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# Ribonucleic Acid Synthesis by Cucumber Chromatin

## DEVELOPMENTAL AND HORMONE-INDUCED CHANGES[1]

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### ABSTRACT

**When intact etiolated 2-day cucumber (*Cucumis sativus*) embryos were treated with indoleacetic acid (IAA), gibberellin $A_7$ ($GA_7$), or kinetin, chromatin derived from the embryonic axes exhibited an increased capacity to support RNA synthesis in either the presence or the absence of bacterial RNA polymerase. An IAA effect on cucumber RNA polymerase activity was evident after 4 hours of hormone treatment; the IAA effect on DNA template activity (bacterial RNA polymerase added) occurred after longer treatments (12 hours). $GA_7$ also promoted template activity, but again only after a prior stimulation of endogenous chromatin activity. After 12 hours of kinetin treatment, both endogenous chromatin and DNA template activities were substantially above control values, but longer kinetin treatments caused these activities to decline in magnitude. When chromatin was prepared from hypocotyl segments that were floated on a $GA_7$ solution, a GA-induced increase in endogenous chromatin activity occurred, but only if cotyledon tissue was left attached to the segments during the period of hormone treatment.**

**Age of the seedling tissue had a profound influence on the chromatin characteristics. With progression of development from the 2-day to the 4-day stage, the endogenous chromatin activity declined while the DNA template activity increased.**

Plant hormones dramatically influence nucleic acid metabolism, particularly RNA synthesis, in a variety of systems (13, 22). Often, hormone-induced increases in RNA production closely parallel, and may be intimately related to, a given physiological response such as cell expansion. Unfortunately, little is known about the mechanisms by which growth hormones influence RNA metabolism. It seems probable, however, that this aspect of hormone action involves the nucleus. Roychoudhury and Sen (20), Maheshwari *et al.* (14), and Cherry (4) showed that the addition of auxin to isolated plant nuclei enhanced nuclear RNA synthesis. Matthysse (16) described a similar effect of auxin on nuclei from soybean and tobacco tissue cultures. Gibberellin $A_3$ enhanced the synthesis of RNA by dwarf pea nuclei when it was included in the isolating medium (11).

Isolated chromatin does not exhibit enhanced nucleic acid synthesis when treated with auxin *in vitro* (18, 21); however, 2,4-D applied to soybean seedlings *in vivo* has been shown to increase the RNA-synthesizing capacity of isolated chromatin (18). The major 2,4-D effect appeared to be manifested at the level of RNA polymerase.

Recently, Matthysse and Phillips (17) have reported the isolation of a protein fraction which, when added to soybean, tobacco, or pea bud chromatin in the presence of auxin, enhanced chromatin-directed RNA synthesis *in vitro*. Their results led them to conclude that the increased rate of RNA synthesis was caused by an increased availability of genome (template) for transcription.

Cucumber seedlings respond well to many plant growth regulators (19). It seemed, therefore, that chromatin from this plant might be a useful material for the study of developmental and hormone-induced changes in RNA synthesis. We describe here the preparation and properties of crude cucumber chromatin.

### MATERIALS AND METHODS

**Plant Materials.** Seeds of *Cucumis sativus* L. cv. National Pickling (Burpee Seed Co.) were soaked for 10 min in 5% Clorox (commerical NaOCl), rinsed vigorously for 3 min with running tap water, and sown on moist cellulose packing material (Kimpak 6223, Kimberly-Clark Co.). The seedlings were kept in darkness at 26 C until they were harvested for chromatin extraction. In a typical experiment, two lots of seeds were planted simultaneously in 30- × 40-cm trays. After 48 hr, the radicle protruded 0.5 to 1.0 cm beyond the seed coat. At this time, the excess water in one tray was decanted. These seedlings were sprayed with 100 ml of a hormone solution, and the tray was returned to the dark incubator. This operation was carried out under a green safelight. After a further 24 hr of incubation, the radicle was 2 to 5 cm in length and the hypocotyl was just beginning to emerge. Hormone-treated and control seedlings were then harvested by separating the embryonic axes from the cotyledons enclosed by the seed coats and placing the axes directly into ice-cold water. After 20 to 30 g of tissue per treatment were collected, the tissue was soaked for 5 min in 1% Clorox, rinsed vigorously with running tap water, and homogenized in a Waring Blendor.

