Phytochemistry, Vol. 28, No. 3, pp. 701–704, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00

ENHANCEMENT OF CHROMATIN AVAILABILITY AND RNA POLYMERASE ACTIVITY IN *CICER ARIETINUM* SHOOTS BY cAMP

A. GROVER and H. C. KAPOOR

Division of Biochemistry, Indian Agricultural Research Institute, New Delhi-110012, India

(*Received in revised form* 17 *August* 1988)

Key Word Index—*Cicer arietinum*; Leguminosae; chick pea; cAMP; RNA polymerase; activation; chromatin availability

Abstract—The effect of cAMP on transcription was studied in chick pea shoots. cAMP was found to increase transcription by increasing chromatin availability as shown by trypsin digestion experiments during short term (1.5 hr) treatment and by activation of RNA polymerase during long term (48 hr) treatment as shown by the altered physiochemical properties of the enzyme.

INTRODUCTION

Consideration of the known functions of cAMP in plant tissues suggested its role as a regulatory agent, similar to its role in animals and micro-organisms where it is known to act as a secondary and a primary messenger respectively [1–3]. Recent mass spectroscopic and IR spectroscopic [4–6] identifications of cAMP and the presence of cAMP-binding proteins [7, 8], cAMP dependent protein kinases [9], enzymes involved in cAMP metabolism [3] and calmodulin [10] have further confirmed the view that this cyclic nucleotide has a regulatory role in plants.

Among various biochemical responses, the involvement of cAMP in regulating nucleic acid synthesis in higher plants has been suggested by number of workers [11–15]. It has been shown to mimic the action of hormones, but until now there has been no report on the mechanism of stimulation of RNA synthesis by this cyclic nucleotide.

The present study is an attempt to understand the mechanism by which cAMP affects RNA metabolism by looking at its effects on RNA polymerase activity and chromatin availability in *Cicer arietinum* shoots.

RESULTS

A short (1.5 hr) treatment of chick pea shoots with cAMP has no effect on the level of RNA polymerase activity but a longer treatment (48 hr) increases the RNA polymerase activity by two-fold (Table 1).

When different fractions of RNA polymerase (Ia, Ib, II and III), after separation on a DEAE-sephadex column, were checked for any stimulation in activity as a result of cAMP treatment for 48 hr, maximum stimulation was observed in RNA polymerase Ia and Ib (*ca* 2-fold) while activities of RNA polymerase II and III were increased by *ca* 20% (Fig. 1).

This increase in RNA polymerase activity by cAMP was not due to synthesis *de novo* but was the result of activation of pre-existing RNA polymerase molecules as suggested by the experiments with inhibitors of RNA and protein synthesis and amino acid analogues (Table 2).

Table 1. Effect of cAMP on RNA polymerase activity in shoots

Treatment	Sp. activity (units/mg protein)	%
Control (H_2O)	0.38 ± 0.02	100
cAMP (10^{-5}M)–1.5 hr	0.40 ± 0.03	105
cAMP (10^{-5}M)–48 hr	0.68 ± 0.02	179

Seeds were germinated in dark at 30° ± 2° in water for three days. cAMP treatment was given for the next 1.5 and 48 hr. Shoots were harvested (25 g) and assayed for RNA polymerase activity. The standard incubation mixture contained 20 μg calf thymus DNA. One enzyme unit = 1 pmol AMP incorporated in 30 min at 27°.

This was further confirmed by studying physicochemical properties such as thermostability and saturation kinetics of the enzyme in control (grown on water) and cAMP treated plants. The pH optimum of the enzyme was shifted from 8 to 9.5 by cAMP treatment. Thermostability of the enzyme was also increased by this treatment (Fig. 2). Saturation kinetics studies with a fixed amount of DNA (1.5 μg) showed that enzyme from cAMP treated shoots exhibited the saturation effect at relatively higher concentration compared to that from the control shoots (Fig. 3).

A short (1.5 hr) treatment of tissue with cAMP did not affect the level of RNA polymerase (Table 1) but increased the transcriptional activity by 20% (Table 3). This could be due to an increase in template availability following cAMP treatment. This was tested by studying the effect of cAMP on transcriptional activity under saturating concentrations of RNA polymerase.

