A PROTEIN INTERMEDIARY IN THE INTERACTION OF A HORMONE WITH THE GENOME*

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Abstract.—The increased rate of RNA synthesis by target cells caused by the plant hormone auxin has been studied as an example of hormonal regulation of transcription. The hormone does not interact directly with chromatin but requires a protein mediator. In the presence of this mediator, auxin increases the rate of RNA synthesis both by isolated plant nuclei and by isolated chromatin. This increased rate of RNA synthesis occurs even in the presence of saturating amounts of RNA polymerase. The hormone and protein do not affect the rate of RNA synthesis if pure DNA is used as the template. The results suggest that auxin plus the protein increase the rate of RNA synthesis by making an increased portion of the genome available for transcription.

Introduction.—It has been shown in several plant and animal systems that the uptake of a hormone by its target organ results in an increased rate of RNA synthesis.[1] That this may be due, in part, to the derepression of genes previously repressed can be inferred from the fact that chromatin isolated from the hormone-treated organ exhibits an increased template activity for RNA synthesis.[2, 3] Since the addition of hormone in the test tube to chromatin isolated from untreated organs does not cause such an increase in template activity, it is probable that an intermediary, not normally isolated with chromatin, mediates the interaction of hormone and chromatin. This intermediary should be contained in the nucleus, since isolated nuclei do respond to hormone addition by increased rate of RNA synthesis.[4] It is reported below that for the plant hormone, auxin, this intermediary is a protein and that in the presence of the purified factor, the addition of hormone to purified chromatin results in increased template activity.

Materials and Methods.—*Nucleic acid synthesis by tissue culture cells:* Tobacco callus cultures of Filner's line XD[5] were maintained on M1D agar medium at 27° in the dark. For experiments the cells were removed from the agar medium and placed in a stainless steel wire basket with $1/16$-in.-square holes in 100 ml of liquid M1D medium without auxin. They were shaken for 2 to 5 hr, and the cells that passed through the basket were used to inoculate flasks containing 200 ml of medium which were placed on the shaker.

Growth was measured by removing 10-ml aliquots of the cultures and determining the packed-cell volume after centrifugation in a clinical centrifuge. Initial liquid culture inocula were between 0.05 and 0.15 ml/10 ml packed-cell volume. After 5 days, 0.5 mg/liter of 2,4-dichlorophenoxyacetic acid (2,4-D) was added to some flasks and 20 μCi of inorganic P-32 was added to all flasks. The cells were collected by filtering on Miracloth 15 to 360 min later. They were broken by sonication in the cold. Amounts of RNA and DNA were determined by the Schmidt-Thannhauser procedure,[6] the orcinol reaction[7] for RNA with yeast RNA as a standard, and the diphenylamine reaction for DNA[8] with calf thymus DNA as a standard. Specific activities were determined by counting aliquots of the RNA or DNA nucleotides in a liquid scintillation counter.

Isolation of nuclei: Tobacco or soybean tissue culture cells were homogenized in a

motor-driven Teflon glass homogenizer and then in a Dounce homogenizer. Pea buds were homogenized for 30 sec in a Waring Blendor at full speed. The isolation medium and differential centrifugation procedure of Kuehl[9] were used. Where required, 0.5 mg/liter of 2,4-D was added to the isolation medium.

RNA synthesis by nuclei: Nuclei were incubated in Kuehl's medium[10] containing 2 μCi ATP(^{14}C) (0.06 μmole) and 10 μg/ml of penicillin at 37° for 10 min for tobacco and soybean and for 5 min for pea bud nuclei. The reaction was stopped by adding 5 ml of cold 10% trichloroacetic acid (TCA) containing 0.04 M sodium pyrophosphate. The precipitate was collected by filtration on membrane filters (Schleicher and Schuell B-6) and washed with three 5-ml portions of TCA. The filters were dried and counted in a liquid scintillation counter.

Isolation of chromatin: Purified pea bud chromatin was prepared by the method of Huang and Bonner.[11] Pea bud DNA was prepared from chromatin by the method of Marmur.[12]

Preparation of RNA polymerase: RNA polymerase was prepared from *E. coli* strain B (General Biochemicals) by the methods of Chamberlin and Berg[13] to the stage of their fraction 3 or 4.

