DNA DEPENDENT RNA POLYMERASE ACTIVITY OF NUCLEI ISOLATED FROM HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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Human peripheral blood lymphocytes in culture rarely undergo division or exhibit a demonstrable synthesis of DNA in the absence of a mitogenic stimulus (Nowell, 1960). The incorporation of precursors into RNA occurs at a low level in normal lymphocytes and upon addition of phytohemagglutinin (PHA), a marked stimulation is observed (Rubin and Cooper, 1965). In order that this process may be studied in detail, a system is described in this communication for the synthesis of RNA (Weiss, 1960) in lymphocyte nuclei obtained from cells not exposed to PHA. In addition to nuclei, the system requires magnesium ion, the four common ribonucleoside triphosphates and ammonium sulfate for maximal activity. Furthermore, utilization of the DNA template for RNA synthesis is prevented by the addition of DNAase, Actinomycin D and RNAase. The incorporation of ribonucleoside triphosphate into RNA proceeds for a relatively long period of time (2 hours) and the product formed is stable under the conditions employed.

MATERIALS AND METHODS

Cell Cultures. Fresh human peripheral blood was obtained from donors at the NIH clinical center and lymphocytes were obtained by a procedure essentially identical to that described by Cooper and Rubin (1965). All cultures were monitored for metaphase figures as a function of PHA stimulation; usually 6-7 percent of the cells were represented by dividing figures (Moorhead, 1964).

Isolation of Nuclei. Lymphocytes (30-60 x 10^6/2.0 ml) were obtained from the growth medium (McCoys or Eagles, MEM) by centrifugation and suspended in 2 mM $MgCl_2$ and 0.4 mM potassium phosphate, pH 6.7 for 1 minute. The sucrose concentration was adjusted to 0.32 M, the suspension was recentrifuged, and the lymphocytes were suspended in 0.32 M sucrose, 0.4 mM potassium phosphate, 2 mM $MgCl_2$ (standard solution) to a concentration of 10 x 10^6 cells/ml. The cells were homogenized in a tight Dounce homogenizer with 15 passes/2 ml; the nuclei were sedimented at 450 x g at 0°C, and then resuspended in standard solution. DNA analyses were performed on aliquots of cell and nuclei suspension, and on individual reaction mixtures. An average total loss of 20 $\pm$ 8 percent of DNA was observed in the preparation of nuclei. Where indicated, crude nuclei were further purified by sedimentation in 2.39 M sucrose, centrifuged, resuspended in standard solution and utilized in the reaction mixture.

Standard Assay Procedure. Nuclei were resuspended to a final concentration equivalent to 7.2-22.0 x 10^6 cells per ml of standard homogenizing solution, as indicated, and 0.2 ml aliquots of this mixture were used in each assay. Unless indicated otherwise each tube contained the following in μmoles in a total of 0.5 ml:

$Mg(Ac)_2$, 7.5; ATP, 0.6; GTP, 0.6; UTP, 0.6; CTP-H^3, 0.06 (specific activity of 159,000, 214,000 or 250,000 dpm/mμmole); ammonium sulphate, 120; and Tris chloride, pH 7.5, 100. All of the ribonucleoside triphosphates were purchased from Schwartz Biochemicals, Orangeburg, New York.

The nuclei were added to the mixture just prior to incubation and the suspensions were maintained at 37° for variable time intervals. The reaction was stopped by the addition of CTP (600 mμmoles) and carrier RNA (2 mg in 0.5 ml of 0.2 M EDTA, pH 7.4). TCA (10%) in 0.02 M sodium pyrophosphate was added and the precipitate was allowed to stand for 10 minutes prior to centrifugation. The tubes were spun at 900 x g for 10 minutes and the precipitate was washed successively with 3 ml portions of

TCA (5%) in 0.02 M sodium pyrophosphate, TCA (5%), ethanol, and a mixture of ethanol, chloroform and ether (2:2:1). The reagents used for stopping the reaction were pre-cooled, and the wash procedure was carried out at 3°C. The precipitates were dissolved in water (0.1 ml) and NCS (1 ml) by agitation on a Vortex mixer and incubation at 40°C. Aliquots (0.5 ml) were counted in a toluene mixture in a Packard liquid scintillation spectrometer with an efficiency of approximately 26 percent. When DNA analyses were carried out on the individual reaction mixtures, the pellets were further washed with 3 ml of acetone, dried, suspended in 0.5 ml of 0.5 N PCA, and heated at 90° for 15 minutes. After cooling and centrifugation, aliquots were removed for use in the diphenylamine reaction. Other aliquots were counted in neutralized Bray's counting solution in the scintillation spectrometer, with an efficiency of about 11 percent. DNA was determined by the diphenylamine procedure of Burton (1956). All assays were performed in duplicate and each experiment represented herein was repeated using a different lymphocyte culture. Although the extent of precursor incorporation varied with nuclei from various lymphocyte donors, the same rates and patterns were observed.

RESULTS

Table 1 shows some of the salt requirements of lymphocyte nuclei for maximal RNA synthesis.

Magnesium ion, as either the acetate or chloride salt gave the maximum stimulation of RNA synthesis at a concentration of 0.015 M (7.5 μmoles/0.5 ml reaction). Potassium ion alone did not stimulate RNA synthesis. Addition of 2-mercaptoethanol resulted in a slight increase in activity and omission of ammonium sulfate resulted in a substantial loss in incorporation of precursor.

