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ISOLATION AND ANALYSIS OF THE PROTEINS OF PLANT NUCLEI: INTERACTION OF HORMONES WITH NUCLEAR PROTEINS IN ISOLATED NUCLEI OF *PHASEOLUS VULGARIS**

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Abstract—Incubation of intact segments or isolated nuclei of bean hypocotyls with IAA-2-^{14}C and kinetin-8^{14}C, showed that these hormones associate with the nuclear proteins. Differences in the degree of association of these hormones with the nuclear proteins were found between the isolated nuclei or intact segments. Partially purified nuclei from undifferentiated hook and the more differentiated lower section of bean hypocotyl displayed quantitative and qualitative changes in nuclear proteins due to hormone treatments. Four of the five nuclear protein fractions of each of the tissues were quantitatively reduced by the hormone treatments. The acidic proteins associated with DNA were increased by IAA but not be kinetin. However, most of the protein electrophoretic patterns were qualitatively unchanged by hormone treatment.

INTRODUCTION

IN 1961, Zaloker[1] proposed that hormones produce their effects primarily by regulating gene activity. Evidence in support of this hypothesis has come from studies of plants,[2-4] insects[5, 6] and mammals.[7-9] That hormones specifically control gene transcription has been demonstrated in some cases, but is still subject to some controversy.[10]

The chemical mechanism by which hormones may regulate DNA-dependent RNA synthesis is not known. One explanation could be that hormones interact with products of regulator genes.[11, 12] While such regulating substances have not yet been found in higher plants, it was shown that in bacteria they are proteins.[13, 14] Certain plant hormones have been shown to affect the rate of synthesis of RNA in isolated plant nuclei.[15-16] Consistent

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[1] M. ZALOKER, *Control Mechanisms in Cellular Processes*, p. 87, Ronald Press, N.Y. (1961).
[2] A. J. TREWAVAS, *Arch. Biochem. Biophys.* **123**, 324 (1968).
[3] L. D. NOODEN, *Plant Physiol.* **43**, 140 (1968).
[4] Y. MASUDA and S. KAMISAKA, *Plant Cell Physiol.* **10**, 79 (1969).
[5] P. KARLSON, *J. Cell. Comp. Physiol.* **66**, *Suppl.* **1**, 69 (1965).
[6] J. CARNEIRO and A. LESSO, *Experienta* **19**, 399 (1963).
[7] F. KENNY, *J. Biol. Chem.* **237**, 3495 (1962).
[8] R. T. SCHIMKEL, E. W. SWEENEY and C. W. BERLIN, *J. Biol. Chem.* **240**, 322 (1965).
[9] C. C. WIDNELL and J. R. TATA, *Biochem. J.* **93**, 29 (1964).
[10] J. R. TATA, *Prog. in Nucl. Acid Res. Mol. Biol.* **5**, 191 (1966).
[11] F. JACOB and J. MONOD, *J. Mol. Biol.* **3**, 318 (1961).
[12] J. MONOD, J. P. CHANGEUX and F. JACOB, *J. Mol. Biol.* **6**, 306 (1963).
[13] J. GALLANT and R. STAPLETON, *J. Mol. Biol.* **8**, 442 (1964).
[14] M. PTASHNE, *Proc. Nat. Acad. Sci. U.S.A.* **57**, 306 (1967).
[15] R. ROYCHOUDHURY, A. DATTA and S. P. SEN, *Biochim. Biophys. Acta* **107**, 346 (1965).
[16] S. C. MAHESHWARI, S. GUHA and S. GUPTA, *Biochim. Biophys. Acta* **117**, 470 (1966).

alterations of electrophoretic patterns of nuclear proteins of bean hypocotyls treated with indoleacetic acid (IAA) have been observed.[17, 18] The fact that some nuclear proteins are specific for a given stage of development of the hypocotyl suggests that they are possibly involved in the process of differentiation and thus gene regulation. Studies into hormonally induced alterations (qualitative and quantitative) of nuclear proteins could increase our understanding of the role of these proteins in metabolism and gene regulation.

