Translational Activity of Messenger Ribonucleic Acid Isolated from Unstimulated and Phytohaemagglutinin-Activated Lymphocytes

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Purified cytoplasmic poly(A)⁺ RNA isolated from unstimulated pig lymphocytes has the same ability to direct translation in a range of cell-free systems as the corresponding mRNA from 20h phytohaemagglutinin-activated lymphocytes. Additional methylation of the mRNA is not required for maximum protein synthesis in the wheat-germ cell-free system. Misleading results are obtained if the mRNA preparations used are not adequately purified, and a method suitable for routine assessment of the degree of purification achieved is described. Cell-free protein-synthesizing systems from unstimulated lymphocytes translate added lymphocyte mRNA with lower efficiency than do comparable systems from phytohaemagglutinin-activated lymphocytes, whatever the source of the mRNA used.

Cultured lymphocytes provide a valuable system for the study of the molecular mechanisms controlling proliferation in eukaryotic cells, as they can be shifted from a quiescent state to one of active proliferation by the addition of mitogenic lectins such as phytohaemagglutinin. Analysis of the sequential biochemical changes occurring before the initiation of DNA replication at 24-36h has shown that there is a 2-fold increase in the rate of protein synthesis as early as 4h after mitogen addition, rising to a 10-fold increase after 20h (Jagus-Smith & Kay, 1976). There is also an increase in the amount of cytoplasmic poly(A)⁺ RNA in this period, but this increase is both smaller and later than the simultaneous increase in protein synthesis (Jagus-Smith & Kay, 1976). There is no change in the rate of polypeptide-chain elongation in this period (Kay *et al.*, 1975), and synthesis of new ribosomes is not required (Kay *et al.*, 1969). This suggests that the increase in the rate of protein synthesis is due to a control operative at the translational level to increase the rate of translation of pre-existing mRNA by pre-existing ribosomes (Kay *et al.*, 1979).

An alternative explanation would be that a substantial part of the poly(A)⁺ RNA present in unstimulated lymphocytes was not functional mRNA, so that the amount of mRNA present in these cells

would be over-estimated by the methods used. Possibilities to be considered include that part of the poly(A)⁺ RNA might be degraded, owing to the higher nuclease activities reported in unstimulated lymphocytes (Kraft & Shortman, 1970; Green, 1977), that it might lack an essential 5'-terminal cap structure (Both *et al.*, 1975), or that it might be rich in mRNA species intrinsically less translatable by lymphocyte ribosomes (Lodish, 1976). However, we report here that poly(A)⁺ RNA carefully purified in good yield from unstimulated lymphocytes is utilized just as efficiently as poly(A)⁺ RNA from 20h phytohaemagglutinin-activated lymphocytes by three different eukaryotic cell-free systems, including systems from lymphocytes. In contrast, cell-free systems from unstimulated lymphocytes utilized any mRNA preparation much less efficiently than did corresponding systems from phytohaemagglutinin-activated cells.

Experimental

Preparation of pig lymphocyte cytoplasmic poly(A)⁺ RNA

The procedures used to prepare and culture lymphocytes from pig blood were identical with those described by Kay *et al.* (1975), except that the cells were cultured at 4×10^6 cells/ml. Cultures were incubated for 20h with or without phytohaemagglutinin-P (15 µg/ml; Difco, Detroit, MI, U.S.A.) before harvesting the cells by centrifugation at 600g for 10min at 37°C. The cell pellets were resuspended in 150mM-KCl/5mM-magnesium acetate/25mM-Tris/HCl (pH 7.6)/0.1% diethyl pyrocarbonate at 2×10^8 cells/ml, and the cells lysed by the addition of 0.1% Triton X-100 and mixing for 2min. The lysate was

Abbreviation used: poly(A)⁺ RNA, poly(A)-containing RNA.

