Volume 5 Number 6 June 1978 Nucleic Acids Research

The relationship between the activities of different pools of RNA polymerases I and II during PHA-stimulation of human lymphocytes

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Received 3 March 1978

ABSTRACT

Following PHA-stimulation of lymphocytes in culture, it is known that nuclear RNA synthesis and the amount of extractable RNA polymerase activity rise in these cells. The relationship between these two phenomena has been examined. Using an in vitro assay system which discriminates between polymerase activity which is "engaged" in nuclear RNA synthesis and a pool of "free" enzyme, the data suggest that the factors regulating the interaction between these two pools of enzyme activity are different for forms I and II RNA polymerases.

INTRODUCTION

The investigation of the regulation of RNA transcription in eucaryotic cells requires an experimental system in which transcriptional rates show a change induced by an agent in vitro which, as nearly as possible, reproduces a similar in vivo stimulus. One such system is the transformation of resting lymphocytes by Phytohaemagglutinin (PHA) which is assumed to resemble the events following antigen binding in vivo. The increase in the rate of RNA synthesis (particularly rRNA and tRNA) which occur during this process has been studied extensively [1]. Although the mechanism of RNA synthetic activation is still obscure, it has been shown that (a) there is an increased RNA polymerase activity in nuclei isolated from PHA-stimulated lymphocytes [2 - 4] and (b) stimulation results in a progressive increase in the level of RNA polymerases I (rRNA), II (putative mRNA) and III (5S and tRNA) which can be extracted from these cells [5]. These findings prompt the question as to whether changes in nuclear RNA polymerase activity are in any way directly related to the apparent 'levels' of RNA polymerase enzyme.

In approaching this problem, account must now be taken of two functional states of RNA polymerases in most (if not all) eucaryotic cells [6,7]: firstly, that which is bound to the chromosome and is involved in RNA synthesis ("engaged" enzyme which can be inhibited in vitro by Actinomycin D) and, secondly, a pool of activity (normally referred to as "free" polymerase) which is not associated with the chromosome and can be identified by its ability to transcribe poly [d(A-T)] in the presence of high concentrations of Actinomycin D [8]. It would be anticipated that the rates of cellular RNA synthesis would be closely related to the concentration of "engaged enzyme activity transcribing the genome rather that to the total concentration of cellular polymerases" [9]. However, contemporary literature with respect to lymphocyte transformation fails to draw a distinction between the different polymerase pools, casting doubt on the interpretability of data which suggests that RNA transcription rates are somehow regulated by gross cellular enzyme 'levels'.

In a reappraisal of the events following PHA-stimulation of resting human lymphocytes, we have examined changes in (a) the endogenous nuclear polymerase activity and (b) the "free" enzyme pool of RNA polymerases I and II and the data indicate that these two phenomena may not always be related directly.

METHODS AND MATERIALS

Lymphocyte Preparation. Blood, taken by venesection into Fenwal Blood packs from healthy, adult human blood donors was subjected to low speed centrifugation and the upper part of the red cells, buffy coat and serum was removed and pooled in batches from 4 - 5 donors; the serum was then removed after a further low speed centrifugation and the resulting "buffy coat residue" stored at room temperature. The buffy coat residues, taken on the same day, were purified in two steps in a method similar to that of Kay et al. [10], omitting the Dextran sedimentation step. Briefly, residues were passed through a large (2.5 x 15 cm) column of absorbent cotton wool at a slow rate to remove polymorphs, macrophages and monocytes and the resulting eluate was layered in batches of 20 - 25 ml over an equal volume of Ficoll-Hypaque (6.35 g Ficoll and 10 g Hypaque per 100 ml, specific gravity 1.078 g/ml) in 50 ml glass centrifuge tubes. These were cent-

rifuged at 360 x g for 25 min in an M.S.E. Super Minor Bench Centrifuge. The cells at the gradient interface were carefully removed, washed once in Phosphate Buffered Saline (PBS, pH 7.4: 0.20 g $NaH_2PO_4H_2O$, 1.92 g Na_2HPO_4, 7.8 g NaCl and 0.4 g KCl dissolved in 1 litre of deionised water) by resuspension and spinning at 360 x g for 10 min and washed again in complete culture medium (see below). The cells were resuspended in complete culture medium and counted in a haemocytometer by dilution in 2% (v/v) glacial acetic acid. They consisted of 95 - 98% small lymphocytes. All operations were carried out at room temperature under sterile conditions.