RNA synthesis by chromatin and RNA polymerase: The complete incubation mixture for RNA synthesis contained in a final volume of 0.25 ml: 10 μmoles of Tris buffer (pH 8.0), 1 μmole of $MgCl_2$, 0.25 μmole of $MnCl_2$, 3 μmoles of β-mercaptoethanol, 0.10 μmole each of CTP, UTP, and GTP, 0.10 μmole of 8-^{14}C-ATP (1 μCi/μmole), chromatin (0.2–30 μg), RNA polymerase (0.8–8 μg), and, if required, 10 μg of bentonite. Samples were incubated at 37° for 10 min. The reaction was stopped by addition of 5 ml of cold 10% TCA containing 0.04 M sodium pyrophosphate. The acid-insoluble material was collected by filtration on membrane filters which were then washed twice with 5 ml of cold TCA. The filters were dried and counted in a liquid scintillation counter. That the radioactive material formed under these circumstances is RNA has been shown by Marushige and Bonner.[14]

Isolation of hormone-reactive protein: Hormone-reactive protein was prepared from tobacco or soybean nuclei isolated in the presence of 2,4-D (0.5 mg/liter) by lysing the nuclei in 0.01 M Tris (pH 8.0) and removing the chromatin by centrifugation at 12,000 $\times$ *g* for 10 min. The supernatant was further purified by chromatography on Sephadex G-200.

To prepare pea bud hormone-reactive protein, the buds were processed as in the procedure for chromatin.[11] The supernatant from the initial centrifugation (4000 $\times$ *g*) was retained and the protein precipitated with $(NH_4)_2SO_4$, 20% of saturation, in the cold for 1 hr. The precipitate was collected by centrifugation at 40,000 $\times$ *g* for 20 min and washed twice with 0.01 M Tris, pH 8.0. The final pellet was suspended in 0.01 M Tris and mixed with an equal volume of a slurry of DEAE-cellulose which was removed by filtration on Miracloth. The filtrate was mixed with an equal volume of CMC-cellulose and the gel collected by centrifugation at 3000 $\times$ *g* for 5 min. The supernatant was discarded and the CMC-cellulose suspended in 0.5 M KCl in 0.01 M Tris, pH 8.0. The gel was again separated by centrifugation and the supernatant retained. The salt was removed by dialysis before the supernatant was assayed for hormone reactivity. The protein appeared to be unstable and preparations only retained their activity for 3–10 days at 4°C.

Assay for RNase: RNase was estimated by incubating the test solution at 37° with P³²-labeled RNA prepared from rat ascites tumor cells by the method of Dahmus and McConnell.[15] The RNA was precipitated after 0, 10, and 45 min with cold TCA and collected on membrane filters which were counted in a liquid scintillation counter.

Assay for ATPase: 0.5 ml of the solution to be assayed was incubated with β-γ-P³²-ATP at 37° for 10 min in the same salt mixture used for RNA synthesis. The reaction was stopped by adding 0.1 ml of 1 N HCl and 0.2 ml of 20% norite in 0.1 N HCl at 4°. The nucleotide phosphates were removed with the norite by low-speed centrifugation, and an aliquot of the supernatant was counted to determine the inorganic phosphate re-

leased. When the ATP alone was incubated under these conditions, about 0.1% of the initial counts were released into the supernatant.

Results.—Effect of 2,4-D on RNA synthesis by tobacco cells: Tobacco tissue culture cells of the line XD isolated by Filner[5] require auxin for growth. If the cells are suspended in an auxin-free medium, growth ceases but the cells do not die (Fig. 1). When auxin (2,4-D, 0.5 mg/liter) again is added to the medium, the cells resume growth. Inorganic $P^{32}O_4$ was added to such auxin-deficient cells together with auxin, and the rate of its incorporation into RNA and DNA was measured. Incorporation into both RNA and DNA by the auxin-treated cultures is double that of the control cultures 15 minutes after auxin addition (Table 1). This higher rate of nucleic acid synthesis is maintained for at least three hours after the addition of auxin.

Effect of 2,4-D on RNA synthesis by nuclei: Nuclei-rich preparations from both tobacco and soybean tissue culture cells and from pea buds respond to the addition of 2,4-D (0.5 mg/liter) by an increase in rate of RNA synthesis varying from 50 to 120 per cent. Tobacco and soybean nuclei respond to the addition of auxin only if isolated in the presence of the hormone. In its absence the factor(s) required for the auxin response is lost from the nuclear preparation. When nuclei were prepared in the presence of auxin and washed once by sedimentation from incubation medium in the absence of auxin, they no longer responded to the addition of auxin by an increase in rate of RNA synthesis. This is
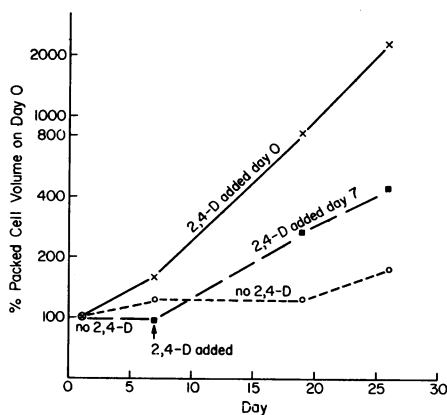