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centrifuged at 300g for 10min at 4°C to remove nuclei, and the supernatant centrifuged again at 12000g for 10min to remove material which sediments alongside mitochondria. Post-mitochondrial supernatants were stored under liquid nitrogen until RNA extraction. The two centrifugation steps are essential, because if the 12000g spin is carried out directly then contamination of the post-mitochondrial supernatant by nuclear material may occur, owing to nuclear lysis. When the two centrifugation steps are used, some additional loss of cytoplasmic material has to be tolerated, but nuclear contamination does not occur. After accumulation of enough post-mitochondrial supernatant, the RNA was extracted as described by Jagus-Smith & Kay (1976). By adding known amounts of ³H-labelled poly(A)⁺ RNA to unlabelled post-mitochondrial supernatants, extracting the RNA and measuring the acid-precipitable radioactivity recovered it was found that the RNA-extraction procedure was 90–100% efficient. The procedure described gave average RNA recoveries of 65 and 75% of total lymphocyte RNA for unstimulated and 20h phytohaemagglutinin-activated lymphocytes respectively.

The isolation of the poly(A)⁺ RNA was carried out by oligo(dT)–cellulose chromatography as described by Aviv & Leder (1972), except that 1 mM-EDTA was included in all buffers. Material eluted by 10mM-Tris/HCl, pH 7.6, in the first passage of the cytoplasmic RNA through the oligo(dT)–cellulose (Collaborative Research, Waltham, MA, U.S.A.) column was made 0.5M with respect to KCl, and the poly(A)⁺ RNA was further purified by a second passage through the column. After this second purification procedure the poly(A)⁺ RNA was precipitated by using the procedure of Osterburg *et al.* (1975), for small amounts of RNA. From [³H]poly(U)-hybridization results, the overall yield of the poly(A)⁺ RNA after these purification procedures is 80–90% of that present in the original cytoplasmic RNA samples. The use of ribonuclease inhibitors during the oligo(dT)–cellulose chromatography of the RNA was avoided for fear of their coprecipitating with the purified poly(A)⁺ mRNA and thus affecting the translatability of the samples. RNA preparations were routinely stored under liquid nitrogen.

[³H]Poly(U) hybridization

The procedure used was based on that of Bishop *et al.* (1974), as amended by Safer *et al.* (1979). The hybridization reaction was carried out in 0.3 ml final volume, except that when fractions from sucrose gradients were assayed the volume was 0.75 ml. [³H]Poly(U) was supplied by Miles Laboratories, Slough, Bucks., U.K.

Preparation and fractionation of [³H]poly(A) chains

Labelled RNA was prepared from lymphocytes that had been cultured for 20h in the presence of [2-³H]adenine (1 μCi/ml; The Radiochemical Centre, Amersham, Bucks., U.K.). Samples of [³H]RNA containing 20000 c.p.m. were subjected to ribonuclease treatment as described by Rosbash & Ford (1974), in order to leave only the poly(A) chains intact. Electrophoresis was carried out on 10cm 13% acrylamide gels at 7mA/gel essentially as described by Loening (1969), by using phosphate buffer in the presence of 0.1% sodium dodecyl sulphate. A marker gel which contained 4S and 5S RNA was run in parallel with the poly(A) gels. The positions of the markers at the end of a run were ascertained by staining with Methylene Blue by the method of Peacock & Dingman (1967). The gels were sliced into 3mm fractions by using a Mickle gel slicer and the RNA was eluted from each fraction with 0.2 ml of Soluene (Packard Instrument Co., Cavenham, Bucks., U.K.) at 37°C for 30min. The radioactivity present was determined as described for [³H]poly(U) hybrids.

Sedimentation of RNA through sucrose gradients

Cytoplasmic RNA samples (0.5mg) were resuspended in acetate buffer (0.1M-NaCl/0.01M-sodium acetate, pH 5.2) and sedimented through 12 ml linear 5–20% (w/v) sucrose gradients in acetate buffer at 20000 rev./min for 20h in a Beckman SW40 rotor at 4°C: 0.42 ml fractions were collected. The positions of marker RNA species (4–5S, 18S and 28S) were ascertained by determining the radioactivity present in fractions of a parallel gradient containing lymphocyte cytoplasmic [³H]RNA.

