J. Biosci., Vol. 5, Number 4, December 1983, pp. 347-353

RNA polymerase activity in isolated nuclei of *Nicotiana sanderae* callus: Characteristics and modulation during differentiation

P. K. ASHA, M. S. SHAILA, C. S. VAIDYANATHAN* and T. RAMAKRISHNAN

Microbiology and Cell Biology Laboratory, and * Department of Biochemistry, Indian Institute of Science, Bangalore 560 012

MS received 18 August 1983; revised 5 October 1983

Abstract. Isolated nuclei from differentiating cultures of *Nicotiana sanderae* showed increased levels of RNA polymerase activity as compared to the nuclei from callus cultures. The RNA synthetic activity was dependent on nucleotide triphosphates and Mg^{2+} and was destroyed by RNase. Maximum activity was obtained in the presence of 50 mM $(NH_4)_2$ SO_4 and α-amanitin inhibited 40% and 55% of the activity in the nuclei from callus and differentiating tissue respectively. The nuclei from differentiating tissue elicited a 3-fold increase in RNA polymerase I and a 4-fold augmentation in RNA polymerase II activities.

Keywords. *Nicotiana sanderae;* RNA polymerase; phytohormones; differentiation.

Introduction

Phytohormones play an important role in root and shoot formation from callus cultures and during seed germination. Although the biochemical events preceding differentiation are not clearly understood, during the development of embryo into root and shoot, enhanced syntheses of RNA and proteins occur (Davies and Larkins, 1973; Chen *et al.,* 1975). In a variety of developing systems, regulation of gene expression has been studied mainly at the transcriptional level (Key 1969; Duda, 1976; Becker, 1979). Nuclear RNA polymerase activity levels undergo dramatic changes during developmental stages such as seed germination (Guilfoyle and Key, 1977) and regeneration of organs (Yu, 1975). However, there are no reports on the modulation of nuclear RNA polymerase activities in a differentiating system. Therefore, attempts have been made to study the regulation of gene expression, specifically at the transcriptional level, in the differentiating callus of *Nicotiana sanderae.* In this communication, we report the characteristics of RNA polymerase activities in the isolated nuclei and the changes in their levels during differentiation.

Materials and methods

Chemicals

2,4-Dichlorophenoxy acetic acid (2,4-D), naphthalineacetic acid (NAA), benzyl

Abbreviations used: 2,4-D, 2,4-Dichlorophenoxy acetic acid;NAA, naphthalene acetic acid; BAP, benzyl aminopurine; MS medium, Murashige and Skoog's medium; MES, 2-(N-morpholinoethane-sulphonic acid); GM, grinding medium, 5 mM MES-NaOH, pH 6.1, 4 mM magnesium acetate, 5 mM, 2 mercaptoethanol, 0.25 M sucrose, 4% gum arabic and 1% Triton X-100.

aminopurine (BAP), Trizma base, 2-(N-morpholinoethane sulphonic acid) (MES), Triton X-100 and nucleotide triphosphates were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. [^{3}H]-UTP (54 Ci/mmol) was from Radiochemical Centre, Amersham, England, the nylon sieves were obtained as a gift from Dr. Gigot, CNRS, Strasbourg, France, and α-amanitin was obtained as a gift from Dr. J. R. Tata, National Institute for Medical Research, Mill Hill, London. All other chemicals were of analytical grade.

Explants

The callus cultures initiated from shoot explants were grown in Murashige-Skoog's (MS) medium (Murashige and Skoog, 1962) supplemented with 1 mg/L 2,4-D and shoot differentiation was obtained in MS medium containing 1 mg/L NAA and 2 mg/L BAP. The cultures were grown at 25-27° C, under 16/8 h light-dark cycles for about 3 weeks.

