AUXIN AND CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE DURING THE ISOLATION OF CHROMATIN FROM *AVENA* COLEOPTILES: EFFECTS ON CELL FREE RNA SYNTHESIS

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SUMMARY

Chromatin from dark grown oat coleoptiles isolated in the presence of indole acetic acid (IAA) or cyclic 3',5'-adenosine monophosphate (cAMP) exhibits a large increase in RNA synthesis over that of chromatin isolated in their absence. The stimulatory effect on RNA synthesis is specific for cAMP since other nucleotides in the isolation medium have no effect on RNA synthesis. Combinations of IAA and cAMP at optimum and suboptimum concentrations in the isolation medium do not result in an additive or synergistic enhancement of RNA synthesis.

We have shown that the auxin, indole acetic acid, stimulates the formation of cAMP in *Avena* coleoptile sections (1). Cyclic AMP also has an auxin-like effect in delaying abscission of debladed *Coleus* petioles (2). There are a variety of plant systems where the addition of an auxin *in vivo* or *in vitro* enhances RNA synthesis in isolated nuclear and chromatin preparations (3, 4, 5). Matthysse and co-workers have reported that nuclei or chromatin isolated in the presence of an auxin in the isolation medium have an increased RNA synthetic capacity over control nuclei in a cell-free RNA synthesizing system (3,6). If cAMP is the intermediate messenger in auxin action, it would be expected that all of the physiological and biochemical effects of auxins should be duplicated by cAMP. In this report it is shown that the effect of auxin in the isolation medium on chromatin template capacity is duplicated by cAMP.

METHODS

Oat (Avena sativa L. var. 'Victory', generously supplied by E.D. Mallough, Canadian Department of Agriculture, Research Station, Regina, Sask.) coleoptiles were grown as previously described (1). Chromatin was isolated from 40 to 50g lots of coleoptiles by the method of Huang and Bonner (7) with the following modifications. $MgCl_2$ and $MnCl_2$ were included in all of the extraction media and buffers at a concentration of 1mM. The final purification step of the chromatin pellet through a 2M sucrose cushion was eliminated. The chromatin pellet which results from the second tris-HCl wash was suspended in 5ml of 0.05M tris-HCl, pH 8.0, containing 1mM $MgCl_2$ and 1mM $MnCl_2$; and aliquots of this suspension were directly used in the RNA polymerase assay. As indicated in the text, various additives were also included in the extraction media. All isolation procedures were carried out at 4C. To inhibit bacterial growth, 200 units/ml penicillin and 200 µg/ml streptomycin sulfate were added to all solutions. All solutions and glassware used were sterile.

The incorporation of labeled ribonucleoside triphosphates into trichloroacetic acid (TCA) insoluble material was used to measure chromatin-bound RNA polymerase activity. The standard assay contained: 20 µmoles tris-HCl, pH 8.0; 1.10 µmole $MgCl_2$; 0.60 µmole $MnCl_2$; 1.0 µmole 2-mercaptoethanol; 0.20 µmole each of CTP, UTP, and GTP or ATP; 0.25 µC [^{14}C] ATP (specific activity, 50 µC/µmole) or 0.0125 µC [^{14}C] GTP (specific activity, 486 µC/µmole); 80 units penicillin; 80 µg streptomycin sulfate; and chromatin, equivalent to 10 µg of DNA, in a final volume of 0.4 ml. The concentrations of $MnCl_2$ and $MgCl_2$ in the reaction mixture were found to be the concentrations for optimum activity. Addition of $(NH_4)_2$ SO_4 or KCl to the reaction mixture inhibited RNA synthesis which is in agreement with reports on other plant systems (8). The assay was routinely run for 20 min at 37C. All reactions were carried out in duplicate The reaction was stopped by the addition of 5 ml of cold 10% TCA. TCA-insoluble material was recovered on Millipore HA 0.45µ filters, washed with cold 10% TCA, dried, and counted in a toluene scintillation fluid (Liquifluor, New England

Nuclear Corp.). As indicated in the text, various inhibitors and additives were also included in the reaction mixture.

The DNA content of the chromatin preparations was measured by the diphenylamine procedure of Dische (9) using calf thymus DNA as a standard. Indole acetic acid was obtained from Eastman Organic Chemical Co., and all unlabeled nucleotides were purchased from Sigma Chemical Co. The α-amanitin was a gift from Prof. Th. Wieland of the Max Planck Inst., Heidelberg.