For some experiments, chromatin was prepared from hormone-treated and control etiolated hypocotyl segments. All manipulations were carried out in a dark room with a green safelight. Hypocotyl segments were prepared from 5-day seedlings by removing the cotyledons and making a second cut approximately 1 cm below the apical hook. In some cases, the cotyledons were left attached during the incubation but removed just prior to

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chromatin extraction. Segments were incubated in the dark in 9-cm Petri dishes containing 6 ml of test solution. Finally, the segments were soaked for 5 min in 1% Clorox, rinsed with tap water, and homogenized by hand in a mortar and then in a glass tissue homogenizer with a loosely fitting pestle.

**Chromatin.** Chromatin was prepared by the methods of Huang and Bonner (7). The chromatin was purified to the "crude chromatin" stage.

**Measurement of RNA Synthesis.** Incorporation of $^{14}$C-nucleoside triphosphates into acid-insoluble material was used as a measure of RNA synthesis. The reaction mixture contained (unless otherwise stated), in micromoles: tris-HCl (pH 7.9), 50; $MnCl_2$, 0.5; GTP, CTP, and UTP, 0.1 each; 0.03 $\mu$c of $^{14}$C-ATP (420 mc/mmole) (New England Nuclear); and chromatin (equivalent to 2–4 $\mu$g of DNA) in a total volume of 0.5 ml. The reaction was initiated by addition of the chromatin and was allowed to proceed for 30 min at 15 C. The reaction was stopped by adding 4 ml of cold 5% trichloroacetic acid. The acid-insoluble precipitates were transferred to glass fiber filter discs (Whatman GF/C, 2.4-cm diameter) and washed three times with 10-ml aliquots of cold 5% trichloroacetic acid, and finally with 10 ml of 95% ethanol. The filters were dried under infrared lamps and counted on a Nuclear-Chicago (720 series) liquid scintillation system. The results were expressed as pmoles $^{14}$C-AMP incorporated per hr per 100 $\mu$g of DNA (specific activity).

**DNA Determination.** For DNA estimation, an equal volume of 1 N $HClO_4$ was added to the chromatin suspension, and the mixture was heated at 70 C for 30 min. Precipitated protein was removed by centrifugation, and the supernatant containing DNA was estimated by the method of Burton (2).

**Bacterial RNA Polymerase.** RNA polymerase was prepared from *Escherichia coli* B cells (General Biochemicals) by a modification of the method of Chamberlin and Berg (3). Purification was taken to stage IV of the procedure.

## RESULTS

**Properties of Cucumber Chromatin.** Chromatin isolated from cucumber seedlings supported the incorporation of $^{14}$C-AMP into an acid-insoluble material *in vitro*. The kinetics of incorporation varied with the reaction temperature (Fig. 1). At 37 C, $^{14}$C-AMP incorporation was essentially complete in 2 to 4 min.

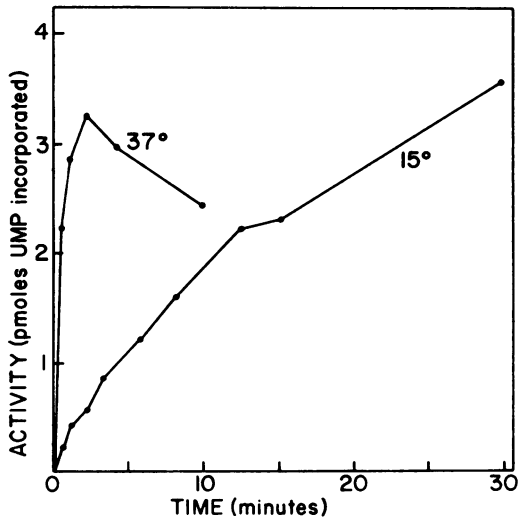


FIG. 1. Kinetics of chromatin-supported RNA synthesis at 37 and 15 C. $^{14}$C-UTP (410 mc/mmole) was used as the labeled substrate. Of chromatin DNA, 5.9 $\mu$g were added per tube.

Table I. *Effects of $10^{-4}$ M IAA on Endogenous and Exogenous RNA Polymerase-fortified Chromatin Activities as a Function of Duration of IAA Treatment*

Control values are averages of 11 experiments.

| Duration of IAA Treatment | Endogenous Chromatin Activity | Exogenous Polymerase-fortified Activity |
|---|---|---|
| *hr* | *% of control* | |
| Control (pmoles/hr·100 $\mu$g DNA) | 97 | 1497 |
| 24[1] | 161 | 118 |
| 18[2] | 164 | ... |
| 12[2] | 156 | 116 |
| 6 | 121 | 103 |
| 4[2] | 121 | 103 |
| 2 | 105 | 103 |

[1] Average of three experiments.
[2] Average of two experiments.

