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Control of Transcription of RNA Rich in Polyadenylic Acid in Human Lymphocytes

(mRNA function/dibutyryl cAMP/phytohemagglutinin/cycloheximide/cortisol)

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ABSTRACT Rapidly labeled polyribosomal RNA rich in poly(A) has been isolated from cultures of highly purified human peripheral blood lymphocytes. Messenger RNA function for this RNA is suggested by its ability to direct [³H]Met-tRNA binding to ribosomes and incorporation of amino acids into protein in a cell-free preparation. Phytohemagglutinin and low concentrations of dibutyryl cAMP (40 nM) increase poly(A)-rich RNA synthesis 40% within 2 hr, and 100–300% by 12 hr; the percent poly(A) content and the size of the poly(A)-rich portion remain constant. Higher concentrations of dibutyryl cAMP (1 mM), which prevent morphological transformation of lymphocytes by phytohemagglutinin, inhibit synthesis of poly(A)-rich RNA in phytohemagglutinin-treated lymphocytes without damaging cells. Cortisol (0.1 mM), which also prevents lymphocyte transformation, inhibits poly(A)-rich RNA synthesis by 80%. Cycloheximide (5 μg/ml), which decreases protein synthesis by 90%, decreases poly(A)-rich RNA synthesis 80% in cells stimulated by phytohemagglutinin. These studies demonstrate that, as part of the early molecular events of their action, phytohemagglutinin and cortisol regulate transcription of adenylate-rich RNA in human lymphocytes, and that similar transcriptional effects can be produced by dibutyryl cAMP.

Several species of messenger RNA (mRNA) (1, 2) and rapidly labeled polysomal RNA in several cell cultures (3–5) contain a covalently bound region that is relatively RNase resistant and rich in adenylate (A-rich RNA). A-rich regions have also been identified in heterogeneous nuclear RNA, and a precursor relationship to cytoplasmic mRNA has been suggested (3, 4, 6). Drug studies and DNA·RNA hybridization data suggest that the poly(A) regions are added after transcription (7), perhaps by a nuclear polyadenylating enzyme (8). Although the function of these A-rich regions remains unclear, their property of adhering to nitrocellulose filters (5, 9) allows selective isolation of mRNA.

Peripheral human lymphocytes respond to phytohemagglutinin (PHA) with morphologic transformation of a large percentage of lymphocytes, increased mitotic index, and increased incorporation of labeled precursors into DNA, RNA, and protein (10–12). An early brief increase in the amounts of lymphocyte intracellular cAMP has been described in PHA-treated human lymphocytes (13). PHA and low concentrations of dibutyryl adenosine 3′:5′-N^6,$O^{2'}$-cyclic monophosphate[(But)$_2$cAMP] (10–100 nM) cause activation of protein phosphokinase and increase in [³H]uridine incorporation into RNA in pig lymphocytes (14). High concentrations of (But)$_2$-cAMP (1 mM) inhibit the lymphocyte response to PHA (15).

In order to study early transcriptional effects of (But)$_2$-cAMP and PHA, we have isolated and characterized rapidly labeled A-rich RNA from human peripheral lymphocytes.

MATERIALS AND METHODS

Preparation of Lymphocytes. A preparation containing 99% human peripheral blood small lymphocytes was isolated (16). Lymphocytes remained 96–100% viable by trypan blue dye exclusion in all incubations.

Incubation and Homogenization. Erythroagglutinating-PHA was obtained from PHA-P (Difco Laboratories) (17). Erythroagglutinating-PHA was used at 2 μg/ml in all experiments, a dose stimulating maximal [³H]thymidine incorporation into DNA. Cortisol (Sigma Chemical Co.) and prostaglandin E_1 (PGE_1) (gift of Dr. John E. Pike, Experimental Chemistry Research Division, The Upjohn Co., Lot No. 10323-JKH-713) were dissolved in ethanol. (But)$_2$-cAMP (P-L Biochemicals Inc.) was extracted five times with ether at pH 2 to remove free butyric acid (18). Replicate lymphocyte culture (2 to 8 × 10^6 cells/ml) were suspended in Eagle's Minimal Essential Medium (Grand Island Biological Co.) containing 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% autologous fresh serum and incubated at 37° in 95% air–5% CO_2, for 24 hr before any experimental additions. In the studies of A-rich RNA synthesis, 50 ng/ml of actinomycin D (Merck L554651-0-10), a concentration sufficient to prevent rRNA synthesis (19, 20), was added to the incubation mixture 30 min to 2 hr before addition of [³H]adenosine (Schwarz–Mann, 24.4 Ci/mmol). Cells were collected and lysed (21). DNA was determined in the presence of 0.1 M NaCl in an Aminco-Bowman spectrofluorometer (22).

