

EARLY EVENTS IN LYMPHOCYTE TRANSFORMATION BY PHYTOHEMAGGLUTININ

I. DNA-Dependent RNA Polymerase Activities in Isolated Lymphocyte Nuclei

BEATRIZ G. T. POGO

From the Department of Cytobiology, The Public Health Research Institute of the City of New York, Inc., New York 10016

ABSTRACT

The DNA-dependent RNA polymerase activities of isolated nuclei from lymphocytes were examined after stimulation with phytohemagglutinin (PHA). The nuclear fraction was prepared with Mg^{++} or Mn^{++} to distinguish between polymerase I (nucleolar) and polymerase II (nucleoplasmic). Distinction between polymerases II and III was obtained by the addition of α -amanitin to the reaction mixture. The results indicated that within 15 min after exposure to PHA the activity of polymerase I increased. Polymerase II activity increased after 1 hr. The enhancement was linear for 6 hr and then leveled off for the subsequent 48 hr. Small increase in polymerase III activity was observed at 48 hr. Inhibition of protein synthesis at the time of exposure to PHA did not prevent the increase in activities during the initial 6 hr. These results imply that the initial increase in enzymatic activities is dependent upon preexisting polymerase molecules and/or factors.

INTRODUCTION

Lymphocyte transformation by phytohemagglutinin (PHA) and other mitogenic agents has been widely studied as a model system in which a resting cell is stimulated to enlarge and divide. The addition of PHA to lymphocyte cultures generates several biochemical changes in the cell. RNA and protein synthesis (1-3), histone acetylation (2), phosphorylation of nuclear proteins (4), the incorporation of phosphate into phosphatidylinositol (5) of glycerol, glucose, and choline into cell lipids (6), and the levels of cyclic adenosine monophosphate (AMP) (7) are stimulated within the first hour after addition of PHA. A concomitant increase in the cell permeability to RNA, protein, and lipid precursors (8, 9), as well as in K^+ trans-

port (10), has been reported, suggesting that some alteration in the cell membrane precedes lymphocyte transformation

Although an increase in RNA polymerase activity in lymphocytes treated with PHA for 18 hr has been demonstrated (11), no information is available either about earlier times after treatment or about the effects on each of the individual polymerases found in eukaryotic cells (12). Taking advantage of the fact that it is now possible to select conditions for assaying the different DNA-dependent RNA polymerase activities in isolated nuclei (13, 14), these activities were studied in nuclei isolated from human peripheral blood lymphocytes at different intervals after

PHA treatment. It was found that soon after PHA stimulation there was an elevation in the RNA polymerase I activity (12, 13), followed by an increase in polymerase II.

MATERIALS AND METHODS

Human peripheral lymphocytes from normal donors were used throughout all experiments. Detailed descriptions of the nutrient media, preparation of lymphocyte cultures, and experimental methods have been published (2, 8).

Isolation of the Nuclear Fractions

Approximately 1×10^7 cells were washed with phosphate-buffered saline (PBS) and then resuspended in 1 ml of 0.01 M Tris-HCl buffer (Schwarz BioResearch Inc., Orangeburg, N.Y./Mann Research Labs Inc., New York) pH 7.8¹ with 1 mM MgCl₂ or 1 mM MnCl₂ and 10 mM KCl (Tris-saline). Swelling of cells was allowed to proceed for 10 min at 4°C. Triton X-100 was added at a final concentration of 0.5% and the cells were disrupted by 10 strokes of a tight-fitting pestle of a Dounce homogenizer, and then the homogenate was centrifuged at 800g for 3 min. The pellet containing the crude nuclear fraction was resuspended in 1 ml of Tris-saline with 0.5% Triton X-100 and 0.1% sodium desoxycholate (DOC). The nuclear fraction was centrifuged at 800g for 3 min and washed with the Tris-saline containing 0.5% Triton X-100 and 0.1% DOC until it appeared to be free of cytoplasmic debris when examined under the phase-contrast microscope. The final nuclear pellet was resuspended in the medium to be used for the RNA polymerase assay.