Isolation and purification of nuclei

Best nuclear preparations, both in terms of yield and transcriptional activity were obtained using the procedure suggested by Dr. Gigot, Institute de Biologie Moleculaire et Cellulaire, CNRS, Strasbourg, France (Personal communication). The tissue was homogenized in a Waring blendor at full speed for 1 min with 3 g/10 ml grinding medium (GM)-5 mM MES-NaOH, pH 6.1,4 mM magnesium acetate, 5 mM 2-mercapto-ethanol, 0.25 M sucrose, 4% gum arabic and 1% Triton X-100. The homogenate was filtered through 4 layers of cheese cloth, 1 layer of lint cloth and passed through a 100 µ nylon screen. The filtrate was centrifuged at 450 *g* for 15 min and the pellet was suspended in GM without gum arabic and was agitated during 20 min and then filtered through a 25 µ nylon screen and subsequently through a 10 µ nylon screen. The filtrate was centrifuged through a 1.2 M sucrose cushion in GM (without gum arabic) at 450 *g* for 15 min. The pellet was suspended in GM (without gum arabic), and was washed three times with the same buffer and the nuclear pellet was suspended in buffer containing Tris-HCl, pH 8.0, 0.25 M sucrose; 4 mM $MgGl_2$ and 5 mM 2-mercaptoethanol. The nuclei thus prepared was free of carbohydrate contaminations, unbroken cells, fragmented particles etc. As observed under phase contrast microscope, the nuclear-preparations were essentially free of membranous material.

When the nuclei were isolated in the presence of the hormones, all the buffers contained the respective hormones used for the growth (1 mg/L 2,4-D for callus and 1 mg/L NAA as well as 2 mg/L BAP for differentiating tissue) of the cultures.

RNA polymerase assay

The assay was a slight modification of the procedure used by Mennes *et al.* (1977) for tobacco nuclei. Reaction was carried out at 26° C for 20 min in a final volume of 200 µl and the reaction mixture contained the following: Tris-HCl, pH 8.0, 40 mM; $MgCl_2$, 5 mM; EDTA, 0.1 mM; 2-mercaptoethanol, 1 mM; KCl 150 mM; bovine serum albumin, 125 µg; ATP, GTP and CTP, 0.3 mM each, unlabelled UTP, 0.02 mM; [^{3}H]-UTP, 10 µCi and nucelar suspension containing 10^5-10^7 nuclei.

At the end of the incubation period, unlabelled UPT was added to a final concentration of 10 mM to dilute out the unincorporated radioactivity (which was ear-

lier found to stick to the RNA unspecifically) and the RNA was precipitated by the addition of 3 ml of ice-cold 5% trichloroacetic acid containing 1% sodium pyrophosphate. The suspension was allowed to stand for 30 min to 1 h and the precipitate was washed (by centrifugation) four times with cold 5% trichloroacetic acid containing pyrophosphate, and once with enthanol. The final pellet was taken in 100 µl of 0.3 N NaOH and incubated at 37°C for 14-16 h, and was subsequently neutralized with perchloric acid. The suspension was counted in 10 ml of aqueous scintillation mixture (250 ml Triton X-100, 500 ml toluene and 3 g 2-diphenyloxazole).

DNA measurements

The nuclei were processed according to Mennes *et al.* (1977) and DNA was estimated by the diphenylamine method as suggested by Giles and Myers (1965).

Results

The kinetics of total nuclear RNA polymerase activity were measured in the nuclei from undifferentiated callus and callus induced to undergo differentiation for 24 h. The rate as well as the extent of RNA synthesis in the nuclei from the differentiating tissue was higher than that from the control callus (figure 1). This could result from more number of RNA polymerase molecules initiating transcription *in vivo,* either due to an initiating-specificity alteration or to an increased number of enzyme molecules.

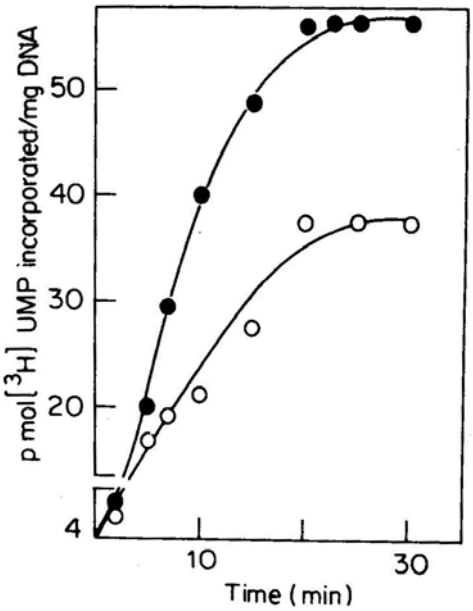

Figure 1. Kinetics of nuclear RNA transportation.(O), Callus;(●), diferentiating tissue.