Lymphocyte Culture. Purified lymphocytes were resuspended in Alpha-MEM, supplemented with 10% (v/v) Foetal Bovine Serum, 100 units/ml Benzylpenicillin and 100 µg/ml Streptomycin Sulphate to a concentration of 1 - 2 x 10^6 cells/ml, distributed in 100 ml aliquots into 75 cm^2 glass culture flasks and incubated at 37°C in a humidified 5% CO_2/air mixture. After overnight incubation, PHA was added to each flask to a final concentration of 2 µg/ml to stimulate the cells. Cells were harvested by combining flasks, spinning at room temperature for 5 min at 360 x g and washing the pelleted cells twice in ice cold PBS. The final cell pellet was counted and was used immediately for isolation of nuclear and 'cytoplasmic' fractions.

Isolation of Nuclear and 'Cytoplasmic' Fractions. Nuclei were prepared by a modiciation of the Hypotonic Method of Woolf et al. [11]. Cells (4 - 5 x 10^8) were resuspended in 3 ml Hypotonic Buffer (containing 0.4 mM potassium phosphate, pH 6.7, 2 mM $MgCl_2$ and 1 mg/ml Bovine Serum Albumin [BSA]) and left for 15 min to swell. Cells were then disrupted by 20 gentle passes in a tight-fitting, Teflon/glass hand homogeniser. Glycerol was then added to 25% (v/v) and mixed with the nuclear suspension by vortexing and was subjected to a further 5 passes in the homogeniser. The suspension was centrifuged at 1500 x g in a Sorvall RC2B Centrifuge using the SM-24 rotor for 10 min at 4°C, the supernatant (referred to as the 'cytoplasmic' fraction) was kept on ice for immediate assay for RNA polymerase activity and the nuclei resuspended in a buffer containing 10 mM Tris/HCl, pH 8.0, 1 mM $MgCl_2$, 1 mg/ml BSA and 25% (v/v) glycerol and counted.

Nuclei were pelleted again at 1500 x g for 10 min and the pellet resuspended in the same Tris/Glycerol buffer to a final concentration of 1 x 10^8 nuclei/ml (the 'nuclear' fraction). Recovery of nuclei was 50 - 60%. All operations were carried out at 4°C.

RNA Polymerase Assays. All assays contained in a final volume of 0.25 ml: 25 mM Tris/HCl, pH 8.0, 0.3 mM each of ATP, GTP and CTP, 0.05 mM [^{3}H]-UTP (325 cpm/pmole), 0.5 mM dithiothreitol, 1 mM $MnCl_2$ with the following additions:
(i) Nuclear polymerase assays: for nuclear RNA polymerase I, the final salt concentration was 25 mM $(NH_4)_2SO_4$ and 200 µg/ml α-amanitin; nuclear polymerase II was that activity sensitive to 1 µg/ml α-amanitin at 200 mM $(NH_4)_2SO_4$. Incubations contained 5 x 10^6 nuclei and were for 60 min at 37°C (see below).
(ii) 'Cytoplasmic' polymerase assays: 'cytoplasmic' polymerase I was assessed at 40 mM $(NH_4)_2SO_4$ in the presence of 200 µg/ml α-amanitin; 'cytoplasmic' polymerase II was that activity sensitive to 1 µg/ml α-amanitin at 130 mM $(NH_4)_2SO_4$. These are the optimal salt concentrations for in vitro RNA synthesis by these enzymes [12]. Calf thymus DNA was added to 40 µg/ml together with 50 µl of the 'cytoplasmic' fraction. Incubations were for 20 min at 37°C (see below).
(iii) Diagnosis of 'free' polymerase in the 'cytoplasmic' fraction: this was based on the system of Yu [6] and Kellas et al. [8]. Assays contained a final salt concentration of 40 mM $(NH_4)_2SO_4$, poly [d(A-T)] at 25 µg/ml and actinomycin D at 200 µg/ml where indicated; the incubations were otherwise as described for the 'cytoplasmic' polymerase above.

Activity was assessed by the incorporation of [^{3}H]-UMP into TCA-precipitable material, collected on Whatman GF/C glass fibre discs and processed according to Chesterton and Butterworth [13].