Preparation and use of the wheat-germ cell-free system

Wheat-germ cell-free systems were prepared as described by Roberts & Paterson (1973), except that the magnesium acetate concentration was 1.25 mM, the ATP concentration 0.1 mM and preincubation was for 30min at 22°C. Incubation conditions for the protein synthesis assay were 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.0, 2 mM-dithiothreitol, 8 mM-phosphocreatine, 1 mM-ATP, 0.2 mM-GTP, 20 μM-amino acids (except methionine), 80 mM-KCl, 0.2 mM-spermidine and 40 μCi of [³⁵S]methionine (The Radiochemical Centre)/ml. Final incubation volumes were 30 μl, of which half was the wheat-germ extract, and incubation was at 23°C for 90min. The final K⁺ and Mg²⁺ concentrations were optimal for protein synthesis directed by lymphocyte poly(A)⁺ mRNA. Protein synthesis was halted by immersion of the tubes in ice. Samples (20 μl) from each incubation were applied to Whatman no. 1 filter-paper circles. The filters were boiled for 10min in 10% (w/v) trichloroacetic acid containing 1 mg of methionine/ml, and rinsed

sequentially with 5% trichloroacetic acid, ethanol and ether before determination of the radioactivity incorporated by counting in a Beckman LS-233 liquid-scintillation counter.

Preparation and use of the rabbit reticulocyte cell-free system

The preparation, preincubation with sparsomycin, incubation conditions, sucrose-gradient-fractionation procedure and determination of the presence of [^{35}S]methionyl-tRNA^{Met} in the 0.22 ml fractions were as described by Kay *et al.* (1978).

Preparation and use of the pig lymphocyte cell-free systems

The preparation of pig lymphocyte cytoplasmic extracts was as described by Kay *et al.* (1975). The final incubation conditions were 80mM-KCl, 4mM-magnesium acetate, 25mM-Tris/HCl, pH 7.6, 6mM-mercaptoethanol, 1mM-ATP, 0.2mM-GTP, 4mM-phosphocreatine, 0.5mg of creatine kinase/ml, 20 μ M-amino acids (except methionine) and 0.5–1.0pmol of [^{35}S]methionyl-tRNA^{Met} per 100 μ l incubation. Half the final incubation volume was pig lymphocyte extract. Incubations were carried out at 37°C for 2min and the mixture was directly layered on to sucrose gradients, the samples being then treated exactly as for reticulocyte cell-free systems.

Results and Discussion

Yield and purity of cytoplasmic poly(A)⁺ RNA

The RNA recovered in the poly(A)⁺ fraction after two cycles of oligo(dT)–cellulose chromatography represented $0.4 \pm 0.1\%$ (s.d. from nine expts.) of the total cytoplasmic RNA from unstimulated lymphocytes and $0.9 \pm 0.2\%$ (s.d. from seven expts.) of the RNA from lymphocytes that had been incubated with phytohaemagglutinin for 20h. The 20h incubation with phytohaemagglutinin increased the total amount of cytoplasmic RNA from 1.1 to 2.3pg per lymphocyte, giving a 4.7-fold increase in the amount of cytoplasmic poly(A)⁺ RNA per cell at this time. This value is in good agreement with the 4-fold increase found by Jagus-Smith & Kay (1976) using a [^3H]poly(U) hybridization method. The increase in protein synthesis at this time was 7–10-fold.

The sedimentation pattern of the cytoplasmic poly(A)⁺ RNA is shown in Fig. 1. The major peak was between 10 and 18S and the size distribution was similar for unstimulated and phytohaemagglutinin-activated lymphocytes. These sedimentation profiles are similar to those reported for labelled human lymphocyte polyribosomal and cytoplasmic poly(A)⁺ RNA (Rosenfeld *et al.*, 1972; Berger & Cooper,

Fig. 1. Size distribution of poly(A)⁺ RNA. RNA samples (0.5mg) from unstimulated (○) or 20h phytohaemagglutinin-activated (●) cells were centrifuged on sucrose gradients, fractionated and the [^3H]poly(U) hybridization to each fraction was determined as described in the Experimental section.

1975), assuming that the poly(A) chain length does not vary as a function of the RNA size (Adesnik & Darnell, 1972). As it has been reported that much of the lymphocyte cytoplasmic poly(A)⁺ RNA has a half-life of more than 20h (Berger & Cooper, 1975), these earlier studies with radiolabelled RNA would represent only the more rapidly labelled component, whereas the data presented here apply to the steady-state population. The size distribution of the steady-state poly(A)⁺ RNA is also compatible with that of the proteins synthesized by activated pig lymphocytes (results not shown).