In callus nuclei, 38 µmol of [^{3}H]-UMP residues were incorporated per mg nuclear DNA, which increased to 56 µmol per mg DNA in the nuclei of differentiating tissue. However, various factors such as the source of plant material, growth condi-

tions, composition of the isolation media, method of homogenization etc, contribute to the RNA synthetic capacity of the nuclei (Mennes *et al.*, 1977).

The divalent cation (Mg^{2+}) concentration for total nuclear RNA polymerase activity was optimal at 5 mM, after which there was a decline in the activity (figure 2). It was observed that maximum activity was obtained at a concentration of about 5×10^6 nuclei in a reaction volume of 200 µl.

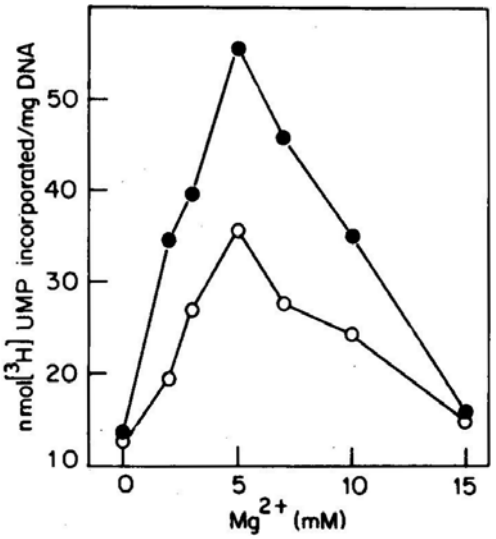

Figure 2. Effect of Mg^{2+} concentration on total nuclear RNA polymerase activity. (O), Callus; (●), differentiating tissue.

The characteristics of the nuclear transcription are given in table 1. In the absence of any one of the nucleotide triphosphates, the activity was approximately 30% and marginal in the absence of Mg^{2+}. The differences in the residual activity of nuclear polymerase(s) from callus and differentiating tissues observed in the absence of one of the unlabelled nucleotide triphosphates could be due to the differences in the levels of endogenous nucleotide pool. In the presence of RNase, more than 90% of the labelled product was destroyed.

Table 1. Characteristics of RNA polymerase activity.

	Total RNA polymerase activity*	
System	Callus tissue	Differentiating tissue
Complete	94.5	409.2
−ATP	34.0	114.0
−GTP	31.0	114.0
−CTP	31.0	113.0
+RNase	4.7	31.0
$-Mg^{2+}$	10.9	12.0

* *P* mol [^{3}H] -UMP incorporated/mg DNA.

The callus (24 h) nuclei were isolated in the presence of 1 mg/L 2,4-D and those from differentiating tissue (24 h) in the presence of 1 mg/L NAA and 2 mg/L BAP.

The effect of ionic strength on nuclear RNA polymerase activity was tested (figure 3) . The activity was maximum at 50 mM $(NH_4)_2$ SO_4 and this activity is assumed to be largely contributed by RNA polymerase I and to a very less extent by the other polymerases (Duda, 1976). At higher concentrations of $(NH_4)_2$ SO_4 (150-200 mM), RNA polymerase II is maximally active, whereas RNA polymerase III and I could contribute only to a very low percentage of the activity observed (Duda, 1976).

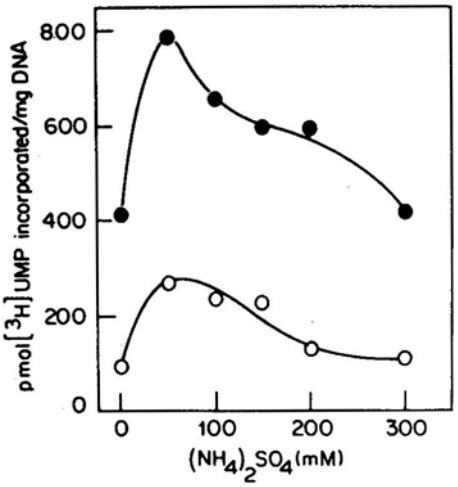

Figure 3. Effect of ionic strength on nuclear RNA polymerase activity. The nuclei were isolated in the presence of the hormones. (O), Callus; (●), differentiating tissue.