Thymidine and Uridine Pulses. [^{3}H]-thymidine (28 Ci/mmole) and [^{3}H]-uridine (16.3 Ci/mmole) were diluted to 20 µCi/ml and 8 µCi/ml respectively. Aliquots containing 2 µCi of each were added to 1 ml lymphocyte cultures containing 1 x 10^6 cells and were incubated as above for either 1 or 2 hours. The reaction was stopped by cooling the tubes to 0°C and adding 5 ml ice-cold 10% TCA, containing 3% $Na_4P_2O_7$ (both w/v). The cells were pel-

etted at 1500 x g for 10 min and washed twice in ice-cold 10% TCA-pyrophosphate before passing onto glass fibre discs and being processed in a similar manner as for RNA polymerase assays.

DNA assay. DNA was measured by the diphenylamine method of Giles and Myers [14].

Materials. Poly [d(A-T)] and α-amanitin were supplied by Boehringer, Mannheim, GmbH; all radioactive materials by the Radiochemical Centre, Amersham, U.K., Actinomycin D by Sigma, London; Alpha-MEM and Foetal Bovine Serum by Flow Laboratories, Irvine, Scotland; Ficoll by Pharmacia, Uppsala, Sweden and Hypaque by Winthrop Laboratories, Surbiton, U.K.; PHA-purified (HA 16-17) was purchased from Wellcome Reagents Ltd., Beckenham, U.K. and Benzyl-penicillin and Streptomycin sulphate from Glaxo, U.K.

RESULTS

Culture Characteristics. The cultures showed the normal characteristics for cells grown under these conditions [1, 15]. The decrease in cell numbers (Fig. 1A) which is routinely observed may arise initially from the trauma of lymphocyte fractionation and subsequently from essential nutrient depletion in cultures at this cell density either in the presence or absence of PHA [15]. Rates of uridine incorporation (Fig. 1B) increased rapidly over 24 hours, reaching a maximum at 48 hours. As lymphocytes were from mixed donors, cultures in the absence of PHA exhibited the characteristics of a mixed lymphocyte reaction on days 3 and 4. This means that the only meaningful time 'control' values are those indicated at the time of addition of the mitogen (referred to as time '0'). This has also been shown to apply to cultures from single donors, due to background stimulation by calf serum and other agents [15]. Thymidine incorporation showed a rapid rise which peaked on day 2 which was also reflected in a virtual doubling of the lymphocyte DNA content at this time (Fig. 1C). Most of the cells therefore were in S-phase by day 2. All parameters showed a decline after 48 hours in stimulated cultures. the sharp and rapid DNA synthetic response may be due to a combination of highly purified PHA preparations, culture at high cell densities (1 - 2 x 10^6 cells/ml) and background allogeneic