The increase in transcription by cAMP was maintained under saturating concentrations of RNA polymerase indicating that this enhancement after 1.5 hr of cAMP treatment was the result of an increase in template availability rather than an increase in RNA polymerase

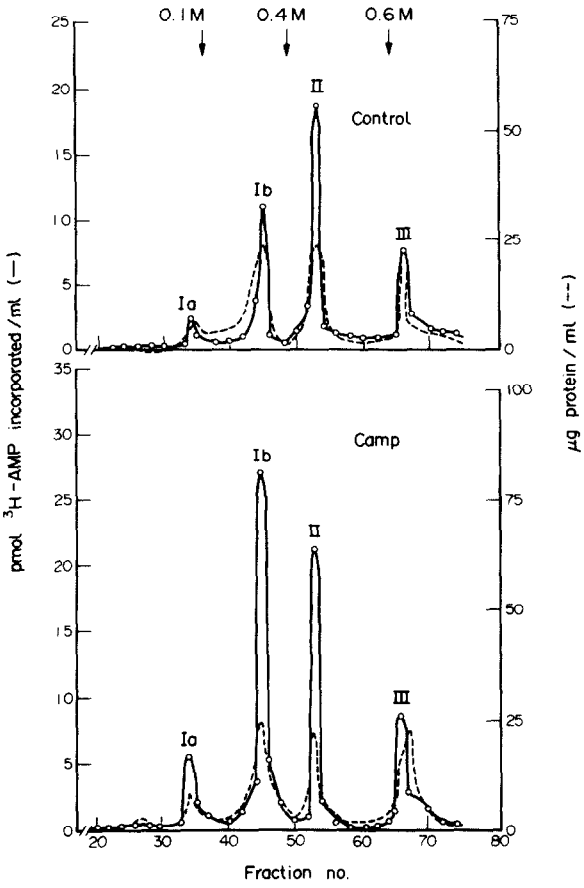

Fig. 1. Elution patterns of RNA polymerases isolated from control and cAMP-treated chick pea shoots, from DEAE-sephadex (A-25) columns. Partially purified enzyme extract, extracted as described in the text, were loaded on to a column (1.5 × 28 cm), washed with buffer III containing 0.01 M $(NH_4)_2SO_4$ and eluted stepwise with the same buffer containing 0.1, 0.4 and 0.6 M $(NH_4)_2SO_4$ (vertical arrows). Fractions of 4 ml were collected and RNA polymerase activity was assayed with the standard reaction mixture containing calf-thymus DNA.

activity (Table 4). This was further confirmed by seeing the effect of trypsin on transcriptional activity of chromatin (Table 5). Predigestion of chromatin with trypsin enhanced RNA synthesis in both control and cAMP-treated tissues by removal of histones but stimulation was less pronounced in the latter where probably cAMP had already removed the histones and made the chromatin more available for transcription.

DISCUSSION

The involvement of cAMP in the regulation of RNA synthesis has been reported in oat coleoptile [13], cowpea [12] and *C. arietinum* [14, 15] but the mechanism by which cAMP stimulates transcription is not clear. It may be due to increased template availability and/or increased specific activity/*de novo* synthesis of RNA polymerase.

In the present study, the effects of cAMP on RNA metabolism were multiple. Stimulation of RNA synthesis during short term treatment is attributed to derepression

Table 2. Effect of transcriptional and translational inhibitors and amino acid analogues on cAMP-stimulated RNA polymerase activity

