GROWTH-RELATED FLUCTUATION IN MESSENGER RNA UTILIZATION IN ANIMAL CELLS

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ABSTRACT

Monkey fibroblasts maintained in culture regulate their levels of intracellular protein throughout the growth cycle by means of variations in the rate of protein biosynthesis. Cytoplasmic mRNA in stationary phase cells was compared to that in exponential phase cells. In stationary phase cells 56% of the cytoplasmic polyadenylated RNA was found in the 40–90S postpolysomal region of sucrose sedimentation gradients, while only 23% was found in this region in exponential phase cells. Analysis of electron micrographs of sectioned exponential and stationary phase cells revealed that this shift in polyadenylated RNA location is accompanied by a loss of polysome-like aggregates of ribosomes. Most if not all of this species of postpolysomal polyadenylated RNA is not being translated by single ribosomes since no detectable amounts of nascent peptide were present in this region. This nonpolysomal polyadenylated RNA is comparable in size to polysomal polyadenylated RNA. The length of the 3′-poly(A) tract was also comparable for these two species. The extent of capping of poly(A)-containing molecules was also comparable for these two species. The template activity of nonpolysomal RNA in a wheat germ extract was comparable to that of polysomal RNA. The peptides produced by these two preparations were of a similar large size. Furthermore, most of the nonpolysomal polyadenylated RNA of stationary phase cells was driven into polysomes in the presence of a low dose of cycloheximide. Therefore, we conclude that the untranslated mRNA that accumulates in stationary phase cells is structurally intact, is fully capable of being translated, and is not being translated due to the operation of a translational initiation block.

KEY WORDS untranslated mRNA · cultured cells · density dependent growth · m^7G poly(A) · in vitro translation

Certain cultured cell types are subject to density-dependent regulation of cell division (48). During a growth cycle, as these cells attain a characteristic high cell density (cell/cm^2), they begin to divide much more slowly. These cells thus can exist in two interchangeable states: the state of rapid cell division (growing or the exponential phase), and the state of slow cell division (resting or the stationary phase). Thus, their growth characteristics are analogous to those of certain cell types within the animal body, for example, hepatocytes, fibroblasts, and kidney cells (7, 11, 19, 39), which can also experience states of slow division or of rapid division depending upon their circumstances.

In cultured animal cells it has been shown that,

as the rate of cell division declines, there is a significant decrease in the rate of ribosomal RNA synthesis (12) and an increase in the rate of ribosomal RNA degradation (12, 37, 66). There is also a decrease in the ribosome content in the cell (3, 15, 41), as well as a decrease in the relative specific activity of elongation factor I and several amino acyl-tRNA synthetases (15). In other words, at a time when the rate of cellular protein synthesis is decreasing, there is a general stepdown of the protein synthetic machinery. Furthermore, the proportion of ribosomes that are not engaged in protein synthesis is increased (15, 36, 37, 49, 50, 59), and also cytoplasmic polyadenylated RNA is accumulated in the nonpolysomal fraction of stationary phase cells (2, 41, 49, 50). These nonpolysomal polyadenylated RNA species and nontranslating ribosomes can be driven into the polysomal fraction in the presence of a low dose of cycloheximide (2, 16, 58). Thus, the accumulation of ribosomes and polyadenylated RNA in the nonpolysomal fraction has led to the postulation that the decline of protein synthesis in resting cells is due to a translational block (59).

However, a divergent conclusion has been reached from experiments with cells resting in growth-limiting concentrations of serum. Under this condition, it was found that most if not all of the cytoplasmic polyadenylated RNA and ribosomes are engaged in translation (25, 38), allowing the conclusion that the extent of translation in resting cells is regulated by the amount of cytoplasmic mRNA.

In this study, we have grown Vero cells with daily medium change. The cells became stationary at a high density with minimal depletion of essential growth-promoting components in a condition more closely analogous to that in living tissues (31). We then asked whether the decrease in protein synthesis observed as cells enter the stationary phase is due to a decrease in the amount or the extent of utilization of mRNA. Since we found both, we asked further whether the nonpolysomal mRNA differed from the polysomal mRNA in structure or function.