The migration on polyacrylamide gels of poly(A) tracts prepared from cytoplasmic RNA by nuclease treatment is shown in Fig. 2. For both preparations most of the poly(A) tracts run as a discrete peak just ahead of the tRNA. When allowance is made for the anomalously low mobility of poly(A) on polyacrylamide gels (Kaufman & Gross, 1974), the mean length of these tracts can be calculated as 52 nucleotides. Taking the average molecular weight of the poly(A)⁺ RNA as 3.5×10^5 (calculated from Fig. 1), that of the poly(A) tracts as 1.7×10^4 (Fig. 2) and the maximum hybridizability of [^3H]poly(U) of the specific radioactivity used in these experiments to poly(A) as 77×10^3 c.p.m./ μ g of poly(A), it can be calculated that 1 μ g of a pure sample of cytoplasmic poly(A)⁺ RNA would hybridize with approx. 3800 c.p.m. of [^3H]poly(U). [^3H]Poly(U) hybridiza-

tion with cytoplasmic poly(A)⁺ RNA prepared by two cycles of oligo(dT)-cellulose chromatography gave 3379–4659 c.p.m./μg of RNA for unstimulated lymphocytes and 3751–4593 c.p.m./μg for 20h-phytohaemagglutinin-activated lymphocytes. Values obtained after a single cycle of oligo(dT)-cellulose chromatography were always below 2500 c.p.m./μg of RNA, and in some experiments they were sub-

Fig. 2. Size distribution of poly(A)⁺ chains of cytoplasmic RNA

[³H]Poly(A) chains obtained by ribonuclease-T₁ and ribonuclease-A digestion of cytoplasmic RNA isolated from unstimulated (○) or 20h-phytohaemagglutinin-activated (●) lymphocytes cultured with [³H]adenine were subjected to electrophoresis on 10cm 13% polyacrylamide disc gels. For further details see the Experimental section.

Fig. 3. [³⁵S]Methionine incorporation into protein by a wheat-germ cell-free system on addition of purified cytoplasmic poly(A)⁺ RNA prepared from unstimulated (○) or 20h phytohaemagglutinin-activated (●) lymphocytes. For procedural details see the Experimental section.

stantially below this value for RNA from unstimulated cells.

The conclusion that at least two cycles of oligo(dT)-cellulose chromatography are necessary to obtain poly(A)⁺ RNA of acceptable purity is in agreement with observations made with other types of cells (Desrosiers *et al.*, 1975; Bantle *et al.*, 1976; Hereford & Rosbash, 1977). After a single cycle over 50% of the RNA retained by the column may be contaminant species, and an even greater contamination may be observed if the intermediate elution step with 0.1 M-KCl is omitted. The earlier report by Hemminki (1975) that poly(A)⁺ RNA represented 3.9% and 4.1% of the cytoplasmic RNA of unstimulated and 20h phytohaemagglutinin-activated lymphocytes respectively is almost certainly accounted for by this technical problem. The low proportions of poly(A)⁺ RNA observed in our studies are comparable with those seen in other cells when care is taken to ensure the purity of the poly(A)⁺ RNA (Desrosiers *et al.*, 1975; Craig *et al.*, 1976). Failure to achieve such purity would completely invalidate comparisons of the biological activities of poly(A)⁺ RNA preparations from different sources.

Translational activity of poly(A)⁺ RNA from unstimulated and phytohaemagglutinin-activated lymphocytes

Purified cytoplasmic poly(A)⁺ RNA preparations from unstimulated and 20h phytohaemagglutinin-

Fig. 4. Formation of 80S initiation complexes in sparsomycin-pretreated reticulocyte cell-free systems by purified lymphocyte cytoplasmic poly(A)⁺ RNA

The incorporation of [³⁵S]methionyl-tRNA^{Met} into 80S initiation complexes was determined after sucrose-gradient centrifugation of cell-free systems incubated with poly(A)⁺ RNA from unstimulated (○) or 20h phytohaemagglutinin-activated (●) lymphocytes as described in the Experimental section.

activated lymphocytes are translated with equal efficiency by a wheat-germ cell-free system (Fig. 3). When mRNA prepared with only a single cycle of oligo(dT)-cellulose chromatography was used in such experiments, a larger amount of mRNA was required to give maximum stimulation, and in some experiments with RNA from unstimulated lymphocytes a lower maximum extent of stimulation was obtained.