RESULTS

The Association of IAA and Kinetin with Nuclear Proteins

Incubation of hypocotyl segments in IAA-^{14}C resulted in a high specific activity associating with the nuclear proteins (Table 1). However, the activity was found in high amounts in the

TABLE 1. THE ASSOCIATION OF LABELED HORMONES WITH PROTEINS FROM VARIOUS CELL ORGANELLES AND NUCLEAR PROTEINS

System	Hormone treatment	Protein fraction cell fraction	μmoles hormone/g protein	
			Hook	Lower hypocotyl
Intact hypocotyl segments	10^{-5} M IAA 2-^{14}C (6 hr) (Incubation)	Cytoplasm	600	
		Microsomes	450	
		Mitochondria	800	
		Nuclei	900	
		Protein fraction		
Intact hypocotyl segments	10^{-5} M IAA 2-^{14}C (6hr) (Incubation)	Total cell soluble protein	750	1200
		ETOH–HCl extracted (chromatin)	800	900
		HCl extracted (chromatin)	500	700
		NaOH extracted (chromatin)	3200	3900
Isolated nuclei	3×10^{-5} M IAA 2-^{14}C (1 hr) (Incubation)	Nuclear sap	170	120
		Acidic ribosomal	60	90
		Acidic ribonuclear	70	50
		Total histone	40	30
		Acidic chromosomal	12	10
	8×10^{-6} M kinetin 8-^{14}C (1 hr) (Incubation)	Nuclear sap	100	110
		Acidic ribosomal	30	65
		Acidic ribonuclear	45	15
		Total histone	30	55
		Acidic chromosomal	5	7

cytoplasmic, microsomal and mitochondrial proteins as well. Part of this activity could be metabolic products of the IAA and not the hormone itself. Further investigation into the various subfractions of the chromatin-proteins showed that most of the activity was found with the alkali-extracted (acidic) proteins associated with the DNA. When isolated nuclei were incubated with IAA-2-^{14}C, various associations became apparent (Table 1). Nuclear

[17] I. V. SARKISSIAN and T. C. SPELSBERG, *Physiol. Plantarum* **20**, 991 (1967).
[18] T. C. SPELSBERG and I. V. SARKISSIAN, *Phytochem.* **7**, 2083 (1968).

sap proteins had the highest amount of radioactivity regardless of the hormone. When IAA (radioactivity) is expressed per unit protein, it is evident that the degree of association of hormone with any nuclear protein fraction depends on the stage of development of the hypocotyl. The much higher amount of IAA associated with nuclear proteins when intact sections of hypocotyl are used as compared to nuclei may be the result of the longer period of incubation (6 hr). It is possible that the hormone was broken down in the intact cells and the label was incorporated into a metabolite which became associated with the nuclear proteins. On the other hand, the intact segments represent a situation more natural than the isolated nuclei, which may include receptor sites or metabolic alterations essential for hormone action. In any case, much more of the label was found associated with nuclear proteins when intact tissues were used as compared to isolated nuclei. As is shown in Table 1, the degree of association of the activity to the proteins was similar between the two tissues and between the two hormones. However, the activity was found to be associated quite differently between the various protein fractions of each tissue.

Effects of Hormones on the Quantity of Nuclear Protein

Table 2 shows the effects of IAA and kinetin on the quantity of each protein fraction of the nuclei of both stages of development. Both levels of IAA caused a reduction in most fractions in the lower hypocotyl and in the hook, 3×10^{-5} M having a much greater effect in reducing the protein centent of the nuclei than did 5×10^{-6} M IAA in the lower hypocotyl. In the hook tissue, the histone fraction was unaffected while the acidic chromosomal proteins were increased in nuclei treated with 5×10^{-6} M IAA and decreased in nuclei treated with 3×10^{-5} M IAA.