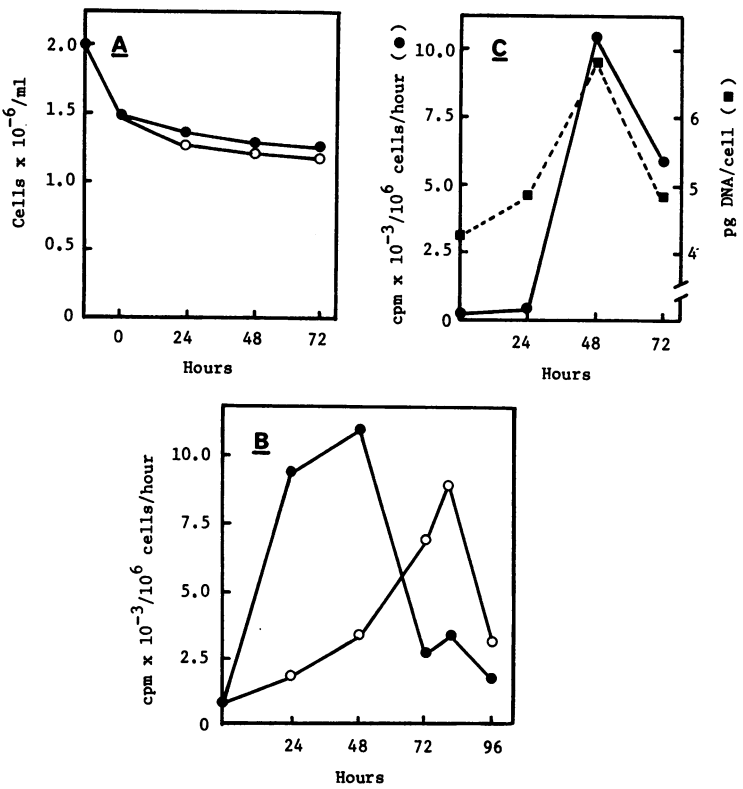

Fig. 1. Characteristics of the Lymphocyte Culture. Lymphocytes were purified from buffy coat residues and cultured as described in Methods. Zero-time refers to the time of PHA addition to the culture medium. A: Cell concentration during culture in the presence (●) and absence (○) of PHA; B: the uptake of [^{3}H]-uridine by cells cultured in the presence (●) and absence (○) of PHA; C: uptake of [^{3}H]-thymidine (●) and cellular DNA content (■) during the culture period in the presence of PHA.

stimulation of cells.

Characteristics of Nuclear and 'Cytoplasmic' Fraction RNA Polymerase Reactions. In this work, we have restricted our attention to forms I and II RNA polymerase activities as it is known that form III, though significant, is a minor component of this system [5]. The cell fractionation system which was used gave rise to a 'cytoplasmic' fraction which was totally devoid of DNA. It will be shown that both nuclear and 'cytoplasmic' fractions contain forms I and II RNA polymerases; the polymerase activity in the 'cytoplasm' is representative of a pool of 'free'

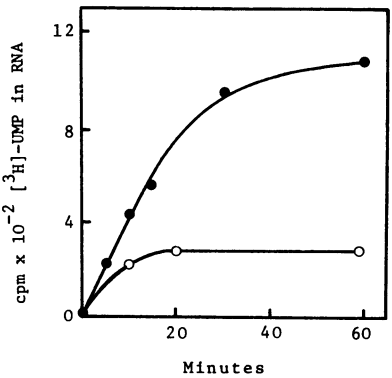

Fig. 2. Time Course of in vitro RNA Synthesis. The time course of in vitro RNA synthesis was monitored in the presence of 200 µg/ml α-amanitin (i.e. form I RNA polymerase only) using 5 x 10^6 nuclei per assay (o——o) or 50 µl 'cytoplasmic' fraction per assay (●——●) derived from lymphocytes cultured for 48 hours in the presence of PHA.

enzyme (i.e., not associated with the chromosome).

(i) Time course of in vitro RNA synthesis. The time course of [^{3}H]-UMP incorporation into RNA in isolated nuclei by the endogenous, chromosome-associated RNA polymerases at all stages of PHA-stimulation had charcteristics similar to those described by Cooke and Kay [3]. For either polymerase, the reaction was essentially complete after 20 min at 37°C with little or no subsequent decay in [^{3}H]-RNA up to 1 hour. On the basis of abundant evidence [see e.g. ref. 16], one can assume that reinitiation of RNA synthesis does not occur to a significant extent in the nuclear transcription system in vitro. The reaction catalysed by the 'cytoplasmic' RNA polymerase using exogenous (purified, nicked) calf thymus DNA as a template was always linear for at least 20 min at 37°C, reaching a plateau by about 60 min. Fig. 2 shows RNA synthesis by form I polymerase in the 2 cell fractions derived from 48 hour, PHA-stimulated lymphocytes, which is a typical example of the difference between these two time courses. Because the kinetics of the nuclear and 'cytoplasmic' RNA polymerase reactions are very different, it is not rational to draw direct comparisons between them. By adopting standard incubation conditions (60 min and 20 min for the nuclear and 'cytoplasmic' polymerases respectively, see Methods), changes in the apparent RNA synthetic potential of the separate cell fractions can be established following PHA-transformation.

Table 1. Actinomycin D Sensitivity of 'Cytoplasmic' RNA Polymerase Activity

Time after PHA stimulation	- Act. D	+ Act. D
Hours	pmole [^{3}H]-UMP / 10^6 cells [a]	
24	0.53	0.53
48	1.26	1.30
72	1.63	1.47

[a] 50 µl aliquots of 'cytoplasmic' fraction were assayed using saturating concentrations of poly [d(A-T)] in the absence or presence of 200 µg/ml Actinomycin D as described in Methods. Data is normalised to RNA polymerase activity derived from 10^6 cells. This assay, in the absence of α-amanitin, includes forms I, II and III RNA polymerases (see text).