FIG. 1.—Growth of tobacco tissue cultures (x—x) in the presence of 0.5 mg/liter of 2,4-D, (O—O) in the absence of 2,4-D, and (■—■) with 2,4-D added after 7 days of growth in its absence.

TABLE 1. *Stimulation by addition of 2,4-D of nucleic acid synthesis in auxin-depleted tobacco tissue culture cells.*

Time of ^{32}P incorporation (min after auxin addition)	Ratio (cpm/μg incorporated auxin-treated)/ (cpm/μg incorporated control)	
	RNA*	DNA†
0–15	2.4	2.5
0–25	3.2	3.0
30–60	2.1	2.0
120–180	2.4	—
150–210	1.7	1.6

* Approximately 2×10^4 cpm or 20 mμM of phosphate were incorporated by the control cells.
† Approximately 2×10^3 cpm or 2 mμM of phosphate were incorporated by the control cells.

due to leakage of the required factor from the nuclei, since if the supernatant from the centrifugation in the absence of auxin is added back to such nuclei, they again become auxin-responsive (Table 2). The substance in the supernatant which confers auxin-responsiveness on the nuclei is heat-labile. It may also be prepared from lysates of tobacco, soybean, or pea bud nuclei. Exclusion chromatography resolves it into two fractions with approximate molecular weights of 20,000 and 200,000. Pea bud nuclei retain the factor even if pelleted from medium lacking hormone.

TABLE 2.   *Stimulation of RNA synthesis in tobacco nuclei by 2,4-D.*

Presence or Absence of Hormone and of Hormone-Reactive Factor in Media Used for:		
Isolation of nuclei	Incubation for RNA synthesis	Incorporation of ^{14}C-ATP into RNA (% control)‡,
—	—	100%§ control
—	+2,4-D	98 ± 7**
+2,4-D	+2,4-D	170 ± 20
—	+S*	110 ± 30
—	+2,4-D + S	170 ± 25
—	+2,4-D + S†	90 ± 20

* S, Supernatant hormone-reactive factor.
† S, Supernatant hormone-reactive factor boiled 10 min.
‡ Averages of three or more experiments.
§ Control nuclei incorporated 200–500 cpm.
** Standard deviation.

Effect of 2,4-D and hormone-reactive protein on RNA synthesis with chromatin as template: Pea bud chromatin can serve as the template for RNA synthesis by *E. coli* RNA polymerase. The addition of auxin (6×10^{-2} μg/ml of 2,4-D) alone to such an RNA-synthesizing reaction mixture is slightly inhibitory (rate of RNA synthesis decreased by approximately 15%). The addition of the hormone-reactive substance alone has no effect on RNA synthesis. However, the addition of both auxin and hormone-reactive substance increases the rate of RNA synthesis by an amount varying in different experiments between 30 and 150 per cent (Table 3). (The use of nucleohistone as a template for RNA synthesis gives similar, although slightly smaller, increases.) If pure DNA is used as the tem-

TABLE 3.   *Effect of tobacco nuclear lysate supernatant preparations on rate of RNA synthesis supported by isolated pea bud chromatin.*

Addenda to RNA synthesis reaction mixture	2,4-D added	Increase in RNA synthesis* (%)
None	—	100
	+	86 ± 4‡
Crude lysate†	—	100
	+	170 ± 25
Purified lysate†	—	100
Light fraction	+	150 ± 20
Heavy fraction	—	100
	+	185 ± 20
Heavy fraction treated with RNase	—	100
	+	150 ± 10

* The control incorporated between 150 and 500 μμmoles ATP-^{14}C.
† Supernatant left after sedimentation of chromatin from lysed nuclei.
‡ Standard deviation.

TABLE 4. *Effect of hormone-reactive protein on RNA synthesis.*

| Source of protein	Template	Increase in RNA synthesis with 2,4-D (0.06 mg/liter) addition (%)
Pea	Pea bud chromatin*	140 ± 15**
Buds	Pea DNA†	92 ± 3
Tobacco	Pea bud chromatin‡	185 ± 20
Nuclei	Pea DNA§	95 ± 1

* 2.1 µg of DNA.
† 0.34 µg of DNA.
‡ 25.5 µg of DNA.
§ 31.9 µg of DNA.
** Standard deviation.

plate, no effect of hormone-reactive substance plus auxin on the rate of synthesis is observed (Table 4).