There was an excellent correlation between the degree of purity of a mRNA population, as determined by its hybridization with [^3H]poly(U), and its translatability. The low stimulations observed with partially purified preparations of poly(A) $^+$ RNA from unstimulated lymphocytes could be due to their contamination by a translational inhibitor such as that reported by Wettenhall & Slobbe (1976).

Fig. 5. Initiation complex formation by cell-free systems from unstimulated (a, b and c) or 20h phytohaemagglutinin-activated (d, e and f) lymphocytes supplemented with purified cytoplasmic poly(A) $^+$ RNA from 20h phytohaemagglutinin-activated lymphocytes

Incubations containing no added mRNA (a, d), 2.2 μg of mRNA (b, e) or 3.9 μg of RNA (c, f) were incubated, centrifuged on sucrose gradients and fractionated as described in the Experimental section.

Translation of the poly(A)⁺ RNA from unstimulated lymphocytes did not require addition of 5'-terminal cap structures by enzymes present in the wheat-germ cell-free system. Addition of 160 μ M-S-adenosylhomocysteine, which inhibits the capping of uncapped mRNA in this system (Both *et al.*, 1975), did not affect the translation of either poly(A)⁺ RNA preparation (results not shown).

Fig. 4 shows that poly(A)⁺ RNA preparations from unstimulated and 20h phytohaemagglutinin-activated lymphocytes also had similar abilities to stimulate formation of 80S initiation complexes in reticulocyte lysates made mRNA-dependent by preincubation with sparsomycin (Darnbrough *et al.*, 1973). A similar comparison was also carried out with cell-free systems from phytohaemagglutinin-activated lymphocytes. Addition of 3.9 μ g of purified poly(A)⁺ RNA from unstimulated lymphocytes (a limiting amount) resulted in the transfer of an additional 1539 c.p.m. from [³⁵S]methionyl-tRNA^{Met} into 80S initiation complexes, compared with 1576 c.p.m. when 3.9 μ g of poly(A)⁺ RNA from activated lymphocytes was used.

In contrast, comparison of initiation-complex formation in cell-free systems from unstimulated and 20h phytohaemagglutinin-activated lymphocytes showed that the systems from unstimulated lymphocytes not only formed fewer endogenous complexes but also utilized added poly(A)⁺ RNA much less efficiently than those from activated cells, whatever the source of the mRNA. Fig. 5 shows that 3.9 μ g of poly(A)⁺ RNA from activated lymphocytes resulted in the transfer of an additional 418 c.p.m. from [³⁵S]methionyl-tRNA^{Met} into 80S initiation complexes when added to a cell-free system from unstimulated lymphocytes, compared with 1576 c.p.m. when added to a comparable system, containing the same number of ribosomes, from activated cells. As hydrolysis of [³⁵S]methionyl-tRNA^{Met} during the brief incubation is negligible, and its dilution by endogenous initiator tRNA is identical in systems from unstimulated and activated cells (results not shown), this confirms the conclusion that the lesser formation of initiation complexes in unstimulated lymphocytes is due to lack of some components other than mRNA.

The mRNA present in unstimulated lymphocytes thus appears, by all the criteria studied here, to be functionally equivalent to that in activated lymphocytes. This work confirms, by a more direct method, that the increase in amount of cytoplasmic poly(A)⁺ RNA after activation is insufficient to account for the increase in protein synthesis observed (Jagus-Smith & Kay, 1976), so that the low rate of protein synthesis in unstimulated lymphocytes must be due to inefficient utilization of the mRNA present in the cells, a conclusion supported by the inefficient utilization of added mRNA by cell-free systems derived from them. This low rate of utilization of the mRNA may be due

to a lack of initiation factors (Ahern *et al.*, 1974), or to the presence of translational inhibitors (Kay *et al.*, 1978).

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