(ii) All 'cytoplasmic' RNA polymerase is in a 'free' form. In the presence of poly [d(A-T)], the actinomycin sensitivity of RNA polymerase activity defines whether or not enzyme is 'free' or is associated with the chromosome (i.e. 'engaged') [6 - 8]. Analyses of 'cytoplasmic' fractions from lymphocytes at various stages in the PHA response using this template showed no significant sensitivity to actinomycin (Table 1). The salt concentration used for this assessment was 40 mM $(NH_4)_2SO_4$ which is optimal for poly [d(A-T)] transcription by forms I and III polymerase but is marginally suboptimal for polymerase II [17]. In subsequent experiments, forms I and II polymerase were assessed using discriminating concentrations of α-amanitin (see Methods).

'Free' RNA polymerase is not restricted to the 'cytoplasmic' fraction: endogenous (chromosome-associated) nuclear polymerase can be inhibited using high concentrations of actinomycin D [8] and poly [d(A-T)]-dependent activity is still evident (data not shown). The RNA synthetic potential of this activity fluctuates during the PHA response in a parallel manner to that in the 'cytoplamsic' fraction and, making reasonable assumptions concerning nuclear volume, it is apparent that 'free' enzyme is not conc-

entrated significantly in either the nuclear or the 'cytoplasmic' fractions which arise from our cell fractionation procedure (which involves hypotonic swelling of the cells in the first instance). However, this situation may not define the in vivo distribution of 'free' polymerase. For the interpretation of the data which follows, it is therefore assumed that the RNA synthetic potential of the 'cytoplasmic' fraction reflects that of the gross 'free' polymerase pool.

Effect of PHA-transformation on Nuclear and 'Cytoplasmic' Forms I and II RNA Polymerase Activities. The capacity for RNA synthesis by either polymerase I or II in nuclei in resting lymphocytes is low but significant (see Fig. 3). Similarly, the amount of 'cytoplasmic' ('free') polymerase activity which is assayable using either calf thymus DNA (Fig. 3) or poly [d(A-T)] (data not shown) is very low. The increase in nuclear form I activity by a factor of 5 and form II activity by about 1.5 over the first 24 hours of PHA stimulation is similar to the effects seen by others [3]. There is a corresponding increase in the RNA synthetic potential of the 'free' polymerase pools and this would account for the relative increase in the amounts of RNA polymerase which Cooke and Brown [2] and Jaehning et al. [5] solubilised from (the nuclei or whole cells of) 24-hour PHA-stimulated lymphocytes. Although synthesis of new enzyme molecules may be the favored interpretation of these findings, the possible activation of pre-existing (inactive) enzyme molecules cannot be excluded.

Subsequent events do not follow this simple relationship. Between 24 and 48 hours, the 'free' form I polymerase pool rises by a factor of 8 but there is no evidence of any further increase in the form I activity in nuclei; nuclear form II activity rises considerably but the 'free' polymerase II pool increases at an even greater rate. Therefore, during this period, there would appear to be no single definable relationship between nuclear function on the one hand and the potential RNA synthetic capacity of the 'free' polymerase pool on the other. The same is true for the events which occur between 48 and 72 hours under the culture conditions used in these experiments: the pool of 'free' polymerases I and II both decline; whereas nuclear form I activity is reduced, nuclear form II activity persists at a high level.

