HORMONAL CONTROL OF CHROMATIN AVAILABILITY AND OF THE ACTIVITY OF PURIFIED RNA POLYMERASES IN HIGHER PLANTS

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1. Introduction

It is now well established that indole-3-acetic acid (IAA) exhibits two types of effects on RNA and protein metabolism in higher plant tissues. For "short" incubation periods (less than 2 hr), this auxin increases incorporation of labelled precursors into heterogeneous nuclear RNA's (HnRNA's) and into short-lived polysomal messenger RNA's (mRNA's) [1, 2], whereas for longer incubation periods (up to 15 hr) there is a marked stimulation of the synthesis of ribosomal RNA (rRNA's) and specific proteins [3]. These effects are independent of the precursor pool size [1]. Moreover, hybridization experiments using unique sequences of plant DNA, strongly suggest the existence of a precursor relationship between HnRNA's and polysomal mRNA's [4]. Taken together, these results argue in favor of a primary effect of indole-3-acetic acid on gene expression. The increased transcriptional activity of isolated chromatin in the presence of IAA and a protein factor [5], as well as the direct interaction of this factor with DNA [6], are in agreement with this view. However, the mechanism by which an auxin stimulates *in vivo* or *in vitro* transcription is not at all clear and might be *a priori* due to three types of phenomena occurring either alone or in combination, namely:

i) increased availability of template;
ii) increased specific activity of nuclear RNA polymerases;
iii) increased amount of nuclear RNA polymerases.

It is the main goal of this paper to present new insights in this problem.

2. Methods

The plant tissues used are obtained from lentil (*Lens culinaris* var Ronde Blonde, Vilmorin). Roots were grown and harvested as previously described [3]. Nuclei were obtained with a method derived from that of Mertelsmann [7]. The average yield of nuclei, based on DNA determination by diphenylamine reaction [8], was routinely 40%. Chromatin and non-histone proteins were prepared according to Baulieu's method [9], except that every solution used contained 5 mM dithiothreitol. The above operations were performed at 4°. Non-histone proteins are chromatographed on DEAE-Sephadex (A-25) columns prepared according to Roeder and Rutter [10] using 50 mM Tris-HCl pH 7.8, glycerol 25% v/v, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol (TGMED buffer) for equilibration. To determine the protein content of chromatographic fractions, the following modifications of the Lowry method [11] were adopted. 2 ml of 10% trichloroacetic acid (w/v) and 100 μg of yeast RNA were added to each fraction (1 ml). The resulting precipitate was washed twice with 5% TCA (2 ml), then dissolved in Lowry solution C (1.25 ml). After 10 min Lowry solution E (100 μl) was added. Absorbance was measured after 30 min at 550 nm. Auxin treatment of the seedlings lasted either 1.5 hr or 14 hr with aerated solutions of 0.25 mM IAA. All the buffers used for RNA polymerase extraction of hormone-treated roots contained 0.1 mM IAA.

RNA polymerase activity was assayed by following the conversion of [^{3}H]UTP into an acid-insoluble material. The standard reaction mixture (0.2 ml) contained 39 mM Tris-HCl pH 7.8, 1.1 mM $MnCl_2$,

0.55 mM KCl, 3 mM NaF, 1.1 mM dithiothreitol, 1 mM phosphoenolpyruvate, 0.5 mM each of ATP, CTP, GTP, 0.0385 mM unlabelled UTP, 1.65 μM [^{3}H]UTP (15 Ci/mmole, CEA), 2 μg pyruvate kinase (Sigma), 5 μg of calf thymus DNA (Calbiochem), enzyme, and ammonium sulfate concentrations as given for each experiment. After incubation at 16° for 30 min, the reaction was stopped by adding 50 μl of a solution containing 80 mM sodium pyrophosphate, 20 μg yeast RNA, and 20 μg bovine serum albumin, then 100 μl 5% sodium dodecylsulfate and 2.5 ml 10% TCA. The unincorporated [^{3}H]UTP was removed by centrifugation and repeated washings of the acid-insoluble precipitate with 2 ml 40 mM sodium pyrophosphate and 5% TCA. The acid-insoluble RNA's were hydrolyzed 20 min at 95° in 0.5 ml 5% TCA. 400 μl fractions of hydrolyzate were placed in vials containing a mixture (8 ml) of dioxane, 100 g/ℓ naphthalene, 5 g/ℓ PPO, 0.30 g/ℓ POPOP, and counted in a Beckman LS-150 liquid scintillation counter. The counting efficiency for ^{3}H was routinely 27%.