Larger quantities of hormone-reactive substance than could be obtained from nuclei were prepared from the supernatant from the first centrifugation in the Huang and Bonner[11] method of making pea bud chromatin. Exclusion chromatography on Sephadex G-200 was found to be useful for separating the high-molecular-weight hormone-reactive factor from lower-molecular-weight materials. A typical elution pattern for the pea preparation is shown in Figure 2, in which amounts of eluting protein are followed by OD_{280} and amounts of hormone-reactive factor by bioassay with chromatin. The preparation contains materials inhibitory to RNA synthesis. Superimposed on such inhibitions are two peaks of promoting activity, with approximate molecular weights of 200,000 and 20,000, respectively. These factors seem to be similar or identical to those prepared from nuclear lysates. Evidently, the stimulatory factors constitute only a small portion of the protein, since the peaks of stimulatory activity do not coincide with peaks of optical density. Rechromatography of the heavy fraction on Sephadex G-200 yields both heavy and light fractions, as does rechromatography of the light fraction, suggesting that the heavy fraction is simply a multimer of the light fraction. The heavy fraction from both pea buds and tobacco was incubated with RNase (20 µg/ml, 25°, 60 min) and rechromatographed to remove the

FIG. 2.—Elution profile on Sephadex G-200 of a typical pea preparation. Molecular weight decreases from left to right. Brackets indicate standard deviations. In the absence of any added fraction, 2,4-D addition causes a 10–30 µµM inhibition in ATP incorporation into RNA.
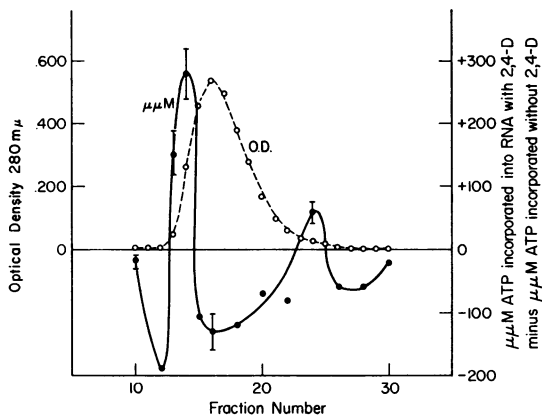
RNase. Activity was recovered with unchanged molecular weight, suggesting that RNA is not a required component of the factor.

The magnitude of the increase in the rate of RNA synthesis is proportional to the amount of factor used. 2,4-D, IAA, and 2,3,6-trichloro-benzoic acid are all active in promoting RNA synthesis in the presence of the factor, but 2,4,6-trichlorophenoxyacetic acid (an antiauxin[16]) is inactive. Thus, the structural requirements for auxin activity in this system seem to be similar to those in the whole plant.

Purified hormone-reactive protein from pea buds contains little, if any, RNase, and there is no difference in the small amount of RNA degraded by the assay mixture in the presence or absence of auxin. Hormone-reactive protein, chromatin, and RNA polymerase were all found to be contaminated with ATPase. The total amount of ATPase in the assay mixture with hormone-reactive protein is sufficient to break down about 10 per cent of the added ATP in ten minutes. There is no effect of auxin on the rate of ATP breakdown. Thus, it seems likely that the effect of auxin and hormone-reactive protein is indeed an effect on RNA synthesis.

To determine whether the increased rate of RNA synthesis in the presence of hormone-reactive protein and auxin is due to an increase in the availability of the chromatin template for transcription, the amount of RNA polymerase in the reaction mixture was increased to saturating levels. The increased rate of RNA synthesis in the presence of auxin was maintained under these conditions, suggesting that the effect of auxin and hormone-reactive protein is indeed on the chromatin.

Discussion.—Specific hormone-binding proteins have been shown to occur in the target organs of several hormones.[17, 18] Hormone treatment has also been shown to increase the template activity for RNA synthesis of chromatin of the cells of the target organ in a number of cases.[2, 3] The present results tend to link these two kinds of observations and provide a basis for a model of hormone-induced derepression in which the hormone interacts with its binding protein, the complex then interacting with chromatin to cause an increase in template activity. The molecular basis for this hormone-induced alteration in chromatin should now be accessible for study.

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