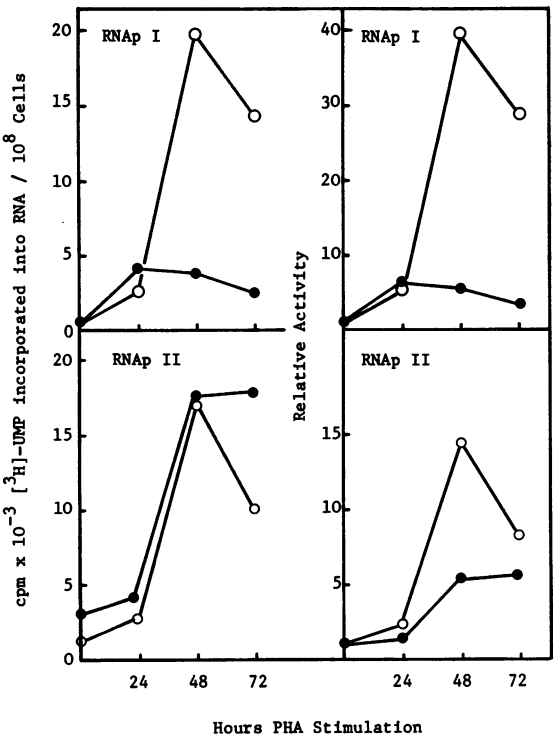

Fig. 3. Effect of PHA-stimulation on Nuclear ('engaged') and 'Cytoplasmic' ('free') RNA polymerases. Nuclear and 'cytoplasmic' fractions were resolved from lymphocytes at various times during the PHA response. Using discriminating concentrations of α-amanitin (see Methods) each fraction was assayed for form I (upper panels) and form II (lower panels). All data has been normalised to show polymerase activity derived from 10^8 cells. The data is presented in two ways for each enzyme: either as gross RNA polymerase activity (left hand panels) or (for consistency with other publications in this area) as changes in polymerase activity relative to time zero (right hand panels). Nuclear RNA polymerase, ●—●; 'cytoplasmic' RNA polymerase, o—o.

DISCUSSION

This data presents an important new dimension to the long catalogue of experiments which have been designed to characterise the effect of PHA-stimulation on lymphocyte RNA metabolism. We have shown that there is no consistent relationship between the transcriptional activity of nuclear RNA polymerases I and II and a 'free' pool of these enzymes which is generated following PHA-stimulation of human lymphocytes. As newly synthesised RNA polymerase is probably distributed between the nucleus and the

extra-nuclear compartment of the cell, studies confined to isolated nuclei [2 - 4] or the solubilisation of RNA polymerases for whole cells [5] present an incomplete picture of the state and organisation of the RNA synthetic machinery which obviously change during PHA transformation. Superimposed on these phenomena are post-transcriptional events which themselves may contribute to the control of the activation process: for example, the resting lymphocyte exhibits a 'wastage' phenomenon whereby a significant proportion of 45-S pre-rRNA is degraded without giving rise to mature 18-S rRNA ; early in the PHA-response, this 'wastage' is reversed [18,19].

There is thought to be an early, protein synthesis-independent activation of rRNA synthesis [4] although this remains controversial [20]. However, within the first 24 hours, it is evident that both the capacity of the nucleus to synthesise RNA rises and (as has been shown here and by others [2,5]) there is a coincident increase in RNA polymerase activity in these cells. This increase in polymerase activity includes the generation of a considerable pool of 'free' enzyme which continues to rise (up to 48 hours) at a rate in excess of that which appears to be required for nuclear function. It is possible that this constitutes the accumulation of macromolecules which are required for daughter cells arising from subsequent cell division, a situation which is thought to pertain in several early developmental systems [21,22]. At the moment, it is not possible to describe the size of the 'free' polymerase pools in stimulated lymphocytes (in terms of numbers of enzyme molecules), neither is it possible to compare the number of polymerase molecules which constitute the endogenous nuclear RNA polymerase activity with the 'free' pool (assessed on the basis of in vitro RNA synthesis using nicked DNA templates).

The data in Fig. 3 form the critical observation that, in the approach to the first S-phase following the PHA-induced transition from G_o, the factors regulating the relationship between functional, nuclear RNA polymerase and the 'free' polymerase pools would appear to be different for forms I and II enzymes. There is other evidence which suggests that, in the case of form I polymerase, rapidly-turned-over auxiliary factors may be required to regulate transcriptional activity of the enz-

yme [23 - 25]: one could speculate that the controlled expression of these factors may be involved in co-opting enzyme from the 'free' pool to become transcriptionally active in nuclei, accounting for the disparate response of nuclear and 'free' pools of form I polymerase to PHA-activation. On the other hand, it has recently been shown that the synthesis of form II polymerase in mammalian cells may be auto-regulatory [26] and it is interesting that, over the first 48 hours, nuclear form II activity rises virtually pari passu with respect to the pool of 'free' enzyme (Fig. 3).

Under the culture conditions of high cell density used in these experiments, cell division does not take place [15,27]. Between 48 and 72 hours, an artificial shut-down in RNA synthesis may begin to take place. This would account for the decline in the 'free' polymerase pools, endogenous form I (rRNA synthesising) polymerase system in nuclei and a plateauing in the rate of nuclear form II polymerase activity.