The ribonucleoside triphosphate requirements for RNA synthesis are shown in Table 2.

Table 1
Salt Requirements for RNA Synthesis

System	Incorporation of CTP-H^3 (mμmoles/mg DNA)
Complete	1.65
-$Mg(Ac)_2$ + $MgCl_2$ (7.5 μmoles)	1.57
-$Mg(Ac)_2$ + KAc (15 μmoles)	0.13
-$Mg(Ac)_2$	0.22
+2-mercaptoethanol (14.4 μmoles)	1.86
-$(NH_4)_2SO_4$	0.36
Complete (Reaction stopped at zero time)	0.11

The complete reaction mixture contained the materials and concentration described for a standard assay under materials and methods. Each reaction mixture contained approximately 3.75×10^6 nuclei and the addition or deletions as indicated above, and was incubated for 30 minutes at 37°C. The reaction was stopped and the precipitate washed, as described in materials and methods, and the incorporation of CMP-H^3 into RNA was measured using a liquid scintillation spectrometer.

Table 2
Ribonucleoside Triphosphate Requirements for RNA Synthesis

Experiment	System	Incorporation of precursors (mμmoles/mg DNA)
A	Complete (CTP-H^3)	1.25
	-GTP	0.48
	-UTP	0.51
	-ATP	0.49
	-UTP,GTP,ATP	0.62
B	GTP-H^3	2.73
	UTP-H^3	1.66
	ATP-H^3	2.35

In experiments A & B, each reaction mixture contained approximately 1.5×10^6 nuclei in a standard assay with the deletions as indicated. In experiment B, each ribonucleoside triphosphate was substituted with the tritium labeled form as indicated above (0.06 μmoles) and supplemented with the three other appropriate ribonucleoside triphosphates (0.6 μmoles, not labeled). The figures shown above were obtained by subtracting the zero time from the 30 minute incorporation values.

The presence of all four of the common ribonucleoside triphosphates was required (Experiment A). Omission of one or more resulted in a marked loss of activity. In experiment B, the incorporation of the other tritiated ribonucleoside triphosphates is shown. GTP-H^3 was incorporated to a greater extent than were the other three ribonucleoside triphosphates.

The rate of incorporation was shown to be linear with time for approximately 15 minutes (Fig 1a). Incorporation continued to increase

for at least 2 hours and the product formed was stable for an additional 2 hours. A similar curve was observed using purified nuclei. The effect of increasing the nuclei concentration in the assay system was examined (Fig 1b). Increases in the nuclei concentration of 2 and 4 fold resulted in the increase in the extent and rate of incorporation of CTP into RNA by approximately 2 and 3 fold, respectively.

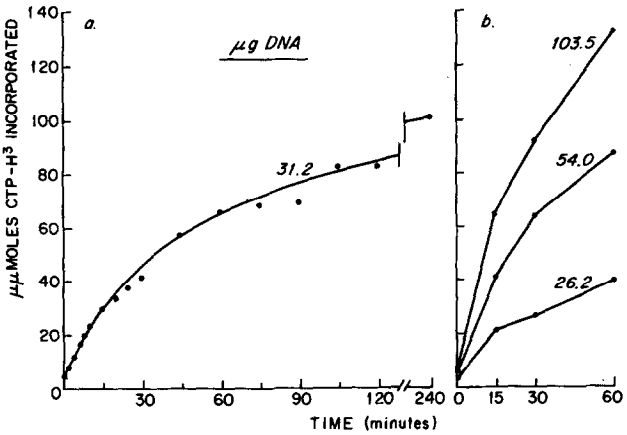

Fig. 1 Incorporation of CTP-H^3 into RNA as a function of time and nuclei concentration. Numbers indicate μg DNA; 6.25 μg DNA/10^6 nuclei.

The effects of adding DNAase, RNAase, and Actinomycin D to the reaction mixture and of boiling are demonstrated in Table 3.

Table 3
Inhibition of RNA Synthesis

System	Incorporation of CTP-H^3 (mμmoles/mg DNA)
Complete	1.30
+DNAase (10 μg)	0.36
+Actinomycin D (10 μg)	0.13
+RNAase (10 μg)	0.17
Boiling at zero time	0.28
Reaction stopped at zero time	0.14

Where indicated commercial DNAase, RNAase and Actinomycin D were added to the tubes immediately after the nuclei addition (3.75 x 10^6 nuclei). The tubes were incubated for 30 minutes at 37°C and the assay performed as described.

Essentially all of the DNA template activity was lost when DNAase, Actinomycin D or RNAase was added to the nuclei. The reaction was also sensitive to boiling which demonstrates that one or more of the requirements for activity are heat labile.

DISCUSSION

The results presented here indicate that nuclei isolated from human peripheral blood lymphocytes may be utilized under the appropriate conditions for the study of DNA-dependent RNA synthesis. In addition to nuclei, the reaction required magnesium ion, the four common ribonucleoside triphosphates and ammonium sulfate for maximal activity. A detailed study of the characteristics of the product formed in lymphocyte nuclei and the relationship of this RNA to that synthesized in culture both in the absence and presence of PHA will be presented elsewhere.

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