TABLE 2. THE EFFECTS OF VARIOUS HORMONE TREATMENTS ON THE QUANTITY OF THE NUCLEAR PROTEINS

Source of nuclei	Hormonal treatment	Nuclear sap	Acidic ribosomal	Acidic ribonuclear	Histone	Acidic chromosomal
Hook	5×10^{-6} M IAA	83 ± 5%	68 ± 3%	96 ± 4%	102 ± 8%	128 ± 12%
	3×10^{-5} M IAA	88 ± 4%	96 ± 3%	90 ± 2%	106 ± 6%	69 ± 10%
	5×10^{-6} M kinetin	62 ± 13%	92 ± 4%	91 ± 3%	101 ± 10%	75 ± 5%
	8×10^{-6} M kinetin	61 ± 10%	91 ± 8%	99 ± 7%	98 ± 8%	79 ± 5%
Lower hypocotyl	5×10^{-6} M IAA	91 ± 8%	94 ± 3%	90 ± 5%	89 ± 9%	108 ± 3%
	3×10^{-5} M IAA	64 ± 5%	85 ± 5%	80 ± 4%	84 ± 5%	81 ± 8%
	5×10^{-6} M kinetin	96 ± 4%	98 ± 3%	58 ± 12%	87 ± 10%	96 ± 8%
	8×10^{-6} M kinetin	95 ± 5%	90 ± 10%	74 ± 8%	68 ± 10%	72 ± 4%

The nuclei were incubated 1 hr in the respective hormone solutions and the nuclear proteins extracted and purified as described. Values are stated in terms of 100% as the value for the level of protein in each fraction from nuclei incubated in solutions without hormone. The variations of five replications are shown.

The increase of acidic chromosomal proteins of hook nuclei with 5×10^{-6} M IAA was much greater than that of the acidic chromosomal proteins of the lower hypocotyl although the responses were in the same direction for both stages of development. In any event, hook and lower hypocotyl nuclei reacted differently to IAA treatment with respect to content of protein after treatments.

Kinetin reduced protein levels of all fractions of nuclei of lower hypocotyl. However, a reduction in protein was caused by kinetin in only two of the five fractions of nuclei of the hook.

Kinetin and IAA have been shown to increase the rates of synthesis of nuclear proteins in plant cells.[6] Although the rate of amino acid incorporation into nuclear proteins was enhanced by hormones, the levels of some nuclear protein fractions were found to be decreased; this suggests a parallel increase in the rate of destruction of these proteins.[2–4] The decrease in the levels of the nuclear proteins described in this paper may be due to the use of isolated nuclei rather than intact tissues in the incubations; the decrease may also be the result of accelerated breakdown of protein in nuclei influenced by hormones.

Qualitative Effects of Hormones on Nuclear Proteins

No alterations of the electrophoretic patterns were observed with the histone, nuclear sap, or acidic ribosomal protein fractions when the nuclei were treated with the two hormones. The changes in quantity of these protein fractions with hormone treatment appear to reflect only a general change in level of all proteins in these fractions and not a loss of gain of any particular fraction.

Acidic Chromosomal Proteins

The acidic chromosomal proteins appeared to be altered only slightly by both IAA and kinetin (Fig. 1). In the lower hypocotyl band "A" was altered in its rate of migration by both hormones. It is of interest that hormone treatment caused band "A" of the lower hypocotyl to migrate at a rate similar to band "A" of the hook. The alteration of the migration of the band by the two hormones suggests a possible transition in the conformation of the protein in that band. Both hormones caused the same type of alteration. The effect appears to be specific to more highly differentiated tissues in that the hormones caused no alterations in the patterns of the acidic chromosomal proteins from the hook. This alteration of the electrophoretic pattern by the hormones seems very minimal. It is, of course, possible that the levels of hormone chosen are simply not effective doses for these experimental conditions.

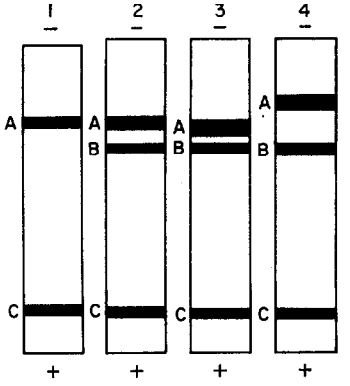

FIG. 1. ACIDIC CHROMOSOMAL PROTEINS OF BEAN NUCLEI.

(1) Nuclei of hook incubated in buffer; (2) nuclei of lower hypocotyl incubated in 5×10^{-6} M kinetin; (3) in 5×10^{-5} M IAA and (4) in buffer. The gels are 15%, pH 8·9. Top: cathode; bottom: anode.

