mg⁻¹ protein)	Relative activity
Control	50 900	1.0
cAMP (10^{-5} M)	132 000	2.6
ATP (10^{-5} M)	72 100	1.4
ADP (10^{-5} M)	67 400	1.3
AMP (10^{-5} M)	75 200	1.5
Adenosine (10^{-5} M)	69 800	1.4
Adenine (10^{-5} M)	65 600	1.3
Dibutryl-cAMP (10^{-5} M)	134 800	2.7

*Seeds were germinated in the dark in the presence of various structural analogues. Enzyme activity was assayed in the DEAE–sepharose eluted fraction.

sand. Treatment of cAMP (10^{-5} M) was done during germination.

Isolation of total RNA. The procedure in ref. [38] was suitably modified. Epicotyls were ground in 2 vol. of extraction buffer (10 mM Tris–HCl, pH 7.5, 2.4% SDS, 100 mM NaCl, 10 mM EDTA). The extract was centrifuged at 12 000 *g* for 15 min. Equal vol. of buffer satd phenol was added to the supernatant, the contents shaken and centrifuged at 10 000 *g* for 10 min. To the aq. phase an equal vol. of phenol $CHCl_3$ (1:1) was added, shaken at room temp. for 15 min and centrifuged at 10 000 *g* for 10 min. The aq. phase was again shaken for 15 min with an equal vol. of $CHCl_3$, centrifuged at 10 000 *g* for 10 min and the aq. phase carefully recovered. An appropriate amount of NaCl was added to the aq. phase to make a final concn of 0.18 M, followed by the addition of 0.16 vol. iso-PrOH. The contents were kept overnight at $-20°$ to facilitate pptn. The RNA was pelleted at 10 000 *g* for 10 min and dissolved in 0.15 M NaOAc (pH 5.7). RNA was again pptd by adding 2–3 vol. EtOH by

keeping overnight at −20° and this step was then repeated. RNA was dried in a vacuum desiccator.

The extracted total RNA was run on agarose–formaldehyde gels [38]. 4 μl RNA (1 mg ml^{-1}) soln, 1.56 μl of MOPS × 10 buffer (0.2 M MOPS (pH 7.0), 50 mM NaOAc, 10 mM EDTA) and 8.88 μl deionized $HCONH_2$ were placed in an Eppendorf tube, heated to 60° for 15 min, and chilled in ice. 5 μl loading buffer (5% glycerol, 1 mM EDTA (pH 8.0), 0.2% bromophenol blue) were added. Electrophoresis was conducted at 40 V with ethidium bromide (0.5 mg ml^{-1}) as stain, and the purity of RNA fraction observed at 302 nm. An intact RNA prepn so obtained was used for poly(A^+)RNA isolation.

Isolation of poly(A^+)RNA. The method in ref. [40] was followed using an oligo(dT) column. 250 mg oligo(dT) was soaked for 15 hr in Tris–HCl (10 mM, pH 7.5). A 2 ml column was prepared and equilibrated with binding buffer which was the same as above except that it contained 0.5 M NaCl. The RNA pellet was dissolved in sterile H_2O, heated at 65° for 15 min and immediately cooled. The concn of RNA soln was made up to 0.5 M NaCl by adding an equal vol. of double strength binding buffer and loaded on the column. 10 ml (20 fractions, each of 0.5 ml) were collected in Eppendorf tubes. This gave poly(A^-)RNA. The column was eluted with 2–3 ml of Tris-HCl, pH 7.5. Six 0.5-ml fractions were collected and pooled. This was poly(A^+)RNA. *A* was measured at 260 nm.

Incorporation of [^{3}H]*uracil into RNA.* Epicotyls of 6-day-old seedlings were cut into small sections and incubated in a mixture comprising 1 mM NaOAc buffer (4.8 M), 10 mM $CaCl_2$, 2% sucrose and 0.5 mg ml^{-1} chloroamphenicol. Flasks were shaken for 2 hr at 30° in the dark. 74 kBq of [^{3}H]uracil (sp. act. 360 GBq $mmol^{-1}$) was added to the medium and the sections incubated for 4 hr in the dark in the presence and absence of indole acetic acid.