RNA Polymerase Assay

The nuclear RNA polymerases were assayed by the incorporation of labeled nucleotides into RNA. Portions of the nuclear fraction containing 20–30 µg of DNA were added to the assay mixture containing different ingredients appropriate to the particular activity being determined. The nuclear fraction prepared in Mg⁺⁺ was used to assay the RNA polymerase I activity (16) with an incubation mixture containing 0.3 M sucrose in 0.01 M Tris-HCl buffer pH 8.0, 4 mM MgCl₂, 0.06 M NaCl, 30 mM 2-mercaptoethanol in a final volume of 0.25 ml. To assay RNA polymerases II and III, the nuclear fractions were prepared in Mn⁺⁺ and the incubation mixture contained 0.3 M sucrose in 0.01 M Tris-HCl buffer pH 8.0, 1.8 mM MnCl₂, 0.06 M NaCl or 0.03 M (NH₄)₂SO₄, 30 mM 2-mercaptoethanol in the same volume. α-Amanitin was added in concentrations ranging from 1 to 20 µg. In all the assays, 0.1 µmole of each unlabeled nucleotide

¹ All pHs were determined at 23°C.

and 0.03 µmoles of ³H-labeled nucleotide adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), or uridine triphosphate (UTP) (SA 20 µCi/µmole) were added. The reaction was allowed to proceed for 10 min at 37°C and then was stopped by adding 10 µmoles of the unlabeled nucleotide and 5 ml of 10% cold trichloroacetic acid (TCA) containing 0.05 M sodium pyrophosphate. The precipitates were collected in Millipore filters (Millipore Corp., Bedford, Mass.), washed twice with 15 ml of 10% TCA, and the radioactivity was determined as described elsewhere (15). In the experiments in which (NH₄)₂SO₄ was added to the incubation mixture at low (0.03 M) and high (0.4 M) ionic strength, both Mg⁺⁺ and Mn⁺⁺ were present at the concentrations indicated above.

To determine the relative incorporation of each nucleotide into RNA, synthesis was allowed to occur using each labeled nucleotide independently under assay conditions already described. Each assay was carried out in triplicate and the entire experiment was repeated on three different lymphocyte preparations, i.e., nine determinations in all.

Nuclear DNA was determined by Burton's procedure (17).

Nucleotides were purchased from Schwarz Bio Research Inc./Mann Research Labs. Inc.; α-amanitin from C. H. Boehringer Sohn, Germany; puromycin-HCl from Nutritional Biochemicals Corporation, Cleveland, Ohio; and Phytohemagglutinin "P" from Difco Laboratories, Inc., Detroit, Mich.

RESULTS

DNA-Dependent RNA Polymerase Activity in Lymphocyte Nuclei at Different Times after Incubation with Phytohemagglutinin

The requirements for the DNA-dependent RNA polymerase activities in isolated lymphocytes nuclei were found to be similar to those described for rat liver nuclei (13, 16). The reactions required either Mg⁺⁺ or Mn⁺⁺, the presence of the four nucleotides, 2-mercaptoethanol, and addition of Na or NH₄ salts at low ionic strength. The reactions were inhibited by actinomycin D, RNase, and DNase. They were linear for 15 min and then reached a plateau.

Nuclear samples were prepared from lymphocytes after exposure to PHA and assayed for RNA polymerase activity. Addition of 10 µg of α-amanitin to the assay mixture of nuclei prepared with either Mg⁺⁺ or Mn⁺⁺ allows one to differentiate somewhat between the three enzymatic activities designated RNA polymerases I, II, and III (12). In the presence of Mg⁺⁺ and