Table 1
Solubilization and isolation of RNA polymerases from lentil roots.

Steps	Total protein (mg)	Specific activity (units/mg protein)	Reovery units
Homogenate	1 950	0.41	810
Soluble-chromatin	13.4	27	364
Non-histone-proteins	2.60	124	323*
DEAE-Sephadex (A-25) chromatography			
Ia	0.015	830	12.5
Ib	0.134	700	93
II	0.205	760	155
III	0.300	155	46.5

* This assay was performed with an incubation mixture (200 μl) containing 100 μg of calf thymus DNA.

Activities assayed under standard conditions with 0.06 M ammonium sulfate and 25 μg/ml calf thymus DNA are expressed in pmoles UMP incorporated in 30 min at 16°. Values are given for 15 g of lentil roots.

3. Results

The various steps involved in the solubilization,

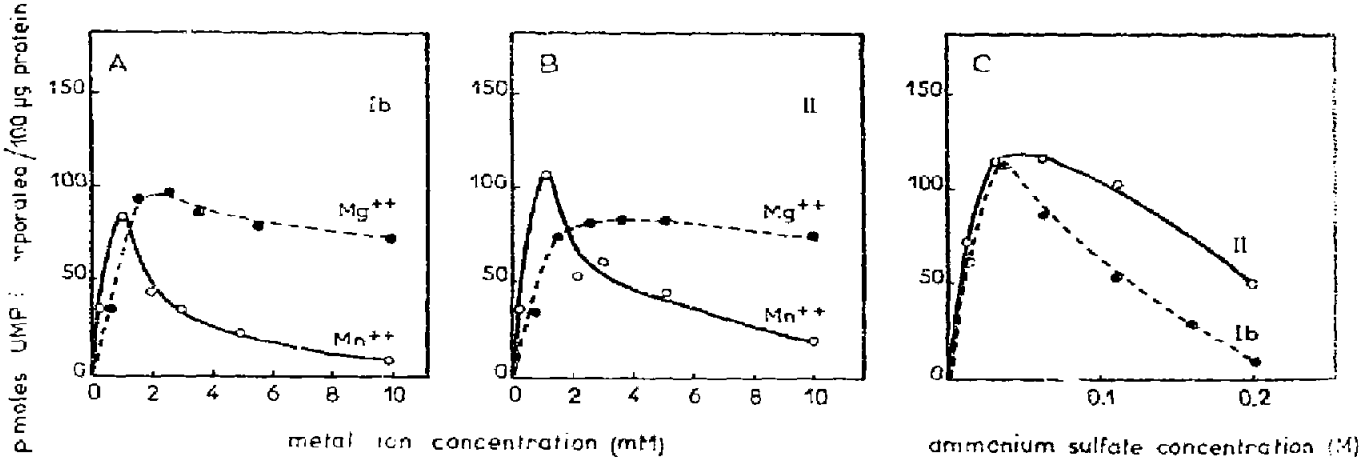

Fig. 1. Effect of divalent cation concentrations and ionic strength in RNA polymerases Ib and II. Assay conditions in A and B were as described in Methods except that the metal ion concentrations were varied as indicated. Each reaction mixture (100 μl) contained 12 mM and 25 mM ammonium sulfate for enzymes Ib and II, respectively. Mn^{2+} effect was tested in the presence of 0.5 mM Mg^{2+}. The amounts of enzymes were 1 μg and 0.6 μg for RNA polymerases Ib and II, respectively. Experiments shown in fig. C were performed in the same conditions, at a fixed concentration of both Mg^{2+} (2.5 mM) and Mn^{2+} (1 mM). Ammonium sulfate concentration was varied as indicated.