Polysome Density Gradients and Isolation of A-rich RNA. Beef adrenal gland cortices were homogenized in 35 mM Tris·HCl (pH 7.5 at 20°)–25 mM KCl–10 mM $MgCl_2$–0.25 M sucrose and centrifuged at 8000 × *g*. The supernatant was adjusted to contain 5% Triton X-100 and 1% deoxycholate. 9 ml of this solution was added as carrier to 1 ml of 8000 × *g* supernatant obtained from the lymphocytes. Polyribosomes were collected, and polyribosomal zonal centrifugation was

Abbreviations: A-rich RNA, rapidly labeled polyribosome-associated RNA containing regions rich in polyriboadenylic acid; (But)$_2$cAMP, dibutyryl adenosine 3′:5′-N^6,$O^{2'}$-cyclic monophosphate; mRNA, messenger RNA; PHA, phytohemagglutinin; PGE_1 prostaglandin E_1.

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done (23). Cl_3CCOOH-precipitated and washed fractions were solubilized in 1 ml of formic acid and counted to no more than 2% error in 10 ml of Bray's solution (24).

Poly(A)-rich RNA was isolated (5, 9), with sequential pH 7.6 and pH 9 extractions. Polyribosomal RNA extracted at pH 9 is referred to as pH 9 RNA, and that portion of the pH 9 RNA retained on a nitrocellulose filter (Millipore Corp.) is referred to as A-rich RNA. For determination of total pH 9 RNA, the extract was precipitated in cold 10% Cl_3CCOOH after addition of 10 μg of carrier RNA, applied to a fiberglass filter (Whatman GF/C), washed with 5% Cl_3CCOOH, and counted in Liqvifluor and toluene. The value was corrected to account for the difference in counting efficiency between this

TABLE 1. *Characteristics of rapidly labeled polyribosomal A-rich RNA and effects of addition of PHA and hormones*

Additions	Labeled precursor	Acid ppt pH 9 polyribosomal RNA (cpm)	Retained on Millipore filter	
			Total	After RNase digestion
Exp. 1A	Adenosine			
None		426	193	92
PHA		2724	1326	595
Exp. 1B	Uridine			
None		432	165	4
PHA		1773	570	12
Exp. 2	Adenosine			
PHA (1-hr label)		342	280	123
PHA (1-hr label, 3-hr chase)		2596	1089	490
Exp. 3	Adenosine			
None		743	255	
PHA		2460	1442	
PHA + cortisol (0.1 mM)		790	273	
Exp. 4	Adenosine			
None		920	405	
PGE_1 (0.1 mM)		506	246	

Additions to each culture containing 2 × 10^7 lymphocytes in Exp. 1, 7 × 10^7 in Exp. 2, 2.5 × 10^7 in Exp. 3, and 4 × 10^7 in Exp. 4 were for 36 hr unless otherwise noted. Actinomycin D (0.05 μg/ml) was added to all incubations 30 min before addition of [^{3}H]adenosine (1.2 μM, 50 μCi/ml) or [^{3}H]uridine (0.5 μM, 50 μCi/ml); in 60 min excess unlabeled adenosine or uridine (50 μM) was added, and incubations were continued for an additional 120 min. In Exp. 2, after incubation for 60 min with [^{3}H]-adenosine unlabeled adenosine was added, and the incubation was stopped or allowed to proceed for an additional 3 hr. Aliquots (200 μl) of polyribosomal pH 9 RNA obtained by sequential extraction were applied to a 45-μm nitrocellulose filter. 200-μl aliquots of pH 9 RNA were precipitated by Cl_3CCOOH (see *Methods*). Digestions with pancreatic RNase (2 μg/ml) + T_1 RNase (50 units/ml) were done in 50 mM Tris·HCl (pH 7.6)–50 mM KCl–1 mM $MgCl_2$ at 37° for 30 min.

and Millipore filters in Bray's solution. Pancreatic RNase (Worthington Biochemical Corp.) and T_1 RNase (Calbiochem), previously incubated at 80° for 30 min in 0.05 M acetate (pH 5.1) to destroy DNase, were used to test for RNase resistance.