Treatment	Sp. activity (units/mg protein)	%
Control	0.46 ± 0.05	100
cAMP (10^{-5}M)	0.89 ± 0.05	193
Act D (10 μg/ml)	0.39 ± 0.04	86
Cordy (5×10^{-4} M)	0.34 ± 0.02	73
5FU (1 mM)	0.29 ± 0.01	62
CHI (10 μg/ml)	0.49 ± 0.01	106
Aa analogues (1 mM each)	0.44 ± 0.02	96
cAMP (10^{-5}M) + Act D (10 μg/ml)	0.85 ± 0.06	185
cAMP (10^{-5}M) + Cordy (5×10^{-4} M)	0.82 ± 0.03	178
cAMP (10^{-5}M) + 5FU (1 mM)	0.74 ± 0.02	161
cAMP (10^{-5}M) + CHI (10 μg/ml)	0.82 ± 0.03	179
cAMP (10^{-5}M) + aa analogues (1 mM each)	0.87 ± 0.03	189

Results represent an average of 3 experiments done in duplicate. Experimental conditions are same as described in the Experimental section.

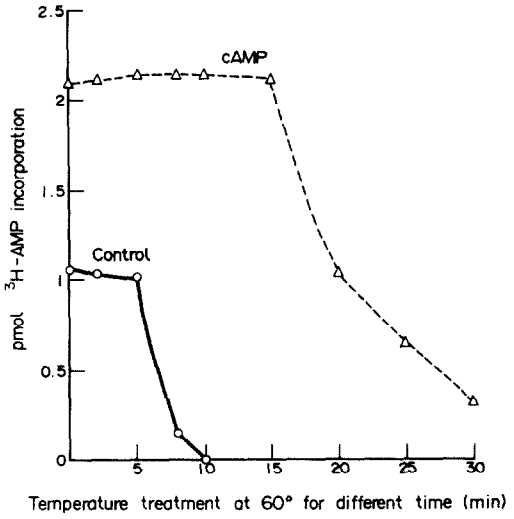

Fig. 2. Thermal denaturation curves of RNA polymerases isolated from control and cAMP-treated chickpea shoots. The enzyme was incubated at 60° for different time intervals and assayed.

of genes (as shown by the trypsin—digestion experiment). During longer treatment, the effects are at least partially explained by stimulation of RNA polymerase I activity. Earlier [15], fractionation of labelled RNA by PAGE revealed a preferential stimulation of rRNA by cAMP. Therefore, it is speculated that the observed stimulation in RNA polymerase I activity is responsible for this increase in rRNA.

Studies done with auxins [16] have also shown quite similar effects to cAMP on template availability and RNA polymerase in *C. arietinum* shoots. It is possible that both cAMP and IAA have a common mechanism of

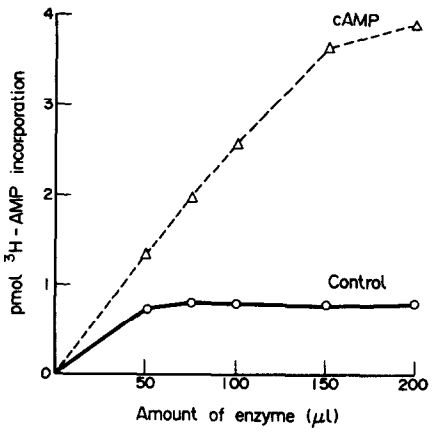

Fig. 3. Saturation kinetic studies on RNA polymerase isolated from control and cAMP-treated shoots. Different amounts of RNA polymerase were assayed with a fixed amount of native chick pea DNA (1.5 μg).

Table 3. Effect of cAMP on transcriptional activity of chromatin

Treatment	pmol AMP incorporated/ 10 μg DNA	%
Control	4.86±0.15	100
cAMP (10^{-5}M)		
1.5 hr	5.85±0.17	120
4.8 hr	9.28±0.74	191

The assay was performed in the standard reaction mixture containing 10 μg of chick pea chromatin instead of calf thymus DNA. Results represent an average of three experiments done in duplicate. Other experimental conditions were the same as described in Table 1.

Table 4. Effect of cAMP on template availability

Treatment	pmol of ^{3}H-AMP incorporated	%
Control	3.89±0.04	100
cAMP (10^{-5}M)		
1.5 hr	4.63±0.05	119
48 hr	5.17±0.21	133

Chromatin equivalent to 1.5 μg DNA was used in the reaction mixture under saturating conditions of RNA polymerase (75 μl of partially purified enzyme). Results represent an average of two experiments done in duplicate. Other experimental conditions were the same as described in Table 1.