Table 2
Effect of trypsin predigestion on RNA synthesis in chromatin isolated from control and auxin-treated (1.5 hr) lentil roots.

Trypsin (μg/ml)	Chromatin from control tissues		Chromatin from auxin-treated tissues	
	(pmoles UMP incorporated/ 10 μg DNA)	Increase (%)	(pmoles UMP incorporated/ 10 μg DNA)	Increase (%)
0	5.980 ± 0.060	–	7.54 ± 0.080	–
10	10.189 ± 0.024	70	12.236 ± 0.306	62
20	10.286 ± 0.411	72	12.139 ± 0.486	61
40	10.419 ± 0.521	74	11.456 ± 0.516	52

Assays were performed as previously described, in a mixture (100 μl) containing 0.038 M ammonium sulfate. Chromatin was incubated 30 min at 15° with trypsin.

isolation, and partial purification of nuclear RNA polymerases of lentil roots are presented in table 1. Four polymerases are obtained from DEAE-Sephadex chromatography (fig. 2). Component Ia comes through with the TGMED wash, and components Ib, II, and III are eluted stepwise with the same buffer containing 0.125 M, 0.250 M, and 0.5 M ammonium sulfate, respectively. The major part of the recovered activity is found in components Ib (30%) and II (51%). The minor components Ia and III represent 4% and 15% of the activity, respectively. From their chromatographic behaviour and by analogy with RNA polymerases of other eukaryotic cells [10, 12–16], it is very likely that enzymes Ia, Ib are of nucleolar origin, and polymerases II and III from the nucleoplasm. Only fractions Ib and II have been studied in the present work since they occur in rather large amounts within nuclei. The specific activity of RNA polymerase preparations Ib and II are 700 and 760, respectively. With regard to the crude homogenate, they have been purified 1700- and 1900-fold, with a recovery of 11% and 19%, respectively.

Both enzymes require divalent cations for activity (fig. 1A, 1B). Whereas the concentration–activity profile for Mn^{2+} is sharp, that obtained for Mg^{2+} is very broad. The various optimum values can be found on fig. 1. The Mn^{2+}/Mg^{2+} activity ratio, at the optimal concentration of each ion, are 0.85 and 1.25 for components Ib and II, respectively. The activity of both enzymes is obviously dependent on the ionic strength, as already observed with other eukaryotic RNA polymerases. Fig. 1C shows the effect of various ammonium sulfate concentrations on the lentil roots enzymes.

RNA polymerase II is strongly inhibited (80%) by α-amanitin, (0.1 μg/ml), whereas enzyme Ib is not. This is also the case with mammalian RNA polymerases of nucleoplasmic and nucleolar origins, respectively [10]. Rifampicin (5 μg/ml) has no effect on either polymerase.

A "short" (1.5 hr) treatment of the tissues with IAA does not affect the level of nuclear RNA polymerases, but increases by about 25% the transcriptional activity of isolated chromatin (table 2). It thus appeared likely that this enhanced activity is related to a hormonal control of chromatin availability for transcription. This idea was tested by using the effect of proteolytic enzymes on transcriptional activity of chromatin [17, 18]. Chromatin from control and auxin-treated (1.5 hr) roots was incubated for 30 min at 15° with trypsin before assay for transcription. An enhancement of RNA synthesis was observed with both chromatins at all the trypsin concentrations used (table 2). Experiments performed with other proteolytic enzymes showed that only those able to cleave arginine and lysine bonds were effective for stimulating template activity of chromatin. These observations indicate that trypsin pre-digestion activates chromatin by removal of histones. Moreover, the results of table 2 show this stimulation to be less pronounced with chromatin obtained from hormone-treated tissues than with homologous chromatin extracted from control. Thus, the above results confirm the idea that chromatin of auxin-treated roots is already in a depressed state and more available for transcription.