Binding of [^{3}H]Met-tRNA to Ribosomes in a Protein Synthesizing Preparation. Beef adrenal cortex ribosomes were prepared (25). The 30,000 × *g* supernatant was incubated at 37° for 40 min in 1 mM ATP, 0.2 mM GTP, 50 μM amino-acid mix, 2 mg/ml phosphoenolpyruvate and 20 international units/ml of pyruvate kinase; after incubation, the solution was adjusted to 5% Triton X-100 and 1% deoxycholate. The ribosomes were isolated and dialyzed for 12 hr against 2 mM Tris·HCl (pH 7.5)–1 mM $MgCl_2$–6 mM 2-mercaptoethanol–0.1 mM EDTA. Ribosomes had an A_{260}/A_{235} ratio of 1.4–1.5. The ribosomes were washed with 0.5 M KCl, and the KCl extract was used as the source of initiation factors. Rabbit liver tRNA (General Biochemicals) was acylated with [^{3}H]-methionine (2.6 Ci-mmol, Schwarz–Mann) with a 70% ammonium sulfate fraction of beef adrenal cortex that contained amino-acid activating enzymes (26). Binding of [^{3}H]-Met-tRNA to ribosomes was measured (27) in an assay volume of 100 μl containing 50 mM Tris·HCl (pH 7.5)–5 mM $MgCl_2$–50 mM KCl–1.3 mM GTP, washed ribosomes (A_{260} = 0.4), 50 μg KCl ribosomal extract, and 35 μg tRNA and A-rich RNA. Incubations were at 37° for 4 min, and were stopped by addition of 2.5 ml of buffer containing 50 mM Tris·HCl (pH 7.5)–160 mM NH_4Cl–12 mM $MgCl_2$. Samples were applied to 0.45-μm nitrocellulose filters, and the filters were washed with 7.5 ml of the above buffer and counted in 10 ml of Bray's solution.

RESULTS

The Presence of Rapidly Labeled A-Rich RNA on the Polysomes of Lymphocytes. Rapidly labeled A-rich RNA from lymphocyte cultures labeled with [^{3}H]adenosine in the presence of actinomycin D (0.05 μg/ml) had the following properties: (*i*) 75-80% of the acid-precipitable radioactivity was in A-rich RNA (Table 1, Exp. 2). (*ii*) 42–50% of the [^{3}H]-adenylate counts were resistant to pancreatic RNase and T_1 RNase digestion; 0–2% of [^{3}H]uridine counts in A-rich RNA were resistant to RNase digestion (Table 1, Exp. 1); 95–97% of [^{3}H]adenylate-labeled RNA not adhering to nitrocellulose filters was rendered acid-soluble by digestion with pancreatic RNase and T_1 RNase, (*iii*) Digestion with 0.1 N KOH (100° for 30 min) solubilized 98–100% of the counts, confirming that labeled DNA was not present. (*iv*) Sucrose density gradients demonstrated a heterogeneous size distribution of A-rich RNA, most sedimenting at 8–14 S; after treatment with pancreatic and T_1 RNase the portion labeled with [^{3}H]-adenosine retained on the filter sedimented at 4–5 S (Fig. 1).

A-Rich RNA Directs [^{3}H]Met-tRNA Binding to Ribosomes. mRNA function for A-rich RNA was suggested by its ability to direct binding of [^{3}H]Met-tRNA to previously incubated ribosomes in the presence of a 0.5 M KCl extract of ribosomes, Mg^{++}, and GTP (Table 2). [^{3}H]Met-tRNA was not bound to the previously incubated ribosomes in the absence of A-rich RNA. A-rich RNA directed the binding of [^{3}H]Met-tRNA 75% as efficiently in the absence of GTP, suggesting that binding directed by internal Met-tRNA was not measured in this assay. In a cell-free protein synthesizing preparation,