Table 5. Effect of trypsin predigestion of RNA synthesis by chromatin isolated from control and cAMP-treated (1.5 and 48 hr) shoots

Trypsin (μg/ml)	Increase in transcription (%)		
	Control	cAMP treated	
		1.5 hr	48 hr
0	—	—	—
10	65	52	42
20	67	51	42
40	70	54	43

The assay was performed as previously described. Chromatin was incubated for 30 min at 15° with trypsin before assay. Results represent an average of two experiments done in duplicate. Other experimental conditions were the same as described in Table 1.

action regarding RNA stimulation. However, in discussing the possible role of cAMP in higher plants, the overall picture that emerges, assigns two different roles for this cyclic nucleotide. One is influenced by the secondary messenger concept where cAMP mediates the action of number of animal hormones [17] while the second view suggests a primary messenger role for it [18]. While it is clear that the cyclic nucleotide is capable of mimicking the effects of plant hormones [12, 15, 16], no significant evidence is available to show that it mediates the effects *in vivo*. Recent studies of Tassi *et al* on *Asparagus* [19] suggest that when attempting to assign a role to cAMP in higher plants, it may well be more relevant to consider its possible similarity with microbial systems where cAMP acts as a primary messenger.

The action of cAMP in stimulating transcription in *C. arietinum* shoots provides further support for the view that cAMP may have a regulatory role in the control of gene expression in plants. However, further work is needed to provide the necessary substantive evidence.

EXPERIMENTAL

Chick pea (*Cicer arietinum* var. Pusa 209) seeds were germinated in dark at 30°±2 in Petri plates containing acid-washed quartz sand. Three-day-old seedlings grown in sterilized dist. H_2O were subjected to different treatments. Chloramphenicol (20 μg/ml) was added to avoid bacterial contamination.

RNA polymerase was extracted from 25 g shoot tissue by the procedure of ref. [20]. Before fractionation, the enzyme was dialysed in buffer II (containing 50 mM Tris–HCl, pH 7.8, 0.1 mM EDTA, 1 mM $MgCl_2$, 10 mM mercaptoethanol and 25% glycerol) and was checked for protein content by the method of ref. [21]. RNA polymerase was fractionated into different components by the method of Sasaki *et al.* [22] except that different elution buffers were used. A DEAE-Sephadex A-25 column (1.5 × 28 cm) was prepared and washed with buffer III [50 mM Tris–HCl, pH 7.8, 10 mM mercaptoethanol, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 0.01 M $(NH_4)_2SO_4$, 25% (v/v) glycerol] *ca* 2–3 times. The sample obtained after dialysis was loaded on the column and washed with 100 ml of buffer III [containing 0.01 M $(NH_4)_2SO_4$] and then eluted stepwise with the same buffer containing 0.1 M, 0.4 M and 0.6 M $(NH_4)_2SO_4$ (50 ml each, at a

flow rate of 60 ml/hr). Fractions (4 ml each) were collected and assayed for their protein content and RNA polymerase activity. Polymerase I, polymerase II and polymerase III were further characterized by studying their sensitivity towards α-amanitin.

RNA polymerase activity was assayed by following the conversion of ^{3}H-ATP into acid-insoluble material. The standard reaction mixture, except where indicated, contained 0.2 mM each of GTP, CTP, UTP, 0.02 M ATP, 20 μg single standard calf thumus DNA, 1 mM $MnCl_2$, 50 mM TES, 10 mM mercaptoethanol, 50 mM $(NH_4)_2SO_4$, 1 μCi ^{3}H-ATP (3000 mCi/mmol) and 50 μl of enzyme extract in a reaction mixture of 0.25 ml. The contents were incubated at 27° for 30 min. The reaction was terminated by following the procedure of Sasaki *et al.* [23]. The contents were pipetted on to a 2.3 cm disc (Whatmann 3 mm). After 1 min the disc was put into a beaker containing 5% TCA with 0.01 M Na pyrophosphate. The disc was washed (× 3) with the above soln for 2 min each. It was then washed twice (1 min each) with Me_2CO to remove any traces of H_2O. The discs were air-dried and placed in 10 ml of Bray's solution [24]. Radioactivity was counted in Packard TRI-CARB liquid scintillation spectrometer.