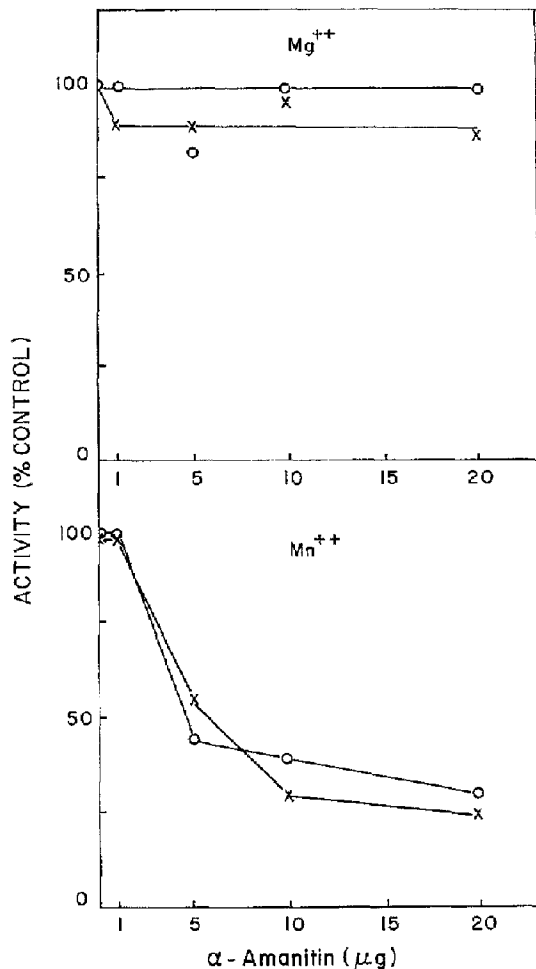


FIGURE 1 Effect of different concentrations of α -amanitin on the RNA polymerase activities of isolated lymphocyte nuclei, isolated in the presence of either Mg^{++} or Mn^{++} . In both instances 0.06 M NaCl was present in the assay mixture. \times — \times , experiment 1; \circ — \circ , experiment 2.

Na, α -amanitin inhibited only 10–15% of the reaction even at very high concentrations (Fig. 1), whereas in the presence of Mn^{++} (with either NaCl or $[NH_4]_2SO_4$ in the incubation mixture) α -amanitin inhibited 70–80% of the activity.² Therefore, the difference between the α -amanitin-resistant and inhibited reactions enables one to estimate the activity of polymerase II (14). The ratio of incorporation of G/U was constant: 0.5

² α -Amanitin added to the tissue culture medium inhibits lymphocyte transformation by PHA (B. G. T. Pogo, unpublished experiments).

in the presence of Mn^{++} and 1.0 in the presence of Mg^{++} .

The Mg^{++} -dependent activity (polymerase I) was quickly increased after addition of PHA, as shown in Fig. 2. Within 15 min of treatment there was a linear increase in the activity that leveled off at about 6–12 hr and then remained constant for the subsequent 48 hr. RNA polymerase II activity showed a lag period of 1 hr and then increased as a function of time in a similar manner to that of polymerase I. On the other hand, no stimulation could be determined in polymerase

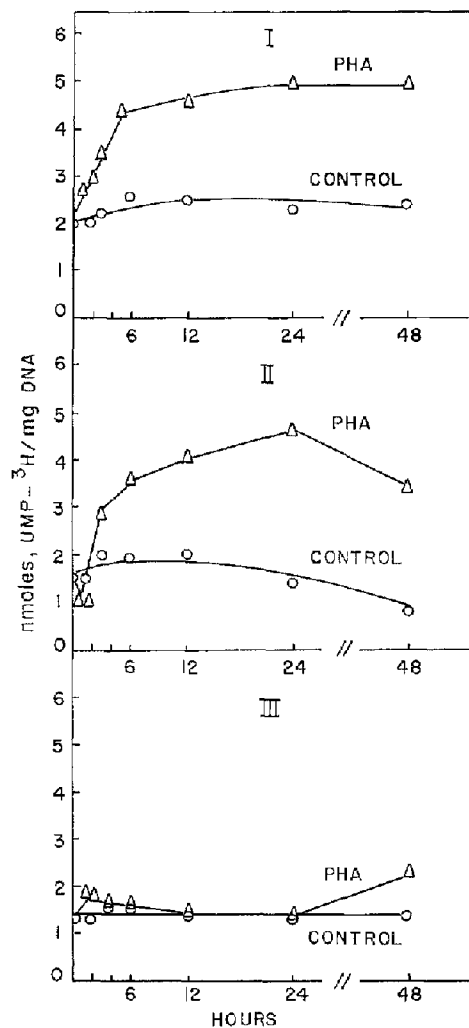


FIGURE 2 RNA polymerase activities of nuclei isolated from lymphocytes exposed for varying periods to PHA. \circ — \circ , control cells; Δ — Δ , PHA-treated cells. UMP, uridine monophosphate.

III activity for the first 48 hr. After that time, however, some increase could be demonstrated.

The Base Composition of the RNA Synthesized at Different Conditions of Incubation

The product synthesized by the nuclear RNA polymerases under various conditions of incubation was studied by measuring the rate of incorporation of the four respective nucleotides. The results of different experiments on the base composition of the products are summarized in Table I.