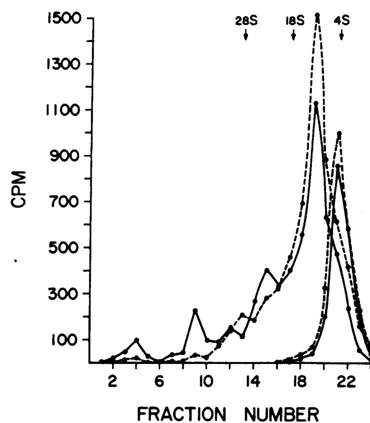

FIG. 1. Sedimentation characteristics of A-rich RNA. Polysomal pH 9 RNA fractions from unstimulated lymphocytes, lymphocytes stimulated for 36 hr with PHA, and equal aliquots incubated with pancreatic RNase (2 μg/ml) + T_1 RNase (50 units/ml) in 50 mM Tris·HCl (pH 7.6)–60 mM KCl–1 mM $MgCl_2$ at 37° for 20 min were adjusted to pH 7.6, mixed with 20 volumes of 0.01 M Tris (pH 7.6)–0.5 M KCl–1 mM $MgCl_2$, and filtered through Millipore filters. The aliquots of unstimulated pH 9 RNA applied to the filters were 5-fold larger to equalize total cpm applied to the filter. The RNA retained on the Millipore filter was eluted for 6 hr with 0.5% sodium dodecyl sulfate– 0.1 M Tris·HCl (pH 9.0) at 4°, mixed with beef adrenal ribosomal and yeast transfer RNA, and subjected to zonal sedimentation at 45,000 rpm for 120 min through a 5-ml 5–20% linear sucrose gradient in 50 mM Tris·HCl (pH 7.6)–50 mM KCl–1 mM $MgCl_2$, or 20 mM Tris·HCl (pH 7.6)–10 mM KCl for the RNase-treated samples. The approximate sedimentation constants of the marker RNA species are indicated. (●——●) A-rich RNA from unstimulated lymphocyte. *Curve* on the right is the RNase-digested aliquot. (●- - -●) A-rich RNA from PHA-stimulated lymphocyte. *Curve* on the right is the RNase-digested aliquot. Sedimentation is from right to left.

A-rich RNA directed incorporation of [^{3}H]leucine into acid-precipitable polypeptides (Abrass, I. B. & Rosenfeld, M. G., in preparation).

Time Course of A-Rich RNA Association with Ribosomes. In these experiments, a 50-fold excess of unlabeled adenosine was added to the culture after 60 min of incubation with [^{3}H]-adenosine and the incubations were continued for an additional 3 hr. There was a 3-fold diminution in the amount of Cl_3CCOOH-precipitable radioactivity in nuclear RNA by 3 hr. A 4-fold increase in polyribosome-associated A-rich RNA occurred by 3 hr (Table 1, Exp. 2), and there was an 8-fold increase in total polyribosome-associated acid-precipitable radioactivity. Similar results were obtained with unstimulated and PHA-treated cultures. Polysomal velocity gradients revealed distribution of RNA labeled with [^{3}H]adenylate throughout the polysomal region (Fig. 2).

Effects of PHA and Hormones. PHA increased [^{3}H]adenylate counts incorporated into total rapidly labeled pH 9 RNA and those retained on a nitrocellulose filter 4- to 10-fold by 36 hr (Table 1). Zonal sedimentation of the A-rich RNA showed that virtually the entire stimulation by PHA involved 8–14S RNA (Fig. 1). PHA increased the percentage of rapidly labeled RNA labeled with [^{3}H]adenylate found associated with polyribosomes but did not alter the polysomal velocity gradient pattern (Figs. 2*A* and 3). The percent resistance to pancreatic RNase and T_1 RNase and the sedimentation value (4–5 S) of the A-rich region were unaltered by PHA (Fig. 1).

To obtain evidence that the stimulatory PHA effect on the incorporation of radiolabeled precursor into A-rich RNA reflected an actual increase in the amount of A-rich RNA synthesized, rather than a change in specific activity of nucleoside pools as cautioned by Peters and Hausen (28), we compared the functional activity of A-rich RNA from unstimulated and PHA-stimulated lymphocytes. A-rich RNA from PHA-treated lymphocytes directed a 3- to 6-fold greater binding of [^{3}H]Met-tRNA to previously incubated ribosomes in a protein-synthesis assay compared to A-rich RNA extracted from an equal quantity (by DNA content) of unstimulated lymphocytes (Table 2).

$(But)_2cAMP$ in low concentrations (10–100 nm) stimulated synthesis of total rapidly labeled polyribosomal pH 9 RNA and A-rich RNA at 12 hr (Table 3); the stimulation was less than that of PHA. This effect was demonstrable within 2 hr after addition of $(But)_2cAMP$ (Table 3, Fig. 2*C*). There was no alteration in the zonal sedimentation characteristics of the A-rich RNA or the poly(A)-rich region isolated after pancreatic RNase and T_1 RNase digestion.