TABLE 1

EFFECT OF IAA AND cAMP ON RNA SYNTHESIS BY AVENA COLEOPTILE CHROMATIN

Concentration of additive in extraction media [M]	Incorporation of [^{14}C] AMP into RNA (% of control)				
	Concentration of additive in reaction mixture[a]				
		10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M
Control	100[b]	-----	------	------	------
IAA					
10^{-5}	120±10[c]	108±24	112±22	117±8	------
10^{-6}	157±9	103±48	127±24	116±26	------
10^{-7}	127±15	114±53	123±6	121±36	------
cAMP					
10^{-3}	113±22	100±14	100±12	107±18	119±16
10^{-4}	103±10	82±21	90±17	81±1	63±11
10^{-5}	162±10	147±4	127±10	110±14	115±21
10^{-6}	158±5	140±24	123±32	110±6	------
10^{-7}	181±12	154±3	167±30	163±8	------

a Incubation was performed as described under METHODS.

b Equivalent to an average of 33.3 pmoles [^{14}C] AMP incorporated per 100μg DNA as determined from duplicate assays in two separate experiments.

c Values represent an average of duplicate determinations for each concentration, in two separate experiments (± standard deviation).

RESULTS

The effect of isolating chromatin in the presence of different concentrations of IAA or cAMP on RNA synthesis is illustrated in Table 1. The addition of IAA to the extraction media at concentrations between 10^{-5} and 10^{-7}M increases the rate of RNA synthesis between 30 and 60% over that of untreated chromatin preparations with an optimal enhancement of RNA synthesis occurring at 10^{-6}M IAA. The addition of cAMP during the isolation procedure at concentrations between 10^{-5} and 10^{-7}M increases the rate of RNA synthesis between 30 and 90%, (an amount which varies in different preparations) over that of untreated chromatin. Optimal stimulation of RNA synthesis is reached at 10^{-7}M cAMP. The addition of different concentrations of cAMP or IAA to the RNA polymerase reaction mixture with chromatin which has been isolated in the presence or absence of cAMP or IAA does not produce any further enhancement of RNA synthesis. The stimulatory effects which result from the inclusion of cAMP or IAA in the extraction media on RNA synthesis are lost if the chromatin preparations are not used immediately after their isolation. In the latter situation the addition

TABLE 2

EFFECT OF DIFFERENT NUCLEOTIDES IN THE EXTRACTION MEDIUM ON RNA SYNTHESIS BY ISOLATED CHROMATIN

Addition to extraction medium[a]	Incorporation of [^{14}C] AMP into RNA (% of Control)
Control	100[b]
cAMP 10^{-7}M	135±8
5'-AMP 10^{-7}M	93.5±5
10^{-6}M	94.5±5
Mixture of 2' and 3'-AMP 10^{-7}M	77±9
ADP 10^{-7}M	84±15

a Incubation was performed as described under METHODS.
b Equivalent to an average of 38.5 pmoles [^{14}C] AMP incorporated per 100 µg DNA as determined from duplicate assays in two separate experiments (± standard deviation).

of cAMP or IAA to the polymerase reaction mixture does produce an enhancement of RNA synthesis (unpublished results).

The data presented in Table 2 show that the stimulatory effect on RNA synthesis is specific for cAMP, since 5'-AMP, a mixture of 2' and 3'-AMP or ADP, in the chromatin extraction media have no effect on RNA synthesis.

The experiments summarized in Table 3 were designed to determine whether the stimulation of RNA synthesis by IAA and cAMP was additive or synergistic. Chromatin samples were extracted in the presence of both cAMP and IAA at optimal and sub-optimal concentrations. The combination of cAMP and IAA at their optimal concentrations, 10^{-7} and 10^{-6}M respectively, does not result in any additive or synergistic enhancement of RNA synthesis above that which is produced by the presence of either compound alone. On the contrary, the rate of RNA synthesis decreased by approximately 15%. Combination of sub-optimal concentrations of each compound also does not enhance RNA synthesis above that which is produced by the optimum concentration of cAMP alone.