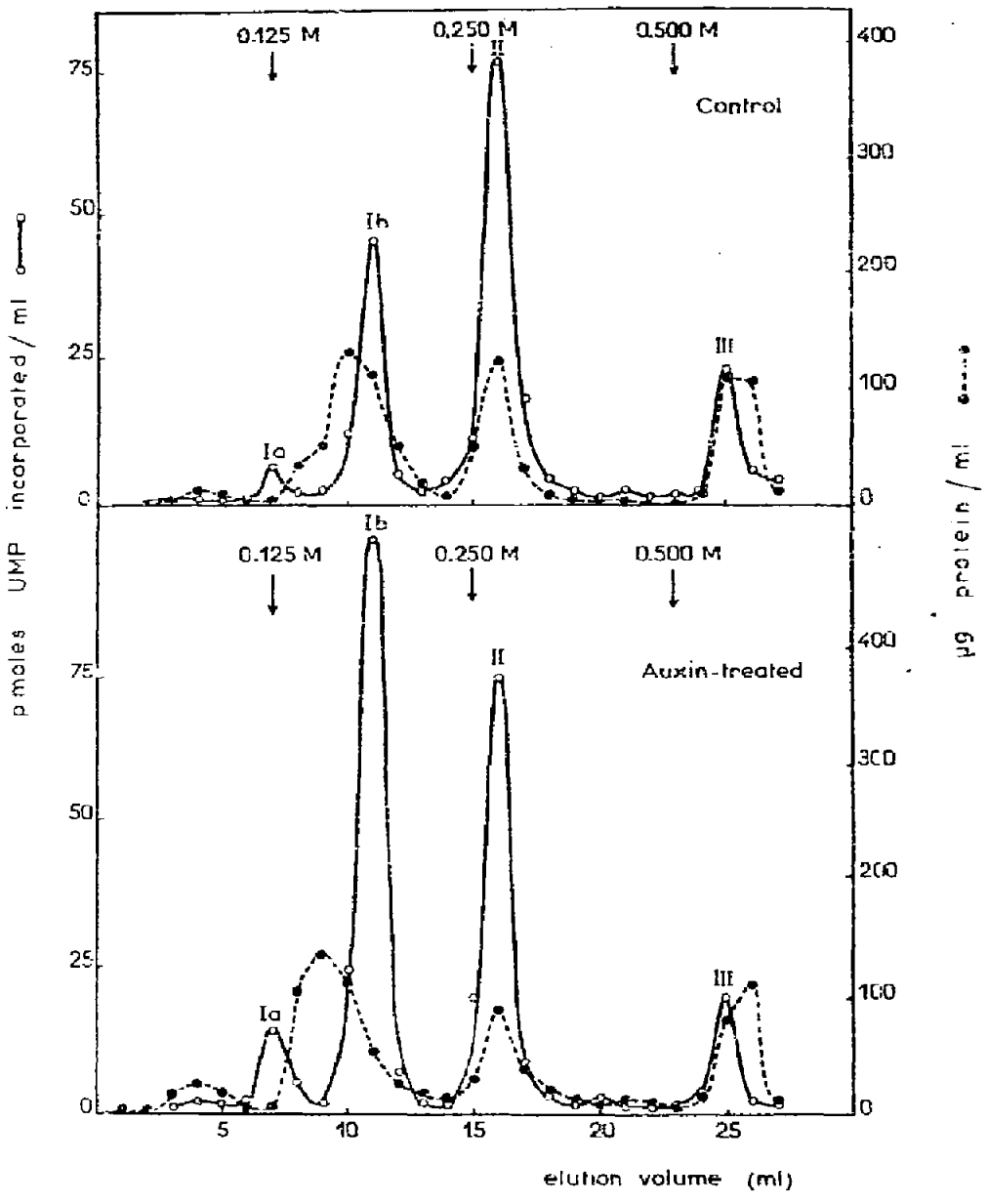

Fig. 2. Elution pattern of control and auxin-treated lentil root RNA polymerases from columns of DEAE-Sephadex (A-25). Non-histone proteins (3.5 ml containing 2.05 mg proteins), extracted as described in the experimental section, were loaded onto a (0.7 × 9 cm) DEAE-Sephadex (A-25) column, washed with 4 ml of TGMED buffer, and eluted stepwise with TGMED containing 0.125 M, 0.250 M and 0.5 ammonium sulfate (vertical arrows). Fractions of 1.0 ml were collected. RNA polymerase activity was assayed with the standard incubation mixture containing 0.06 M ammonium sulfate and calf thymus DNA 25 μg/ml.