At 15 C, net incorporation continued to increase for at least 30 min. All subsequent assays were run at 15 C to minimize the effect of chromatin-associated ribonuclease.

Bonner *et al.* (1) have shown that purified pea chromatin can be transcribed by added RNA polymerase prepared from *E. coli*. At saturating levels, *E. coli* RNA polymerase increased the rate of cucumber chromatin-directed RNA synthesis 5- to 20-fold (10).

Chromatin-directed RNA synthesis was strongly dependent on the presence of a divalent cation ($Mn^{2+}$ or $Mg^{2+}$) and all four of the RNA precursor nucleotides (Johnson and Purves, unpublished results). The reaction was very sensitive to such inhibitors as pyrophosphate, actinomycin D, ribonuclease, and deoxyribonuclease, but cycloheximide exerted no effect (10). These observations led us to believe that cucumber chromatin exhibited the capacity to support RNA synthesis and could be used to study the effects of plant hormones on this activity.

**Effects of Auxin.** Chromatin-directed RNA synthesis was not affected by the inclusion of IAA in the reaction mixture or in the buffers used during chromatin extraction (10). As in the case of 2,4-D and soybean hypocotyl segments (18), however, chromatin isolated from cucumber embryonic axes pretreated with $10^{-4}$ M IAA exhibited an enhanced capacity to synthesize RNA, in either the presence or the absence of saturating levels of *E. coli* RNA polymerase (Table I). IAA-induced promotions of endogenous chromatin activity (no added polymerase) were similar in magnitude after 12, 18, or 24 hr of hormone treatment; the IAA effect was prominent, but smaller, after only 4 hr of treatment. The stimulatory effect of IAA on DNA template activity (measured with saturating levels of *E. coli* RNA polymerase) was only marginal after 4 to 6 hr of hormone treatment but reached 116 to 118% of control activity after 12 to 24 hr. Typical saturations of chromatin (from control and 12-hr IAA-treated plants) with *E. coli* RNA polymerase are shown in Figure 2.

**Effects of Gibberellin.** The effect of $10^{-4}$ M gibberellin $A_7$ on endogenous and bacterial RNA polymerase-fortified chromatin activities is shown in Table II. A gibberellin effect at the level of DNA template was not apparent after 12 hr of GA treatment; after 24 hr, however, the template activity resulting from GA treatment was 114% of control activity. The GA-promoted endogenous chromatin activity was substantial after 12 hr of treatment (140% of control); however, after 24 hr of treatment, at which time a gibberellin effect on template activity was clearly noticeable, the GA-enhanced endogenous chromatin activity

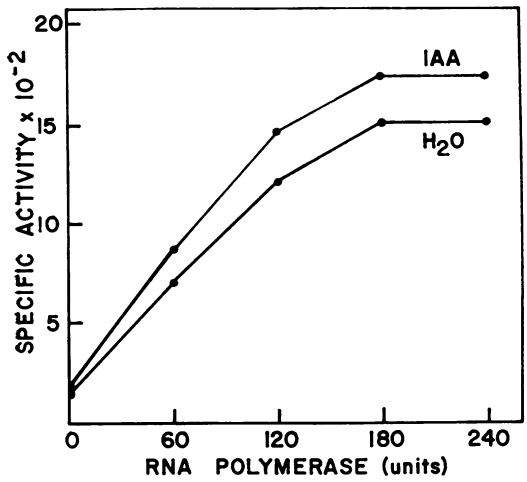


FIG. 2. *E. coli* RNA polymerase saturation of chromatin prepared from control and auxin-treated 72-hr seedlings. Auxin treatment: $10^{-4}$ M IAA for 12 hr. Specific activity: pmoles $^{14}$C-AMP incorporated per hr per 100 μg DNA. RNA polymerase unit: 1 mμmole $^{14}$C-AMP incorporated per hr per mg protein using salmon sperm DNA as a template, under conditions described by Chamberlin and Berg (3).