Acidic Ribonucleoproteins

Hormone treatment altered the electrophoretic pattern of acidic ribonucleoproteins in a manner specific to both stage of development and hormone. When nuclei from the lower hypocotyl were incubated with either level of kinetin, band "A" was eliminated (Fig. 2A). The same band was not affected when the nuclei were incubated with IAA. In contrast,

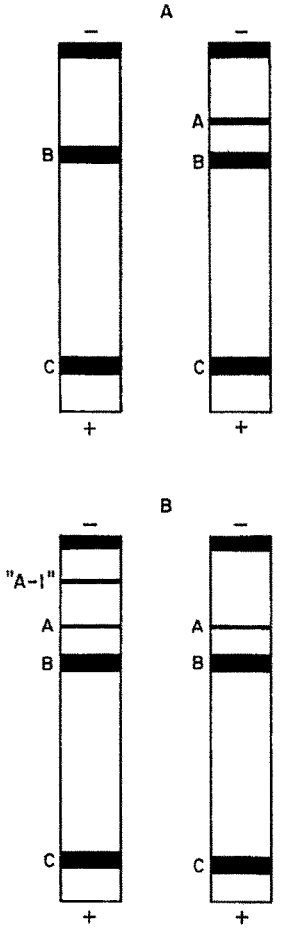

FIG. 2. ACIDIC RIBONUCLEOPROTEINS OF NUCLEI OF LOWER HYPOCOTYL.
Incubated in (A) buffer (right) and 5×10^{-6} M kinetin (left) and (B) in a buffer (right) and 5×10^{-6} M IAA (left). The gels are 15%, pH 8·9. Top: cathode; bottom: anode.

incubation with 5×10^{-6} M IAA caused the appearance of a new band, "A-1". This band was never found after kinetin treatment. As in the case of acidic chromosomal proteins, the patterns of the acidic ribonucleoproteins from the hook nuclei were not altered by the hormone treatments of nuclei.

The differential response of acidic ribonucleoproteins from the lower hypocotyl to the two hormones is of interest. The elimination of band "A" in the ribonucleoprotein patterns by kinetin treatment creates a pattern resembling that of the hook acidic ribonucleoprotein.

There appears to be a dedifferentiation of nuclei caused by kinetin with respect to the elctrophoretic patterns. The effect of IAA is the reverse of that of kinetin suggesting different mechanisms of action for the two hormones.

DISCUSSION

It has been shown[19] that each protein fraction of the nuclei upon gel electrophoresis displayed patterns which were specific for the particular stage of development. This specificity suggests that these proteins may play a role in differentiation. Whether their role in development is an active one as enzymes or as gene regulators, or a passive one as structural proteins is not known. That hormones do activate RNA synthesis in cells is known in plants.[2-4] Recent evidence has shown that IAA and other auxins can activate RNA synthesis in isolated plant cell nuclei.[20, 21] In these studies, auxins were bound to nuclear components within 1 min.[21] If the special receptor molecules in nuclei are lost during isolation of the nuclei, then the hormone action by IAA (i.e. activation of RNA synthesis) is lost.[20]

The qualitative and quantitative response of these nuclear proteins to the hormone treatments indicates a possible interaction of these components. Whether such an interaction is essential for hormone action remains to be determined. Specific hormone receptor proteins have been found and partially characterized in mammalian systems.[22, 23] However, in plants such investigations have not been as numerous. These experiments suggest that IAA and kinetin do bind to the nuclear proteins especially when the hormones are applied to intact tissues instead of isolated nuclei. The nuclear proteins with the highest specific activity of the label were the acidic proteins associated with the DNA; this was only found when the IAA 2-^{14}C was incubated with intact tissue and not with the isolated nuclei. These acidic chromosomal proteins and not the histones may be the receptor molecules involved in the accumulation of the auxins and kinetins with the deoxyribonucleoproteins observed by Fellenberg.[24, 25]

Our studies with isolated nuclei however, indicate that the other nuclear proteins are also complexed with IAA and kinetin. The differences in association of these hormones with the nuclear proteins in intact tissues or isolated nuclei cannot be explained at present. The possible metabolic alterations of the hormones by the intact cells, the effect of binding of hormones to cytoplasmic protein receptors[22, 26] as well as the general differences in environment of the nuclei during hormone treatment may explain the differences observed. Variations in the methods of isolation of nuclei have been explained as the cause of different responses of nuclei of tobacco tissue culture cells to IAA or 2,4-D.[20]

Since IAA has been shown to not only increase the rate of RNA synthesis but also to cause the appearance of some new species of RNA,[2] speculation can be made that the nuclear proteins are involved in the regulation of gene activity. The tissue specificity of these proteins and the association of the labeled hormones with them indicates a possible link in the regulation of RNA synthesis.