If the incubation period of the tissues in hormone solutions is longer (14 hr), then the levels of "nucleolar" polymerases Ia and Ib, but not those of "nucleoplasmic" enzymes II and III, are increased (fig. 2). In order to determine whether the increased level of RNA polymerase Ib was due to an increased synthesis of the enzyme or to a change in its specific activity, the following experiment was done. Activity of purified enzyme Ib,

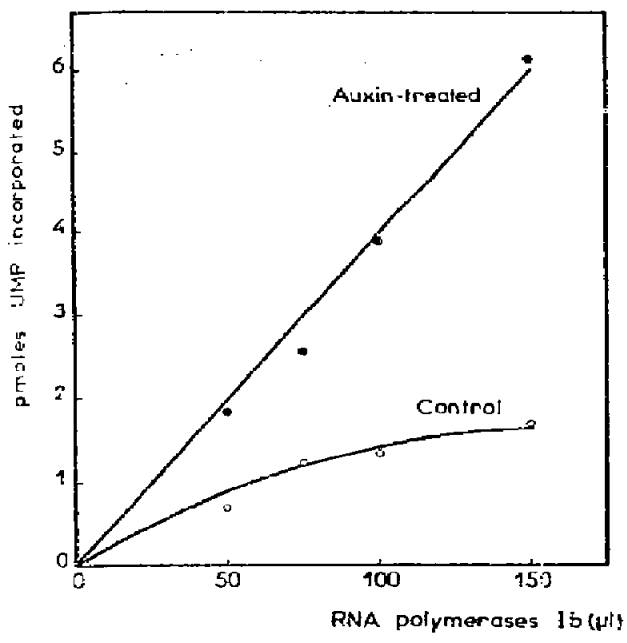

Fig. 3. Relationship between enzyme concentration and activities of RNA polymerases Ib extracted from control and auxin-treated lentil roots. Aliquots of peaks Ib eluted from DEAE-Sephadex (A-25) columns were assayed for RNA polymerase activity in an incubation medium (200 µl) containing 0.09 M ammonium sulfate and a fixed amount of native lentil DNA (1.5 µg).

extracted from control and auxin-treated roots, was measured using varying amounts of polymerase Ib and a fixed quantity of lentil DNA (1.5 µg). The difference of activity for these two homologous RNA polymerases Ib is more pronounced at high than at low enzyme concentrations (fig. 3). Since lentil DNA is saturated at lower concentrations of polymerase Ib when the enzyme is isolated from control than from auxin-treated tissues, it is very likely hormone treatment induces some conformational change of RNA polymerase Ib leading to an increased specific activity. Similar results have been observed with nucleolar polymerases obtained from control and hydrocortisone-treated rat liver [6].

4. Discussion

Various RNA polymerases have already been isolated and partially purified from plant material [12, 14]. However, the only extensive purification has been effected from coconut milk [12]. The specific activities of the polymerase preparations obtained from lentil roots and coconut nuclei are about the same. The present report thus seems to be the first to be concerned with the extensive purification of RNA polymerases from higher plant tissues. The polymerases extracted from lentil roots exhibit obvious similarities to their counterparts from animal tissues. However, with respect to salt dependency and divalent cation requirements, "nucleolar" and "nucleoplasmic" RNA polymerases from lentil roots resemble each other more than do the homologous enzymes obtained from animal cells. It is thus impossible to differentiate the activities in whole chromatin or in "solubilized polymerase preparation" simply by varying Mn^{2+}, Mg^{2+} or ammonium sulfate concentration, as was done with animal materials [19–21]. Isolation and partial purification of RNA polymerases appear to be prerequisite steps for establishing the mechanism of auxin control at the transcriptional level.