In high concentration (1 mM), $(But)_2cAMP$ inhibited A-rich RNA synthesis in PHA-stimulated lymphocytes (Table 4). Addition of $(But)_2cAMP$ for the entire incubation period abolished the PHA effect on A-rich RNA. The cell count, DNA content, and cell viability as assessed by trypan blue dye exclusion were unaltered. Removal of $(But)_2cAMP$ by washing the cells at 3 hr or 30 hr of a 36-hr incubation completely restored the PHA stimulation of A-rich RNA but did not restore the effect on [^{3}H]thymidine incorporation into DNA at 36 hr. By 96 hr, however, the PHA effect on [^{3}H]thymidine incorporation into DNA was entirely restored. Addition of $(But)_2cAMP$ at 32 hr of a 36-hr incubation did not diminish PHA stimulation of A-rich RNA synthesis, but decreased [^{3}H]thymidine incorporation into DNA 40–50%.

TABLE 2. *Initiation assay with rapidly labeled polyribosomal A-rich RNA*

Addition	[^{3}H]Met-tRNA bound (cpm/mg of ribosomal protein)
None, 0.5 M KCl ribosomal extract omitted	30
None	33
Unstimulated lymphocyte A-rich RNA (40 μl)	320
PHA-stimulated lymphocyte A-rich RNA (40 μl)	1186

Lymphocytes prepared from one unit of peripheral blood were divided into two equal portions and incubated for 36 hr with either no addition or 2 μg/ml PHA. The pH 9 RNA obtained by sequential extraction was collected on Millipore filters, eluted with 0.5% sodium dodecyl sulfate in 0.1 M Tris·HCl (pH 9), and extracted 3 times with water-saturated phenol. The ether was removed by bubbling with N_2. DNA content of unstimulated and PHA-stimulated lymphocyte cultures were equal. A_{260} could not be determined for the RNA eluted from the Millipore filter as the values were below the sensitivity of the method. In control incubations, 40 μl of 0.1 M Tris·HCl (pH 9) extracted through the entire procedure for preparation of A-rich RNA replaced the 40 μl of A-rich RNA.