[19] T. C. SPELSBERG and I. V. SARKISSIAN, *Phytochem.*, in press.
[20] A. G. MATTHYSSE, *Plant Physiol.* **43**, S-42 (1968).
[21] K. J. TAUTVYDAS and A. W. GALSTON, *Fed. Proc.* **28**, 902 (1969).
[22] E. V. JENSEN, T. SUZUKI, T. KAWASHIMA, W. E. STUMPF, P. W. JUNGBLUT and E. R. DESOMBRE, *Proc. Nat. Acad. Sci. U.S.* **59**, 632 (1969).
[23] T. ERDOS, *Biochem. Biophys. Res. Commun.* **32**, 338 (1968).
[24] G. FELLENBERG, *Planta* **84**, 195 (1969).
[25] G. FELLENBERG, *Planta* **84**, 324 (1969).
[26] I. V. SARKISSIAN and T. C. SPELSBERG, *Nature* **214**, 319 (1967).

It should be mentioned that nuclei which were further purified using concentrated sucrose solutions of 2·0 M or which were allowed to age for several hours did not respond to the hormone treatments.

EXPERIMENTAL

The preparation of segments of shoots of 7- to 8-day-old bush beans (*Phaseolus vulgaris*) has been described.[18] In one series of experiments the undifferentiated hypocotyledonary hook and a maturing section of the lower hypocotyl were incubated for 6 hr in 10^{-5} M IAA-2-^{14}C (sp. act. 0·5 mc/mM) in 0·05 M phosphate buffer, pH 7·0. The tissues then served as sources of nuclear proteins.

Isolation and Purification of Nuclei

All isolation procedures were carried out at 4° and were similar to those described in the preceding paper[19] except for the final purification of the nuclei. The nuclei were purified by layering the crude suspension on 1·4 M sucrose in TKMC buffer and centrifuging for 30 min at 16,000 *g* in a swinging-bucket rotor. The sedimented nuclei were suspended in 18 ml of TKMC buffer for use. Routine observations of nuclei with the microscope revealed some contaminating debris in such preparations. Further purification in higher concentrations of sucrose as described in the preceding paper apparently damaged the nuclei since they failed to respond to hormone treatments as observed by gel electrophoresis and quantitative analysis.

Incubation of Nuclei

Suspensions of nuclei were separated into three equal volumes and placed into three sterilized flasks at 4°, one with 0·6 ml of water, one with 0·6 ml of a specified amount of IAA in TKMC buffer and one with an amount of kinetin in 0·6 ml of TKMC buffer. The flasks were gently warmed to room temperature and were then placed on a shaker. After 1 hr the contents of the flasks were quickly cooled to 0–1° and centrifuged 10 min at 10,000 *g*. The sedimented nuclei were resuspended in TKMC buffer and were centrifuged again. Following the second sedimentation the nuclei were ready for analysis.

Analytic Procedures

The following protein fractions were extracted from the nuclei: nuclear sap, acidic ribosomal, histone, acidic chromosomal and acidic ribonuclear.[19] Protein quantity was determined by the method of Lowry and co-workers[27] and spectrophotometrically.[28] Standards for neutral and acidic proteins were established with egg albumin; commercially prepared calf thymus histone was the standard for histones. Methods of polyacrylamide gel electrophoresis have been described.[18] Amounts of IAA- and kinetin-^{14}C associated with nuclear protein were determined by measuring radioactivity of 0·7 ml of each protein suspension in a scintillation spectrometer. Total proteins of mitochondria, cytoplasm, microsomes, and nuclei were extracted by the method of Shih *et al.*[29] which is a modified version used by other investigators.[30,31] The protein was quantitated and compared with the radioactivity to determine the specific activity as described above.

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[27] O. H. Lowry, J. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
[28] O. Warburg and W. Christian, *Biochem. Zeit.* **310**, 384 (1941).
[29] S. C. Shih, G. A. Jung, and D. C. Shelton, *Crop Science* **7**, 385 (1967).
[30] G. Schmidt and S. J. Tannhauser, *J. Biol. Chem.* **161**, 83 (1945).
[31] R. W. Wannenmaker, W. L. Banks and W. H. Wunner, *Anal. Biochem.* **11**, 320 (1965).