Table 2. Effect of α-amanitin on nuclear RNA polymerase activity.

Tissue	Concentration of α-amanitin	RNA polymerase activity[a]
Callus	0	225.0
	0.1	176.0
	1.0	168.0
	2.0	156.0
	5.0	140.0
	10.0	135.0
Differentiating	0	590.7
	0.1	579.0
	1.0	360.0
	2.0	343.8
	5.0	315.3
	10.0	265.0

[a] *p* mol of [^{3}H]-UMP incorporated/mg DNA.

The inhibition by α-amanitin at various concentrations was tested. The nuclei were isolated in the presence of the hormones. Assays were done in the presence of 150 mM ammonium sulphate, at various concentrations of α-amanitin. The assay mixture with ammonium sulphate and α-amanitin was preincubated for 10 min after which [^{3}H]-UTI was added. The per cent inhibition at the highest concentration of α-amanitin (10 mM) was 40% and 55% in callus and differentiating tissue respectively..

The effect of α-amanitin, a potent inhibitor of eukaryotic transcription, on total nuclear RNA polymerase was studied. Because α-amanitin inhibits RNA polymerase II activity, its effect was tested in the presence of 150 mM $(NH_4)_2$ SO_4. The results showed that 55% of the total activity was inhibited by 10 µg/ml α-amanitin in the case of nuclei from differentiating tissue, as against 40% inhibition of nuclei from callus tissue (table 2).

RNA polymerases I and II in the nuclei were quantitated by means of their differential sensitivity to α-amanitin, as well as their altered ionic strength requirements (Widnell and Tata, 1966; Hentschel and Tata, 1977). RNA polymerase II is measured by subtracting the residual activity in the presence of α-amanitin at 150 mM $(NH_4)_2$ SO_4 from total activity obtained at 150 mM $(NH_4)_2$ SO_4 (without α-amanitin). The total activity in the presence of 50 mM $(NH_4)_2$ SO_4 (without α-amanitin) represents RNA polymerase I activity. It was observed that the differentiating tissue contained elevated levels of both the polymerases and that the enhancement in RNA polymerase II was higher (4 times) than that of RNA polymerase I (3 times) activity (table 3).

Table 3. RNA polymerases I and II in the nuclei of callus and differentiating cultures.

Tissue	RNA polymerase I	RNA polymerase II
	p-mol [^{3}H]-UMP incorporated/mg DNA	
Callus	267.9	84
Differentiating	793.8	322

The amounts of RNA polymerases I and II present in the nudei were quantitated by virtue of their differential sensitivity to α-amanitin (as given in the text) and by the effect of $(NH_4)_2$ SO_4 requirements. The nuclei were isolated in the presence of the hormones.

Discussion

The present studies on RNA synthesis in *N. sanderae* cultures show that differentiation of callus into shoot is preceded by an increased RNA polymerase activity and this enhancement is reflected in the activities of RNA polymerase I as well as II. It has long been known that analogous systems such as hormone-treated seedlings, and plant organs elicit increased RNA content and *in vitro* RNA polymerase activity as compared to their control counterparts (Key, 1969; Chen *et al.,* 1975). Studies using carrot suspension cultures (Sengupta and Raghavan, 1980; Fujimura *et al.,* 1980) showed that the content as well as the rate of synthesis of RNA increases during somatic embryogeny. Similarly, *N. sanderae* cultures show raised levels of RNA synthesis at very early stages of differentiation (24 h). This could be due to the increased levels of nuclear RNA polymerases observed at 24 h after induction of differentiation.