The two effects of hormone treatments on RNA metabolism, namely the enhanced syntheses of HnRNA's and rRNA's are likely due to different types of mechanisms. The increased synthesis of HnRNA's and shortlived mRNA's, for "short" hormone treatments, is due only to a derepression of gene batteries [22], as suggested by the above results (table 2). On the other hand, the stimulation of rRNA synthesis during "long" hormone treatments, is at least partially explained by the stimulation of a "nucleolar" RNA polymerase Ib.

Estrogen action in animal target cells is characterized by a stimulation of nucleolar RNA polymerase activities followed by an increase in rRNA and protein synthesis [23]. According to Baulieu et al. [24] this activation of nucleolar enzymes could be induced by a "key intermediary protein" (kip) translated from short-lived mRNA. Results presented in the present report are compatible with these views. The early effect of auxin on template capacity of chromatin could be concerned with the turning on of genes coding for effector(s) of "nucleolar" RNA polymerases. The two effects of auxin treatment, namely the increased template activity of chromatin and the stimulation of nucleolar RNA polymerases would thus be functionally linked. Investigations are now in progress to isolate a soluble auxin-induced protein effector that is able to enhance the specific activity of purified RNA polymerase Ib.

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References

[1] R. Miassod, P. Penon, M. Teissere, J. Ricard and J.P. Cecchini, Biochim. Biophys. Acta 224 (1970) 423.
[2] P. Penon, Proceedings of Symposium on Nucleic Acids and Proteins in Higher Plants (Tihany, Hungary, 1971) in press.
[3] P. Penon, J.P. Cecchini, R. Miassod, J. Ricard, M. Teissere and M.H. Pinna, Phytochem. 9 (1970) 73.
[4] M. Teissere, P. Penon, J. Ricard and G. Ratle, FEBS Letters 23 (1972) 65.
[5] A.G. Matthysse and C. Phillips, Proc. Natl. Acad. Sci. U.S. 63 (1969) 897.
[6] H. Mondal, R.K. Mandal and B.B. Biswas, Nature New Biology 240 (1972) 111.
[7] R. Mertelsmann, European J. Biochem. 9 (1969) 311.
[8] K. Burton, Biochem. J. 62 (1956) 315.
[9] A. Alberga, N. Massol, J.P. Raynaud and E.E. Baulieu, Biochemistry 10 (1971) 3835.
[10] R.G. Roeder and W.J. Rutter, Nature 224 (1969) 234.
[11] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[12] H. Mondal, R.K. Mandal and B.B. Biswas, European J. Biochem. 25 (1972) 463.
[13] P. Chambon, F. Gissinger, J.L. Mandel, Jr., C. Kedinger, M. Gniazdowski and M. Meihlac, Cold Spr. Harb. Symp. Quant. Biol. 35 (1970) 693.
[14] G.C. Strain, K.P. Mullinix and L. Bogorad, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2647.
[15] R.G. Roeder and W.J. Rutter, Proc. Natl. Acad. Sci. U.S. 65 (1970) 675.
[16] E.M. Sajdel and S.T. Jacob, Biochem. Biophys. Res. Commun. 45 (1971) 707.
[17] J.H. Frenster, Nature 206 (1965) 680.
[18] J. Katz, P. Krone, W. Troll, A. Blaustein and M. Levitz, Endocrinology 90 (1972) 1147.
[19] C.C. Widnell and J.R. Tata, Biochim. Biophys. Acta 129 (1966) 478.
[20] T.L. Hamilton, C.S. Teng and A.R. Means, Proc. Natl. Acad. Sci. U.S. 59 (1968) 1265.
[21] F.L. Yu and P. Feigelson, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2177.
[22] J. Bonner, M.E. Dahmus, D. Fambrough, R.C. Huang, K. Marushige and D.Y.H. Tuan, Science 159 (1968) 47.
[23] B.W. O'Malley, W.L. McGuire, P.O. Kohler and S.G. Korenman, Rec. Progr. Horm. Res. 25 (1969) 105.
[24] E.E. Baulieu, A. Alberga, C. Raynaud-Jammet and C.R. Wira, Nature New Biology 236 (1972) 236.