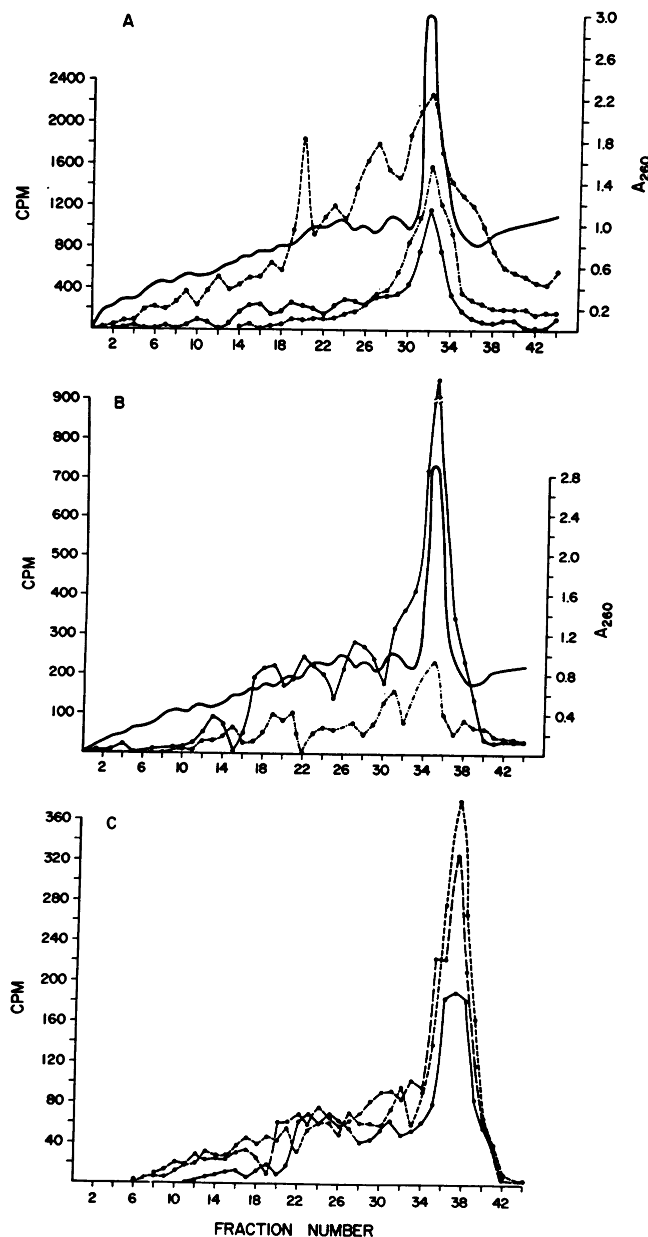

FIG. 2. Distribution of rapidly labeled RNA in polyribosome density gradients. (*A*) At 36 hr, unstimulated, PHA-treated, and PHA + cortisol (0.1 mM)-treated lymphocyte cultures (6.4 × 10^7 cells in 10 ml) were incubated for 1 hr with 0.05 μg/ml of actinomycin D, then incubated with [^{3}H]adenosine (2 μM, 500 μCi) for 60 min. Polyribosomes were subjected to zonal sedimentation after addition of carrier beef adrenal polyribosomes at 22,500 rpm for 150 min in an SW27 Spinco rotor through 28 ml of a 10–30% linear sucrose gradient in 0.01 M Tris·HCl (pH 7.6)–25 mM KCl– 1 mM $MgCl_2$. Fractions were collected and precipitated with Cl_3CCOOH. (——) A_{260}, (●– – –●) PHA (2 μg/ml), (●——●) unstimulated, (●—·—·●) PHA (2 μg/ml) + cortisol (0.1 mM). (*B*) 6.4 × 10^7 unstimulated lymphocytes and 1.3 × 10^8 PGE_1 (0.1 mM)-treated lymphocytes were incubated for 36 hr, and polysomal density gradients were run as described above. (——) A_{260}, (●——●) unstimulated, (●—·—·●) PGE_1 (0.1 mM). (*C*) Hormones were added to cultures of 2 × 10^7 lymphocytes in 10 ml 15 min after the addition of [^{3}H]adenosine (1.2 μM, 500 μCi), and incubations were continued for an additional 105 min. Polyribosomes were subjected to zonal sedimentation as described above. (●——●) unstimulated, (●– – –●) $(But)_2cAMP$ (10 mM), (●-----●) PHA.

TABLE 3. *Stimulation of rapidly labeled polyribosomal RNA by dibutyryl cAMP*

Addition (hr in culture)	Acid-precipitable polyribosomal pH 9 RNA (cpm/120 μg DNA)	Polyribosomal pH 9 RNA retained on Millipore filter (cpm/120 μg DNA)
None	576	220
$(But)_2cAMP$ (40 nM), 2 hr	806	270
$(But)_2cAMP$ (40 nM), 12 hr	1728	471

$(But)_2cAMP$ was added for 2 or for 12 hr to lymphocyte cultures containing 1.5 × 10^8 lymphocytes. [^{3}H]Uridine (2.5 μM, 500 μCi) was added 30 min after addition of actinomycin D (50 μg/ml), and incubations were stopped after 120 min with chilling and the addition of excess unlabeled uridine (50 μM). Results of one of four experiments giving similar results are expressed as cpm pH 9 RNA, labeled with [^{3}H]uridine, in 200-μl aliquots per 120 mg lymphocyte DNA.

Incubation of unstimulated lymphocytes for 36 hr with PGE_1 (0.1 mM), which increases the concentration of lymphocyte intracellular cAMP (12), (Table 1) or with cortisol (0.1 mM) markedly inhibited A-rich RNA synthesis. Incubation with cortisol (0.1 mM) for 3 hr caused minimal or no alterations of A-rich RNA synthesis; addition of cortisol during the extraction procedure had no effect. Incubations of 2–4 hr with PGE_1 (0.1–100 μM) increased A-rich RNA synthesis 40–70%. Polyribosomal velocity gradients showed minimal qualitative alterations (Fig. 3*B*), but the distribution of rapidly labeled RNA was altered (Fig. 2*A* and *B*).

Effect of Inhibition of Protein Synthesis on A-Rich RNA Synthesis. Addition of cycloheximide (5 μg/ml) for 8 hr resulted in a 90–95% decrease of [^{3}H]leucine incorporation into protein but there was no change in cell count, DNA content, or cell viability as assessed by trypan blue dye exclusion. Addition of 5 μg/ml cycloheximide decreased by 80–90% the stimulation of rapidly labeled polyribosomal A-rich RNA by PHA at 30 hr (Table 5). Cycloheximide (5 μg/ml) similarly inhibited the stimulation of A-rich RNA synthesis produced by addition of PHA for 6 hr.