In the presence of Mg^{++} , both control and PHA-stimulated nuclei synthesized mostly GC-rich RNA, suggesting that the ribosomal genes were being transcribed. In the Mn^{++} mixture, A and U were preferentially incorporated into the RNA. When α -amanitin was added to the assay mixture, the base composition in the Mn^{++} -incubated nuclei shifted towards an RNA product enriched in adenylic acid in both controls and PHA-treated cells. The possibility that a homopolymerization reaction was taking place in the isolated nuclei in the presence of α -amanitin was investigated by following incorporation of each nucleotide in the absence of the other three in the reaction mixture. Under these conditions, only incorporation of adenylic acid was observed, but, it was reduced by 50%. This result suggested that a polymer of adenylic acid was synthesized. The relationship between poly A synthesis and the nature of the RNA being transcribed as a whole is being investigated further.

Effect of Puromycin on Polymerase Activities

The increase in RNA polymerase I and II activities could be due to *de novo* synthesis of polymerase molecules (or factors), or could occur because preexisting enzyme molecules become functional. To test the first possibility, puromycin was added at the time of PHA addition, and the RNA polymerase activities were measured. In a parallel experiment, puromycin at 20 μ g/ml inhibited 90% of the amino acid incorporation within 1 hr (2). As can be seen in Fig 3, the increase in RNA polymerase activities was not blocked by puromycin until 6 hr had elapsed. Therefore, the initial increase in the activities was probably due to enzyme molecules that were already present in the cell.

Further evidence for that interpretation was obtained by experiments in which high ionic strength $(NH_4)_2SO_4$ concentration was used. In this condition, more template should become available for the enzymes to copy (18). It can be seen that the nuclei from control cells were highly stimulated by 0.4 M $(NH_4)_2SO_4$, whereas the PHA-treated nuclei were stimulated less. This would imply that more template was available as a result of PHA treatment (Table II).

Addition of calf thymus DNA to the incubation mixture slightly increased the incorporation of nucleotides 6 hr after induction with PHA. This result would suggest that at this time more polymerase molecules were free and available for binding to the template. As previously shown (see

TABLE I
Relative Nucleotide Incorporation by Isolated Lymphocyte Nuclei

Hours after the addition of PHA	Nucleotide incorporated	Control cells				PHA-treated cells			
		Mg^{++}		Mn^{++}		Mg^{++}		Mn^{++}	
		—	α -Amanitin	—	α -Amanitin	—	α -Amanitin	—	α -Amanitin
6	AMP	0.25	0.32	0.27	0.40	0.23	0.30	0.37	0.33
	CMP	0.29	0.21	0.22	0.24	0.27	0.28	0.20	0.26
	GMP	0.27	0.27	0.20	0.16	0.35	0.22	0.17	0.14
	UMP	0.19	0.20	0.31	0.21	0.15	0.20	0.25	0.27
24	AMP	0.25	0.32	0.29	0.49	0.22	0.25	0.28	0.56
	CMP	0.25	0.20	0.23	0.21	0.26	0.17	0.23	0.11
	GMP	0.30	0.36	0.24	0.10	0.26	0.33	0.23	0.10
	UMP	0.20	0.12	0.24	0.21	0.24	0.25	0.26	0.23

Nucleotide incorporation (relative values) by isolated lymphocyte nuclei.

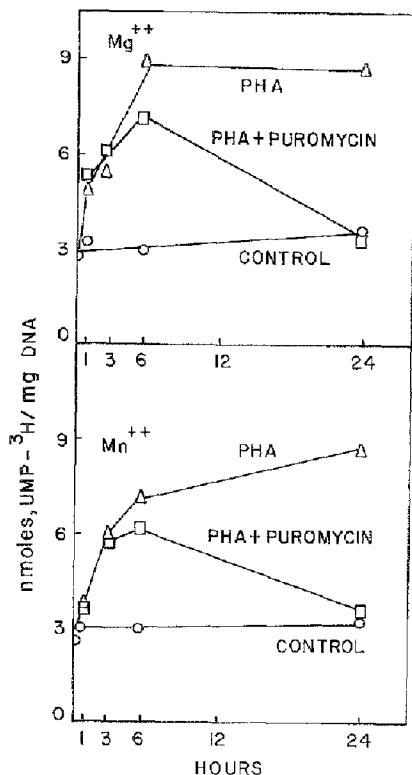


FIGURE 3 Effect of puromycin on the RNA polymerase activities of isolated lymphocyte nuclei. The cells were incubated with PHA and 20 $\mu\text{g}/\text{ml}$ of puromycin for different periods. Δ — Δ , cells incubated with PHA; \square — \square , with PHA and puromycin; and \circ — \circ , control cells.