MATERIALS AND METHODS

Cell Growth

Vero cells were purchased from American Type Culture Collection. They were grown in Dulbecco's modified Eagle's medium (DME) (Gibco, Grand Island Biological Co., Grand Island, N. Y.) plus 10% (vol/vol) calf serum (Flow Laboratory, Rockville, Md.) at 37°C in the presence of 5% CO_2 and 100% humidity. When exponential phase cells and stationary phase cells were compared, the cells were plated at a density of 1×10^4 cells/cm^2 and the growth medium was replaced daily. 3–4-day cultures at a density of $5–10 \times 10^4$ cells/cm^2 were used as exponential phase cells. 10–11-day cultures were used as stationary phase cells. These cells had reached the saturation density of $\sim 1 \times 10^6$ cells/cm^2 by day 8 or 9.

Radioactive Labeling

When cells were pulse labeled with [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.; 1,000Ci/mmol), unlabeled methionine was deleted from the DME, although trace amounts were provided by undialysed calf serum. To label the nascent peptide chain, regular growth medium was removed and prewarmed medium containing [^{35}S]methionine at 50 μCi/ml was added. Cells were incubated at 37°C for 2.5 min. At the end of this time, the monolayers were washed twice with ice-cold phosphate-buffered saline (150 mM NaCl, 4 mM KCl, and 9.5 mM phosphate, pH 7.2) and processed immediately for sucrose gradient analysis. The trichloroacetic acid-precipitable radioactivity in each fraction was determined by scintillation counting.

When cells were labeled with $^{32}PO_4$ (New England Nuclear, Boston, Mass.; carrier free), the concentration of orthophosphate in the growth medium was reduced to 1/100 of the regular level. Cells were grown in the presence of $^{32}PO_4$ at 50 μCi/ml for 24 h. Under this condition, no reduction in the amount of polysomes was detected as compared to cells grown in the absence of $^{32}PO_4$, although the generation time of growing cells was slightly increased (30 h as compared to 22–24 h).

Cell Fractionation

Monolayers were washed twice with ice-cold phosphate-buffered saline. Hypotonic lysis buffer (15 mM NaCl, 15 mM Tris-HCl, pH 8.3, and 16.5 mM $MgCl_2$ with 0.2% (vol/vol) diethylpyrocarbonate added immediately before use) was added. Cells were removed from plastic dishes with a rubber scraper, and the plasma membrane was disrupted in a stainless-steel piston homogenizer (clearance 0.015 inch). After nuclei and cell debris were centrifuged into a pellet at 1,000 *g* for 7 min, the supernate was adjusted with 10% (vol/vol) NP-40 (Shell Chemical Co., Chicago, Ill.) to 0.5%. The final volume of cell extract was 1.5-fold that of the lysis buffer applied. The entire operation was carried out at 4°C. Sucrose gradients of 15–50% (wt/vol) with a sucrose cushion of $^1/_{25}$ of the volume of 70% (wt/vol) were routinely used to separate ribosome subunits and monosomes from polysomes. All sucrose solutions were made in 10 mM NaCl, 10 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, saturated with diethyl pyrocarbonate. The

optical profile of each gradient was monitored with an ISCO UV-flow analyser. (ISCO, Lincoln, Neb.).

RNA Preparation

Whole cytoplasmic RNA was extracted from cytoplasmic extracts with phenolchloroform-isoamyl alcohol (50:48:2) in the presence of 0.1 M NaCl, 0.5% sodium dodecyl sulfate (SDS), 15 mM EDTA, and 10 mM Tris-HCl, pH 7.6, and precipitated twice in 2.5 vol of absolute ethanol at −20°C for 15 h each. It was then dissolved in a small amount of autoclaved water and dried by lyophilization.

Nonpolysomal and polysomal RNA, extracted from material sedimenting at 40–90S and >90S, respectively, were prepared from the appropriate sucrose gradient fractions. Fractions were collected into tubes containing a sufficient amount of 10% (wt/vol) SDS to make an approximate final concentration of 0.5%. The pooled fractions were adjusted to 0.1 M NaCl, and then extracted with phenol-chloroform-isoamyl alcohol (50:48:2) as described above.