Thermostability was tested by heating the enzyme at 60° in a water bath for different specified periods before assay. Chromatin was prepared by the method of ref. [25]. DNA was estimated by the method of [26]. Transcriptional activity of chromatin was measured as described above for the enzyme assay, except that the standard reaction mixture contained 10 μg of isolated chromatin instead of calf thymus DNA. Saturation experiments were done with 1.5 μg of chromatin and 75 μl of the enzyme extracted from the same system. In trypsin predigestion experiments the chromatin was incubated with 0, 10, 20 and 40 μl of trypsin at 15° for 30 min. before measuring the transcriptional activity.

REFERENCES

1. Brown, E. G. and Newton, R. P. (1981) *Phytochemistry* **20**, 2453.
2. Francko, D. A. (1983) *Adv. Cyclic Nucleotide Res.* **15**, 97.
3. Newton, R. P. and Brown, E. G. (1987) in *Hormones, Receptors and Cellular Interactions* (Chadwick, C. M. and Garrod, D. R., eds), p. 115. Cambridge Univ. Press.
4. Narayanan, A., Vermeersch, J. and Pradet, A. (1970) *C. R. Acad. Sci.* **27**, 2406.
5. Salomon, D. and Mascarenhas, J. P. (1971) *Life Sci.* **10**, 879.
6. Janistyn, B. (1981) *Z. Naturforschung* **36**, 1973.
7. Brown, E. G., Edwards, M. J., Newton, R. P. and Smith, C. J. (1980) *Phytochemistry* **19**, 23.
8. Endress, R. (1983) *Phytochemistry* **22**, 2147.
9. Kato, R., Uno, I., Ishikawa, T. and Fujii, T. (1983) *Plant Cell Physiol.* **24**, 841.
10. Anderson, J. A., Charbonneau, H., Jones, H. P., Mc Cann, R. O. and Cormier, M. J. (1980) *Biochemistry* **19**, 3113.
11. Kapoor, H. C. and Sachar, R. C. (1976) *Experientia* **32**, 558.
12. Kapoor, H. C. (1987) *Phytochemistry* **26**, 31.
13. Srivastava, A. K., Azhar, S. and Krishnamurti, C. (1973) *FEBS Letters* **33**, 239.
14. Salmon, D. and Mascarenhas, J. P. (1972) *Biochem. Biophys. Res. Commun.* **47**, 134.
15. Grover, A. and Kapoor, H. C. (1987) *Indian J. Exp. Biol.* **25**, 333.
16. Grover, A. (1985) Ph.D. thesis. Indian Agricultural Research Institute, New Delhi-110012, India.
17. Rall, T. W., Sutherland, E. W. and Berthet, J. (1957) *J. Biol. Chem.* **224**, 463.
18. Makman, R. S. and Sutherland, E. W. (1965) *J. Biol. Chem.* **240**, 1309.
19. Tassi, F., Restivo, F. M., Puglisi, P.O- and Cacco, G. (1984) *Physiol. Plant.* **60**, 61.
20. Mehta, S. L., Lodha, M. L., Naik, M. S. and Singh, J. (1975) *Phytochemistry* **14**, 2145.
21. Lowry, O. H., Rosenbrough, N. J., Forr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
22. Sasaki, Y., Tomi, H. and Kamikubo, T. (1979) *Plant Sci. Letters* **14**, 355.
23. Sasaki, Y., Sasaki, R., Hashizume, T. and Yamada, Y. (1973) *Biochem. Biophys. Res. Commun.* **50**, 785.
24. Bray, G. C. (1960) *Anal. Biochem.* **1**, 279.
25. Huang, R. C. and Bonner, J. (1962) *Proc. Natl Acad. Sci. U.S.A.* **48**, 1216.
26. Burton, K. (1956) *Biochem. J.* **62**, 315.