Fig 3), at this critical period protein synthesis was required to maintain the increase in RNA synthesis (Table III)

DISCUSSION

The results reported in this paper indicate that increase in the activity of the DNA-dependent RNA polymerases is one of the earliest responses of the cell to the mitogenic agent.

The Mg^{++} -dependent RNA polymerase activity was stimulated immediately after the cells were exposed to PHA. On the other hand, the Mn^{++} -dependent RNA polymerase activity was stimulated after a lag period of 1 hr. The third RNA polymerase activity, which is Mn^{++} -dependent but not inhibited by α -amanitin, remained unstimulated by the mitogenic agent.

Although protein synthesis was not required for the initial increase, it was necessary for the maintenance of the stimulated RNA polymerase activities. These results indicate that the number of enzymatic molecules (or factors) necessary for the initial stimulation in RNA synthesis pre-existed within the nonactivated lymphocytes.

Three mechanisms by which PHA may increase the RNA polymerase activities can be postulated: (a) the amount of RNA polymerase is controlled by affecting the synthesis of the enzymes or some of their subunits, (b) the availability of the DNA template is altered, and (c) the function of some factor(s) necessary for the binding of the enzyme to the template is affected.

The first possibility assumes that the amount of

TABLE II
Effect of Different $(\text{NH}_4)_2\text{SO}_4$ Concentration on RNA Polymerase Activity
(nmoles ^3H -UMP/mg DNA per 10 min)

Hours after the addition of PHA	Experiment number	Control			PHA		
		0.40 M	0.03 M	0.03	0.40 M	0.03 M	0.03
2	1	3.3	1.6	2.1	3.0	2.3	1.3
	2	1.8	1.4	1.3	2.5	1.8	1.4
6	1	2.7	1.4	1.9	2.9	2.6	1.1
	2	8.7	2.0	4.4	8.4	6.6	1.3
24	1	2.3	1.5	1.8	5.0	6.0	0.8
	2	4.0	1.3	3.1	8.8	8.0	1.1

Effect of varying $(\text{NH}_4)_2\text{SO}_4$ concentrations on the RNA polymerase reaction of control and PHA-stimulated lymphocyte nuclei.

TABLE III
RNA Polymerase Activity
(nmoles ³H-UMP/mg DNA per 10 min)

Hours after the addition of PHA		Control cells		PHA-treated cells	
		Mg ⁺⁺	Mn ⁺⁺	Mg ⁺⁺	Mn ⁺⁺
2	—	3.8	5.0	5.4	5.6
	+ DNA	3.9	4.0	5.4	6.6
6	—	3.6	5.5	7.0	7.7
	+ DNA	3.8	4.4	8.5	7.9
24	—	3.8	5.0	10.8	10.2
	+ DNA	7.9	6.4	11.2	12.6

Effect of adding exogenous template on RNA polymerase activities of control and PHA-stimulated lymphocyte nuclei. 20 μg of native calf thymus DNA was added to the assay mixture described in Materials and Methods.

RNA polymerase is the limiting factor within the cell. However, this could be ruled out because inhibitors of protein synthesis failed to affect the initial stimulation of the enzymes.

The possibility that more sites in the DNA template are available is suggested by the experiments using high ionic strength in the enzyme assay. Contradictory interpretations can be drawn from these results since it was shown that the isolated Mg⁺⁺-dependent RNA polymerase was inhibited while the isolated Mn⁺⁺-dependent RNA polymerase was stimulated by high concentrations of salts (12). Nevertheless, Hirschhorn et al. (19) and B. G. T. Pogo (unpublished results) have observed increases in template activity after addition of exogenous DNA-dependent RNA polymerase to nuclei isolated from PHA-treated lymphocytes.

Before the third possibility, concerning the function of some factor(s) required for the binding of the enzyme to the template, can be considered, the study of isolated enzymes from normal and stimulated lymphocytes is necessary.

The results reported here indicate that RNA polymerase I was the earliest synthetic activity to be stimulated after lymphocytes were exposed to PHA. Activation of RNA polymerase I has been also demonstrated after cells were stimulated by hormones (20–23), by surgical removal of a segment of the organ (24, 25), or by a medium enriched in amino acids (26).

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