The Isolation of Polyadenylated RNA

Polyadenylated RNA was isolated by a one-step procedure (33). Specifically, the postpolysomal and polysomal regions of sucrose gradients were collected into 10% SDS and adjusted to a final concentration of 0.4 NaCl, 0.5% SDS, and with a sucrose concentration of lower than 20%. The mixture was subsequently subjected to poly(U)-affinity chromatography. Poly(U) (Sigma Chemical Co., St. Louis, Mo.) was immobolized to GF/C glass fiber filters (Whatman, Inc., Clifton, N. J.) by the method of Sheldon et al. (54) at 500 μg/filter. Four filters were then cut into 1-mm² pieces and packed into a siliconized Pasteur pipet with a bed volume of ~0.75 ml, as described by Silverstein et al. (55). After extensive washing with elution buffer (70% (vol/vol) formamide, 5 mM EDTA, 10 mM Tris-HCl, pH 7.6, and 0.5% SDS) and equilibration with hybridization buffer (0.4 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.6, and 0.5% SDS), the column has the capacity to bind more than 500 μg of poly(A). Although the upper limit of the binding capacity has never been tested, this capacity is more than 1,000-fold in excess of what is actually required in our isolation procedure. The chromatography is routinely carried out at 37°C. The elution of poly(U) from the glass fiber has been tested over a period of 48 h and found to be undetectable. When polyadenylated RNA was to be isolated, the sucrose solution was passed through the column at 10 ml/hr. Then the column was washed with 50 ml of hybridization buffer at the same flow rate. The polyadenylated RNA was eluted with 3 ml of elution buffer over a period of 30 min. 4 M NaCl was added to the eluted material to give a final concentration of 0.2 M, and the RNA was precipitated in the presence of 3 vol of absolute ethanol for 15 h at −20°C. With this procedure, the recovery of polyadenylated RNA is over 95%. In isolating ^{32}P-labeled polyadenylated RNA, 50 μg of *Escherichia coli* tRNA was added as carrier during ethanol precipitation.

The Size Determination of Polyadenylated RNA and Poly(A)-Fragments

To determine the length of poly(A), RNA samples were digested with ribonuclease A (RAF, Worthington, Biochemical Corp., Freehold, N. J.) at 20 μg/ml and ribonuclease T_1 (RT1, Worthington) at 20 U/ml in the presence of 0.3 M NaCl, 30 mM Tris-HCl, pH 7.4, and 20 mM EDTA, at 37°C for 30 min. At the end of digestion, 10% SDS was added to a final concentration of 1% followed by *E. coli* tRNA to 50 μg/ml as carrier, and 3 vol of absolute ethanol. Precipitation was carried out at −20°C for 15 h.

The size of polyadenylated RNA and poly(A)-fragments was determined by polyacrylamide gel electrophoresis as described by Bishop et al. (4). Gels were polymerized in 0.5 × 13.2 cm glass tubes. RNA samples were dissolved in 5 mM Tris-acetate, pH 7.2, 2 mM EDTA, 0.2% SDS, and 50% (vol/vol) formamide, and heated at 75°C for 5 min before being loaded on gels. Bromphenol blue was used as the tracking dye. Electrophoresis was carried out at 90 V. At the end of the run, the gels were extruded from the glass tubes, placed in a rectangular quartz cell, and scanned in a Gilford gel scanner at 260 nm to locate the positions of various RNA size markers. To determine the electrophoretic mobility of polyadenylated RNA, gels were frozen and sliced into 0.125 cm thick pieces. Groups of six adjacent slices were soaked in 0.5 ml of 0.375 M NaCl, 0.0375 M Tris-HCl, pH 7.4, and 0.5% SDS at 37°C overnight to elute the RNA. The poly(A) content in each fraction was determined by [^{3}H]poly(U) hybridization.

^{3}H-Poly(U) Hybridization

^{3}H-Poly(U) with a specific activity of 300 cpm/ng was prepared by the method of Bishop et al. (5) and used throughout this sudy. Hybridization was performed as described by Rosbash and Ford (46). Namely, hybridization was carried out in 0.3 M NaCl, 0.03 M Tris-HCl, pH 7.4, and 0.5% SDS. The amount of [^{3}H]poly(U) used was 5–10-fold in excess of the estimated amount of poly(A) in the reaction. After being incubated at 45°C for 10 min, the reaction mixtures were chilled on ice, and diluted with 10 vol of ice-cold 0.3 M NaCl, and 0.03 M Tris-HCl, pH 7.4. Ribonuclease A was added to a concentration of 20 μg/ml, and the mixture was incubated for 10 min. at 0°C. Ribonuclease A-resistant radioactivity was precipitated by adding trichloracetic acid, to 12%, in the presence of carrier. The precipitate was collected on Millipore filters (Millipore Corp., Bedford, Mass.) and counted in a scintillation spectrophotometer.