Table II. *Effects of $10^{-4}$ M $GA_7$ on Endogenous and Exogenous RNA Polymerase-fortified Chromatin Activities as a Function of Duration of $GA_7$ Treatment*

Control values are averages of six experiments.

| Duration of $GA_7$ Treatment | Endogenous Chromatin Activity | Exogenous Polymerase-fortified Activity |
|---|---|---|
| *hr* | *% of control* | |
| Control (pmoles/hr·100 μg DNA) | 91 | 1310 |
| 12 | 142 | 103 |
| 12 | 138 | 100 |
| Average | 140 | 102 |
| 24 | 95 | 106 |
| 24 | 135 | 120 |
| 24 | 122 | 119 |
| 24 | 120 | 111 |
| Average | 118 | 114 |

Table III. *Effects of $10^{-4}$ M $GA_7$ on Chromatin Activities as a Function of Presence or Absence of Cotyledons*

Chromatin was extracted from 5-day seedlings. The 24-hr incubation was carried out with hypocotyl segments ± cotyledons. All values represent percentages of water control activity. Control values are averages of five experiments.

| | Endogenous Chromatin Activity | | Exogenous Polymerase fortified Activity | |
|---|---|---|---|---|
| | + cotyledons | − cotyledons | + cotyledons | − cotyledons |
| | *% of control* | | *% of control* | |
| Control (pmoles/hr·100 μg DNA) | 67 | 44 | 321 | 320 |
| | 145 | 87 | 127 | ... |
| | 108 | 106 | 97 | 87 |
| | 113 | 88 | 87 | 102 |
| | 117 | ... | 89 | ... |
| | 88 | 108 | 106 | 104 |
| Average | 114 | 97 | 101 | 98 |

Table IV. *Effects of Kinetin on Endogenous and Exogenous RNA Polymerase-fortified Chromatin Activities as a Function of Duration of Kinetin Treatment*

Control values are averages of seven experiments. Concentration of kinetin was 10 mg/liter.

| Duration of Kinetin Treatment | Endogenous Chromatin Activity | Exogenous Polymerase-fortified Activity |
|---|---|---|
| *hr* | *% of control* | |
| Control (pmoles/hr·100 μg DNA) | 62 | 441 |
| 12 | 136 | 109 |
| 12 | 173 | 136 |
| Average | 154 | 122 |
| 18 | 120 | 111 |
| 24 | 100 | 92 |
| 24 | 119 | 111 |
| 24 | 110 | 84 |
| 24 | 115 | 109 |
| Average | 111 | 99 |

had dropped to 118% of control. GA-induced promotion of DNA template and endogenous chromatin activities associated with the breaking of dormancy in hazel seeds has been reported by Jarvis *et al.* (8, 9). These authors, however, found that GA effected an increase in DNA template activity prior to its enhancement effect on endogenous chromatin activity.

$GA_7$ elicited no apparent growth response over a 24-hr period when it was applied to intact 2-day etiolated cucumber seedlings. However, Katsumi *et al.* (12) have shown that etiolated cucumber hypocotyl segments excised from 5-day seedlings exhibited an increased rate of elongation in response to GA, provided some cotyledon tissue was left attached. It was of interest, then, to determine whether the chromatin activity of cucumber hypocotyls paralleled the GA-induced growth response. The results are shown in Table III. Chromatin isolated from GA-treated segments with cotyledons attached during the period of treatment had an enhanced endogenous chromatin activity. The GA effect was lost if cotyledons were absent during the treatment. There was no GA-enhanced DNA template activity in either the presence or the absence of cotyledons. Thus, only the GA effect on endogenous chromatin activity reflects the hormone's influence on elongation.

**Effects of Cotyledons.** Chromatin prepared from water-treated hypocotyl segments with cotyledons attached during the treatment had a consistently greater capacity for endogenous chromatin-supported RNA synthesis than did similarly treated segments without cotyledons (Table III). The activity of the former was on the average 152% of that for the latter. The presence or absence of cotyledons during the 24-hr treatment period had no effect on the DNA template activity.

**Effects of Kinetin.** The effect of kinetin (10 mg/liter) treatment of intact cucumber seedlings on endogenous and bacterial RNA polymerase-fortified chromatin activities is shown in Table IV. In each experiment, chromatin was isolated from 72-hr embryonic axes following a water or hormone treatment period of 12, 18 or 24 hr. A 12-hr kinetin treatment increased the endogenous chromatin activity to 154% of the control activity, while the template activity was increased to 122% of control. Longer kinetin treatments produced smaller enhancements. After 24 hr

Table V. *Effects of Seedling Age on Endogenous and Exogenous RNA Polymerase-fortified Chromatin Activities*

All values are expressed as percentages of activity of chromatin isolated from 48-hr embryonic axes. Each line represents a separate experiment. The 48-hr values are averages of six experiments.