DISCUSSION

The early events by which hormones acting via cAMP exert their effects are not completely understood. ACTH and cAMP stimulation of cortisol synthesis in the adrenal gland is dependent upon new protein synthesis but not on the synthesis of new RNA, suggesting regulation at the level of translation of mRNA (29, 30). Subsequent studies have suggested similar translational control for hormones acting via cAMP in many target tissues (31–33). The time of onset and extent of transcriptional effects by hormones acting via cAMP remain undefined. The early effects of $(But)_2cAMP$, PHA, and cortisol on mRNA synthesis in human peripheral lymphocytes were studied to clarify early transcriptional effects.

We have demonstrated rapidly labeled polyribosomal RNA rich in poly(A) in human peripheral lymphocytes. Both PHA and $(But)_2cAMP$ (10–100 nM) stimulated the synthesis of A-rich RNA. mRNA function was suggested for lymphocyte polyribosomal A-rich RNA by its ability to bind [^{3}H]Met-tRNA to ribosomes in a protein synthesis initiation assay and to direct incorporation of amino acids into protein in a cell-free preparation. A-rich RNA extracted from PHA-stimulated cells directed a 3- to 6-fold increased binding of [^{3}H]Met-tRNA to ribosomes compared to A-rich RNA extracted from an equal number of unstimulated lymphocytes. The fact that $(But)_2cAMP$ (10 nM) and PHA produced similar increases in A-rich RNA labeled with either adenosine or uridine suggests

TABLE 4. *Effect of concentrations of $(But)_2cAMP$ on the PHA stimulation of poly(A)-rich RNA synthesis*

Addition	Time of medium change (hr)	[^{3}H]-Thymidine incorporation into DNA (cpm)	Polyribosomal pH 9 RNA retained on Millipore filter (cpm/150 μg DNA)
Exp. 1			
None (control)	No change	3,776	732
PHA	No change	20,800	5591
PHA	3	10,400	3287
PHA	30	20,180	2757
PHA + $(But)_2$-cAMP (1 mM)	No change	3,658	679
PHA + $(But)_2$-cAMP (1 mM)	3	3,623	3021
PHA + $(But)_2$-cAMP (1 mM)	30	4,061	2715
Exp. 2			
None (control)	No change	3,756	1377
PHA	No change	21,090	7680
PHA + $(But)_2$-cAMP (1 mM) added at 32 hr	No change	11,630	7947

Additions to each culture containing 2.3×10^7 lymphocytes in Exp. 1 and 3.2×10^7 lymphocytes in Exp. 2 were made at zero time. Medium changes were made twice at the times indicated by centrifugation at $500 \times g$, aspiration, and replacement with fresh medium containing PHA but no $(But)_2cAMP$. At 34 hr, actinomycin D (0.05 μg/ml) was added to all cultures; 1 hr later [^{3}H]adenosine (1.2 μM, 30 μCi/ml) was added, and 60 min thereafter unlabeled adenosine (50 μM) was added, and incubations were continued for an additional 2 hr. Aliquots (400 μl) of polyribosomal pH 9 RNA obtained by sequential extraction were collected on Millipore filters. [^{3}H]Thymidine incorporation into DNA was measured at 36 hr on 1 ml of each culture. [^{3}H]-Thymidine (2 mCi, 6.7 Ci/mmol) was added for 2 hr. The cells were then washed in 0.8% saline, precipitated in 5% Cl_3CCOOH, frozen and thawed, sonicated, collected on fiberglass filters, washed three times with 5% Cl_3CCOOH, and counted in 10 ml of Bray's solution.

that increased adenylylation of RNA (8, 34) synthesized before addition of $(But)_2cAMP$ or PHA is not involved in their early effects. PHA and $(But)_2cAMP$ did not alter the sedimentation characteristics or the percentage of RNase-resistant adenylate residues of the A-rich region isolated from rapidly labeled A-rich RNA.

In high doses (1 mM), the continued presence of $(But)_2$-cAMP suppressed A-rich RNA synthesis in unstimulated and PHA-stimulated cells. The continued presence of $(But)_2$-cAMP was required for maintenance of this effect. Thus, either $(But)_2cAMP$ acts as a competitive inhibitor of the mediator of PHA, or its effect is mediated by a protein with a short half-life. Late addition of $(But)_2cAMP$ (1 mM) did not suppress the PHA effect on A-rich RNA synthesis although it suppressed the increased incorporation of [^{3}H]thymidine into DNA, suggesting an early event in the PHA effect which, once completed, renders its effect on A-rich RNA synthesis irreversible by $(But)_2cAMP$. $(But)_2cAMP$ (1 mM) has no effect on the binding of PHA to the lymphocyte membrane (14).