TABLE 3

EFFECT OF COMBINING cAMP AND IAA IN THE EXTRACTION MEDIUM ON RNA SYNTHESIS BY ISOLATED CHROMATIN

Addition to extraction medium	Incorporation of [^{14}C] AMP into RNA (% of control)	
	Expt. 1	Expt. 2
Control	100[a]	100[b]
cAMP 10^{-7}M	143±5[c]	215±20
10^{-8}M	------	216±20
IAA 10^{-6}M	136±6.5	190±17
10^{-7}M	------	168±15
10^{-7}M cAMP + 10^{-6}M IAA	112±2.5	152±15
10^{-8}M cAMP + 10^{-7}M IAA	------	202±25

a Equivalent to 39.0 pmoles [^{14}C] AMP incorporated per 100 µg DNA as determined by duplicate assays.
b Equivalent to 40.0 pmoles [^{14}C] AMP incorporated per 100 µg DNA (duplicate assays).
c Standard deviation.

TABLE 4

REQUIREMENTS OF AVENA CHROMATIN-BOUND RNA POLYMERASE ISOLATED IN THE PRESENCE OR ABSENCE OF cAMP

System[a]	Incorporation of [^{14}C] AMP into RNA (% of control)	
	Control	cAMP (10^{-7}M)
Complete System + [^{14}C] ATP	100[b]	132
Complete System + [^{14}C] GTP	100[c]	127
+ actinomycin D, 10 µg/ml, [^{14}C] ATP	10	15
+ actinomycin D, 10 µg/ml, [^{14}C] GTP	20	20
Minus (CTP, UTP, GTP) + [^{14}C] ATP	2	3
+ ribonuclease, 50 µg/ml, + [^{14}C] ATP	8	14
+ α-amanitin, 20 µg/ml, + [^{14}C] ATP	53	40
+ rifampin, 20 µg/ml, + [^{14}C] ATP	97	135

a Reaction mixture as described under METHODS.
b Equivalent to 59 pmoles [^{14}C] AMP incorporated per 100 µg DNA.
c Equivalent to 50 pmoles [^{14}C] GMP incorporated per 100 µg DNA.

That a DNA-dependent RNA polymerase activity was being studied is shown by the data in Table 4. The enzyme activity is dependent on the presence of the four ribonucleoside triphosphates. Both [^{14}C] ATP and [^{14}C] GTP are equally utilized as substrates in the polymerization reaction. The inclusion of either actinomycin D at 10 µg/ml or RNase (50 µg/ml), to the polymerase reaction mixture inhibits the accumulation of acid-insoluble product. The addition of α-amanitin, (20 µg/ml) to the polymerase assay produces a 50 to 60% inhibition of RNA synthesis from both cAMP treated and untreated chromatin preparations. The presence of rifampin, 20 µg/ml, in the reaction mixture had no significant effect on RNA synthesis indicating the absence of any bacterial or mitochondrial contamination.

The reaction kinetics of the RNA polymerases from cAMP treated and untreated chromatin preparations are shown in Fig. 1. In both preparations the reaction is essentially complete by 20 min.

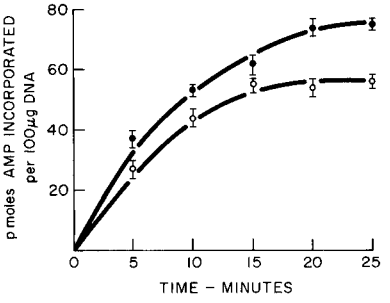

FIGURE 1

The kinetics of RNA synthesis by chromatin with its associated RNA polymerase isolated in the presence (—●—) or absence (—o—) of 10^{-7}M cAMP.

DISCUSSION

Chromatin prepared with cAMP in the isolation solutions has an increased capacity for RNA synthesis, similar to that obtained with IAA. If the effects of cAMP and IAA were via different mechanisms one would expect that when the two compounds were combined at their optimum concentrations, an additive effect would be seen on RNA synthesis. This has not been found to be the case. Our results thus suggest that the effects of cAMP and IAA on RNA synthesis are not brought about by different mechanisms. Auxins seem to stimulate RNA synthesis *in vitro* through a class of mediator proteins by either increasing the amount of available template for transcription or by enhancing the activity of the endogenous RNA polymerase (3, 5, 6, 10). Although there is not enough information available at present to fit cAMP into the molecular scheme of auxin action, it is not inconceivable that some sort of cAMP mediated activation of an enzyme(s) via a phosphorylation is involved.

The results reported in this paper lend further support to information already in the literature (1, 2, 11), that auxin effects in plant systems are mediated through cAMP.

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