| | Endogenous Chromatin Activity | | Exogenous Polymerase-fortified Activity | |
|---|---|---|---|---|
| | 72 hr | 96 hr | 72 hr | 96 hr |
| 48 hr (pmoles/hr·100 μg DNA) | 129 | | 815 | |
| | 71 | 60 | 109 | 111 |
| | 81 | 41 | 131 | 123 |
| | 68 | 46 | 110 | 125 |
| | 59 | ... | 116 | ... |
| | 77 | 37 | 151 | 188 |
| | 65 | 36 | ... | ... |
| Average | 70 | 44 | 123 | 137 |

of treatment with kinetin, the endogenous chromatin activity was only 111% of control, while the kinetin effect on template activity, obscured somewhat by variability, seemed to have disappeared completely.

**Changes during Normal Development.** The capacity of chromatin to synthesize RNA was dependent upon the developmental age of the seedlings from which it was extracted. Table V indicates that chromatin prepared from 48-hr embryonic axes had a significantly greater endogenous chromatin activity than did that extracted from 72- or 96-hr seedlings. The lowest endogenous chromatin activity was obtained with extracts of 96-hr plants. Although the endogenous chromatin activity decreased with increasing age of the seedlings, the DNA template activity of the chromatin increased with seedling age (Table V).

## DISCUSSION

Of the two chromatin-dependent RNA synthesis activities monitored throughout this study, the largest and most reproducible effects *in vivo* of IAA, $GA_7$, and kinetin were the promotions of endogenous chromatin activity. Under optimal conditions, hormone-induced endogenous chromatin activities reached 161, 140, and 154% of control activity as a result of treatments with IAA, $GA_7$, and kinetin, respectively. In contrast, maximal levels of DNA template activities effected by IAA, $GA_7$, and kinetin were 118, 114, and 122% of control activity, respectively. All three plant hormones, therefore, are capable of enhancing both DNA template availability and endogenous chromatin activity to varying degrees.

If it is assumed that *E. coli* RNA polymerase transcribes only those portions of the cucumber genome that are available to cucumber RNA polymerase, and that the bacterial RNA polymerase is not more effective than cucumber RNA polymerase in utilizing cucumber DNA for transcription *in vitro*, then it follows that cucumber RNA polymerase limits the endogenous level of chromatin-supported RNA synthesis. Under such circumstances, hormone-enhanced endogenous chromatin activity would result from increases in the level or enzymatic activity of cucumber RNA polymerase. It is possible, however, that application of plant hormones *in vivo* may in some manner confer more stability to cucumber RNA polymerase during the chromatin extraction procedure, possibly through an effect on the binding of RNA polymerase to chromosomal material. The likelihood of such an explanation is lessened by the observation that the presence of IAA during the chromatin extraction, and 2-hr IAA treatment periods, did not appreciably increase chromatin activity.

If cucumber RNA polymerase is indeed the rate-limiting factor in the endogenous chromatin system, then hormone-induced stimulations of the endogenous chromatin activity could not be manifested through an increase in DNA template availability. This is true if it is assumed that the rate of cucumber RNA polymerase-mediated transcription is not greater in areas of the chromatin genome made available through hormone action. Nevertheless, the hormones did effect an expansion of the DNA template availability as measured with *E. coli* RNA polymerase. In the case of IAA and $GA_7$, however, hormone-enhanced endogenous chromatin activity preceded any significant hormone influence on the DNA template activity. Thus, it would appear that the initial mechanism by which these hormones influence RNA synthesis involves cucumber RNA polymerase, and the hormonal effect on DNA template availability is probably a secondary effect.

It was not the over-all purpose of this investigation to relate the influence of hormones on chromatin activity to hormone-induced growth responses. In fact, the intact 48-hr cucumber seedlings did not undergo an increase in fresh weight or elongation in response to IAA or $GA_7$ at concentrations of $10^{-4}$ M or less (Johnson and Purves, unpublished results). Presumably, the growth reaction in this young tissue is saturated with an endogenous supply of these growth regulators. Since the treatment of 48-hr seedlings with IAA, $GA_7$, or kinetin did enhance chromatin activity, this enhancement must reflect the action of the hormones at some level of development other than growth.

During the period of seedling development from the 48-hr to the 96-hr stage, numerous metabolic changes must occur. These changes are probably related to the observed alterations in chromatin activity. There was an initial high endogenous chromatin activity at the 48-hr stage which declined as development progressed. At the same time, however, there was an increase in DNA template activity. Similar findings have already been reported for frog (5, 6) and sea urchin (15) embryos.

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