Cycloheximide in doses inhibiting protein synthesis by 95% suppressed A-rich RNA synthesis in the PHA-stimulated lymphocyte at both early and late time-points to levels approximating those found in the unstimulated lymphocyte,

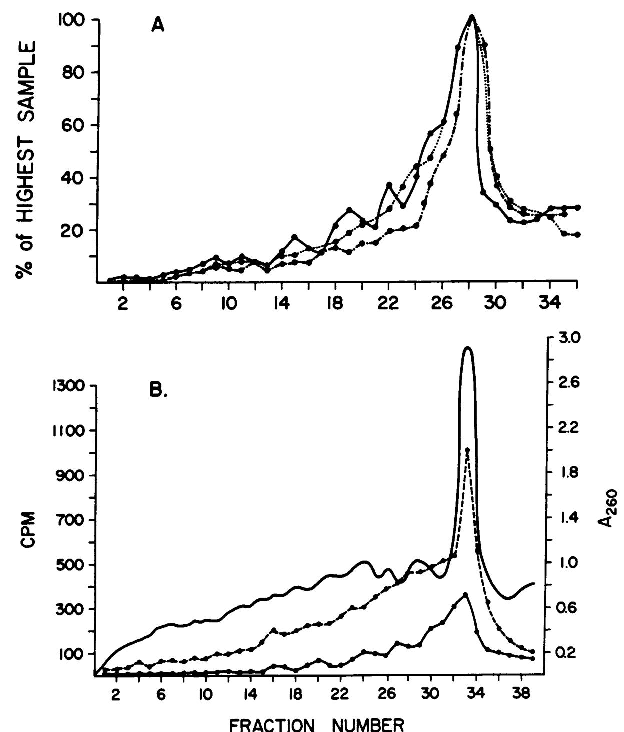

FIG 3. Polysomal velocity gradients of lymphocyte polyribosomes labeled with [^{14}C]uridine. Lymphocyte cultures (4×10^7 cells in 10 ml) with no addition or with PGE_1 (0.1 mM), cortisol (0.1 mM), or PHA were incubated for 30 hr with [^{14}C]-uridine (20 μM, 0.20 mCi), and polyribosomal density gradient centrifugations were done. Polyribosomal patterns in (*B*) are expressed as Cl_3CCOOH-precipitable cpm per fraction; in (*A*) as percentage of the highest acid-precipitable cpm to enable comparison of qualitative differences in the patterns. (*A*) (●——●) unstimulated, (●- - -●) PGE_1 (0.1 mM), (●—·—●) cortisol (0.1 mM). (*B*) (———) A_{260}, (●- - - - -●) PHA-stimulated, (●——●) unstimulated.

TABLE 5. *Effect of cycloheximide on lymphocyte A-rich RNA synthesis*

Addition	Cycloheximide (5 μg/ml)	Polyribosomal A-rich rapidly labeled RNA adhering to Millipore filter (cpm [^{3}H]adenylate/ 100 μg DNA)
None		342
None	+	274
PHA		2575
PHA	+	639

Cultures containing 3.4×10^7 lymphocytes were incubated for 36 hr with or without PHA. Cycloheximide (5 μg/ml), a dose inhibiting protein synthesis by 95%, was added 4 hr before the addition of [^{3}H]adenosine. Actinomycin D (0.05 μg/ml) was added 30 min before addition of [^{3}H]adenosine (1.2 μM, 500 μCi), and incubations were continued 60 min. 200-μl aliquots of pH 9 polyribosomal RNA obtained by sequential extraction were applied to 45-μm nitrocellulose filters. Similar results were obtained with A-rich RNA labeled with [^{3}H]uridine.

suggesting that protein synthesis is required for PHA-induced stimulation of transcription. This may reflect the necessity of synthesis of a specific protein that mediates the stimulation of transcription in addition to a relatively short half-life of enzymes normally required for transcription, such as RNA polymerase, because a quantitatively similar inhibition of A-rich RNA synthesis is not seen in unstimulated lymphocytes. Chromatin template activity is not increased by medium change in fibroblast cultures in the absence of new protein synthesis (35).

These data suggest that cAMP, hormones acting via cAMP, and PHA have an early transcriptional effect, increasing synthesis of mRNA within 2 hr. This increase in transcription depends upon intact protein synthesis.

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