The response of resting lymphocytes to PHA is complicated by differential effects of the mitogen on different sub-classes of lymphocytes. The lymphocyte preparative technique used in this work (see Methods) yields a population of cells which is predominantly T-lymphocytes (i.e. monocyte depleted and partially depleted in B-cells). T-lymphocytes are the stimulated cells but recent work has shown that 'suppressor-' and 'helper-' T cells (T_γ and T_μ respectively [28,29]) differ in their individual response with respect to the optimal concentration of PHA and the response of a mixed population of T_γ and T_μ cells is synergistic rather than being a simple additive phenomenon [29]. Future efforts to analyse specific events in the induction process are therefore going to require the resolution of lymphocyte sub-populations.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council (grant no. 976/316/C). The authors wish to express their appreciation to Dr. J.E. Kay for his assistance in establishing the lymphocyte culture technology and to Dr. Davies and the Staff of the Regional Blood Transfusion Service, Edgeware, for the supply of buffy coat residues.

REFERENCES

1. Ling, N. and Kay, J.E. (1975) Lymphocyte Stimulation, 2nd edn. North Holland Publishing Co.
2 Cooke, A. and Brown, M. (1973) Biochem. Biophys. Res. Commun. 51, 1042-1047
3 Cooke, A. and Kay, J.E. (1973) Exp. Cell Res. 79, 179-185
4 Pogo, B.G.T. (1972) J. Cell Biol. 53, 635-641
5 Jaehning, J.A., Stewart, C.C. and Roeder, R.G. (1975) Cell 4, 51-57
6 Yu, F.-L. (1974) Nature (London) 251, 344-346
7 Yu, F.-L. (1975) Biochim. Biophys. Acta 395, 329-336
8 Kellas, B.L., Austoker, J.L., Beebee, T.J.C. and Butterworth, P.H.W. (1977) Eur. J. Biochem. 72, 583-594
9 Yu, F.-L. (1977) J. Biol. Chem. 252, 3245-3251
10 Kay, J.E., Ahern, T., Lindsay, V.J. and Sampson, J. (1975) Biochim. Biophys. Acta 378, 241-250
11 Wolff, J.S., Langstaff, J.A., Weinberg, G. and Abell, C.W. (1967) Biochem. Biophys. Res. Commun. 26, 366-371
12 Chambon, P. (1975) Annu. Rev. Biochem. 44, 613-638
13 Chesterton, C.J. and Butterworth, P.H.W. (1971) Eur. J. Biochem. 19, 232-241
14 Giles, K.W. and Myers, A. (1965) Nature (London) 206, 93.
15 Stewart, C.S., Cramer, S.F. and Stewart, P.G. (1975) Cell. Immunol. 16, 237-250
16 Gilboa, E., Soreq, H. and Aviv, H. (1977) Eur. J. Biochem. 77, 392-400
17 Schwartz, L.B., Sklar, V.E.F., Jaehning, J.A., Weinmann, R. and Roeder, R.G. (1974) J. Biol. Chem. 249, 5889-5897
18 Cooper, H.L. (1969) J. Biol. Chem. 244, 1946-1952
19 Cooper, H.L. (1969) J. Biol. Chem. 244, 5590-5596
20 Dauphinais, C. and Waithe, W.I. (1977) Eur. J. Biochem. 78, 189-194
21 Roeder, R.G. (1974) J. Biol. Chem. 249, 249-256
22 Davidson, E.H. (1976) Gene Activity in Early Development, 2nd edn. Academic Press Inc.
23 Lampert, A. and Feigelson, P. (1974) Biochem. Biophys. Res. Commun. 58, 1030-1038
24 Gross, K.J. and Pogo, A.O. (1974) J. Biol.Chem. 249, 568-576
25 Cereghini, S. and Franze-Fernandez, M.T. (1974) FEBS Lett. 41, 161-165
26 Somers, D.G., Pearson, M.L. and Ingles, C.J. (1975) Nature (London) 253, 372-374
27 Andersson, J., Coutinho, A., Melchers, F. and Wa anabe, T. (1977) Cold Spring Harbor Symp. Quant. Biol. 41, 227-236
28 Moretta, L., Webb, S.R., Grossi, C.E., Lydyard, P.M. and Cooper, M.D. (1977) J. Exp. Med. 146, 184-200
29 Moretta, L., Ferrarini, M., Mingari, M.C., Moretta, A. and Webb, S.R. (1976) J. Immunol. 117, 2171-2174