Sekiya and Yamada (1978) observed that when tobacco cells cultured *in vitro,* were induced to differentiate into shoots, there was an initial decrease followed by an enhancement in the soluble RNA polymerase activity. However, a 3-4 fold enhancement in the activities of nuclear RNA polymerases I and II was elicited by the differentiating callus of *N. sanderae* during early stages. Although evidence indicates that there is an overall increase in the synthesis of all species of RNA as a

response to hormone-induced developmental changes, reports from several laboratories indicate that RNA polymerase I is mainly involved (Guilfoyle and Hanson, 1973; Chen *et al.*, 1975; Rizzo *et al.*, 1977). However Guilfoyle *et al.* (1980) have recently observed a 10-fold and a 6-fold augmentation in RNA polymerases. I and II respectively as a consequence of treatment with 2,4-D on soybean tissue. Similarly, Burkhanova *et al.* (1980) have also reported that BAP could bring about an increase in RNA polymerases I and II activities in the nuclei from barley protoplasts by a factor of 2.6 to 2.8 and 2.2 to 2.5 respectively. The present study shows that the nuclei from tissues that were induced to differentiate by treatment with NAA and BAP, contain 3-fold and 4-fold high levels of RNA polymerases I and II respectively. This could be due to increased enzyme synthesis, activation of pre-existing RNA polymerases, changes in the compartmentation of free and chromatin-bound enzyme, increased template availability, acting either alone or in combination. In an attempt to distinguish between these possibilities, we have demonstrated that there is a shift in the ratio of chromatin bound/free enzyme molecules occurring during differentiation (to be published elsewhere). However, to understand the modulation of the RNA polymerases, their fractionation and subsequent analysis of the transcription products are required.

Acknowledgements

Financial assistance from Department of Atomic Energy, Trombay, and Council for Scientific and Industrial Research, New Delhi, are gratefully acknowledged. The authors thank Dr. G. Lakshmi Sita for her help.

References

Becker, W. M. (1979) in *Nucleic Acids in Plants,* eds T. C. Hall and J. W. Davies (Florida: CRC press, Inc.) Vol. 1, p. 111.

Burkhanova, E. A., Fedina, A. B., Dmitrieva, G. N. and Kulaeva, O. N. (1980) *Dokl. Akad. Nauk* (*USSR*), **252,** 488.

Chen, Y., Lin, C., Chang, H., Guilfoyle, T. J. and Key, J. L. (1975) *Plant Physiol.,* **56,** 78.

Davies, E. and Larkins, B. A. (1973) *Plant Physiol.,* **52,** 339.

Duda, C. T. (1976) *Ann. Rev. Plant Physiol.,* **27,** 119.

Fujimura, T., Komamine, A. and Matsumoto, H. (1980) *Physiol. Plant.,* **49,** 255.

Giles, K. W. and Myers, A. (1965) *Nature* (*London*), **206,** 93.

Guilfoyle, T. J. and Hanson, J. B. (1973) *Plant Physiol.,* **53,** 110.

Guilfoyle, T.J. and Key, J. L. (1977) *Biochem. Biophys. Res. Commun.,* **74,** 308.

Guilfoyle, T. J., Olszeski, N. and Zurfluh, L. (1980) in *Genome Organization and Expression in Plants,* ed. C. J. Leaver (New York: Plenum Press) p. 93.

Hentschel, C. C. and Tata, J. R. (1977) *Dev. Biol.,* **57,** 293.

Key, J. L. (1969) *Ann. Rev. Plant Physiol.,* **20,** 449.

Mennes, A. M., Voogt, E. and Libbenga, K. R. (1977) *Plant Sci. Lett.,* **8,** 171.

Murashige, T. and Skoog, F. (1962) *Physiol. Plant,* **15,** 474.

Rizzo, P. J., Pedersen, K., and Cherry, J. H. (1977) *Plant Sci. Lett.,* **8,** 205.

Sekiya, J. and Yamada, Y. (1978) *Agric. Biol. Chem.,* **42,** 485.

Sengupta, C. and Raghaven, V. (1980) *Exp. Bot.,* **31,** 347.

Widnell, C. C. and Tata, J. R. (1966) *Biochem. Biophys. Acta,* **123,** 478.

Yu, F. L. (1975) *Biochem. Biophys. Res. Commun.,* **64,** 1107.