Primer RNA extraction. The primer RNA was extracted following the procedure in ref. [41]. 5 g of 6-day-old frozen epicotyls were homogenized in 5 vol. buffer-satd phenol (buffer containing 0.5% SDS, 0.025 M Na_2EDTA and 0.075 M NaCl, pH 8.0). The contents were chilled in ice and centrifuged at 6500 *g* for 30 min at 4°. The aq. phase was removed carefully, concn of NaCl made to 0.2 M and overlayered with an equal vol. cold 95% EtOH. DNA was spooled out and RNA was pptd by addition of 95% EtOH and stored at −20° for 15 hr.

Isolation of poly(A^+)polymerase. The method described in ref. [33] was followed, with slight modification. 5 g chilled tissue was homogenized in chilled Tris–HCl buffer (25 ml, 50 mM, pH 8.0) containing 2-mercaptoethanol (5 mM) and polyvinylpyrrolidone (4% w/v). The homogenate was spun at 10 000 *g* for 15 min at 4° and the supernatant subjected to $(NH_4)_2SO_4$ precipitation (0–50%). The contents were centrifuged at 10 000 *g* for 15 min at 4° and the protein pellet was suspended in 3 ml Tris–HCl buffer (50 mM, pH 8.0) and desalted on a Sephadex G-25 column (1.5 × 12 cm). The desalted fraction was then loaded onto a DEAE–sepharose column [30] equilibrated in buffer containing 20 mM Tris–HCl (pH 8.0) and 15 mM KCl. The column was washed with 1 column vol. of the above buffer and eluted with 15–200 mM KCl gradient. Fractions collected around 60 mM KCl were collected and used for subsequent poly(A^+) polymerase studies.

Estimation of enzyme activity. Assay was done according to the method in ref. [36]. The assay mixture contained [^{3}H]ATP (4 μCi, sp. act. 3 000 μCi $mmol^{-1}$), unlabelled ATP (0.4 μmol), Tris–HCl buffer (100 μmol, pH 8.0), 2-mercaptoethanol (2 μmol), $MnCl_2$ (2 μmol), primer RNA (1 mg) and DEAE–sepharose fraction (1 mg protein) in a final vol. of 500 μl. The reaction mixture was incubated at 33° for 30 min and the reaction was terminated by adding equal vol. TCA (10%) containing Na pyrophosphate (2 mM). 500 μg BSA was added as a carrier protein at the time of terminating the reaction. The TCA precipitable fraction was collected on a Whatman (3 mm) filter. The ppt was washed 10 × with a 5-ml aliquot of chilled TCA (5%) and finally rinsed with a mixture of $EtOH/Et_2O$ (1:1, 10 ml) and Et_2O (15 ml). Filter paper disks were then dried at 60° and the radioactivity determined.

Polyacrylamide gel electrophoresis. The DEAE–sepharose fraction from cAMP-treated and H_2O controls were fractionated on 10% PAGE containing primer RNA (1 mg) prepared by the method in ref. [42]. Gels were pre-washed electrophoretically (2 mA gel^{-1}) with Tris–glycine buffer (pH 8.3) containing 2-mercaptoethanol (5 mM) for 1 hr at 4°. The DEAE–sepharose fraction (1 mg protein) was loaded onto the gels and electrophoresed at 4° for 1 hr. Thereafter incubation mixture (120 μl) containing [^{3}H]ATP (4 μCi, sp. act. 3 000 μCi $mmol^{-1}$), unlabelled ATP (0.4 μmol), $MnCl_2$ (2 μmol), 2-mercaptoethanol (2 μmol) and Tris-HCl buffer (100 μmol, pH 8.0) were loaded on each column and again electrophoresed at 33° for 30 min. Protein was estimated by the method in ref. [43].

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