The Measurement of 5'-Capping in the Polyadenylated RNA

^{32}P-labeled polyadenylated RNA was dissolved in 0.3 ml of autoclaved water. 2 μl were removed and added to 0.5 ml of 0.3 M NaCl, 30 mM Tris-HCl, pH 7.4, 20 mM EDTA, and 10 μg of RNAse A and 10 U of RNAse T_1 were added. After incubation at 37°C for 30 min, trichloracetic acid precipitable radioactivity was determined in the presence of carrier, and used as a measure of the amount of poly(A) in the RNA preparation.

To the rest of the sample, sodium acetate, pH 4.5, and EDTA were added to a final concentration of 50 mM and 20 mM, respectively. Complete enzymatic digestion of the RNA was achieved with 10 μg of RNAse A, 10 U of RNAse T_1 and 30 U of RNAse T_2 (Sankyo Co., Tokyo) at 37°C for 15 h. Additional fresh enzymes were added 2 h before the termination of the digestion. DEAE-sephadex (A-25, Pharmacia Inc., Piscataway, N. J.) chromatography was performed as described by Perry and Kelly (43). 0.7 × 20 cm columns with 8 ml of DEAE-sephadex equilibrated in 20 mM Tris-HCl, pH 7.6, and 7 M urea were used. The RNA digest was mixed with 150 μg of oligo(A) as charge markers, and loaded on the column in 4 ml of 20 mM Tris-HCl, pH 7.6, and 7 M urea (Tris-urea buffer). 40 ml of 0.135 M NaCl in Tris-urea buffer was used to wash through the mononucleotides and dinucleotides, then the more highly charged material was eluted with 85 ml of a linear gradient of 0.14–0.4 M NaCl in Tris-urea buffer. With this procedure, the material eluting between -5 and -6 charge was essentially free of contamination by lower charged material. At the end of the chromatography, 1 M NaCl in Tris-urea buffer was used to wash the column; little radioactivity was detected in the wash. The optical profile of the eluate was measured by a LKB Uvicord II UV monitor (LKB Instruments, Inc., Rockville, Md.). 1.97-ml fractions were collected. 0.4-ml aliquots were taken to measure the amount of radioactivity. The flow rate was 10 ml/h throughout the chromatography. The material with charge -5 to -6 was further treated with nuclease P1 and bacterial alkaline phosphatase, and the digestion product was shown to contain the dinucleotide m^7GpppX by thin-layer chromatography in the presence of known standards (P-L Biochemicals, Inc., Milwaukee, Wis.) (data not shown), according to the procedure of Schibler and Perry (52).

In Vitro Translation in Wheat Germ Extract

The wheat germ extract was prepared essentially as described by Marcu and Duddock (40), except that 20 mM KCl, 30 mM K acetate, 5 mM HEPES-KOH, pH 7.6, 1 mM Mg acetate, 0.1 mM $CaCl_2$, 0.2 mM spermidine, and 1 mM dithiothreitol were used at the step of Sephadex G-25 chromatography. Small aliquots were stored at −70°C. The translation reaction contains: 6 mM HEPES-KOH, pH 7.6, 0.3 mM ATP, 0.024 mM GTP, 1.92 mM creatine phosphate, 6 μg/ml creatine phosphokinase, 1.45 mM Mg^{+2}, 111 mM K^+, 0.02 mM Ca^{+2}, 0.12 mM spermidine, 2 mM dithiothreitol, and 30 μM each of the 19 amino acids and the 20th amino acid isotope-labeled. 10 μl of wheat germ extract (1.9 OD_{260}) was used in a 50 μl reaction. The reaction was carried out at 29°C. The amino acid incorporation was linear for at least 75 min, and continued for more than 120 min. Translation was terminated by adding 0.4 ml of 0.1 M KOH. This material was incubated for an additional 20 min at 29°C, then 2 ml of 12% (wt/vol) trichloracetic acid was added. It was then allowed 10 min on ice. The precipitates were collected on GF/A glass fiber filters, washed with absolute ethanol, and processed for scintillation counting.

When the translation products were to be analyzed by gel electrophoresis, [^{35}S]methionine (New England Nuclear, 600 Ci/mmol) was used. 1-μl aliquots of the reaction were added to 0.4 ml of 0.1 M KOH to determine the trichloroacetic acid-precipitable radioactivity. To the rest of the reaction, 12 μl of 0.25 M Tris-HCl, pH 6.8, 5% SDS, 0.7 M mercaptoethanol and 20% glycerol was added. After being boiled for 2 min, the reaction mixture was subjected to electrophoresis on a 10% SDS polyacrylamide gel (34).

The Preparation of Thin Sections for Electron Microscopy

The cells were scraped off the monolayer, then washed with cold phosphate-buffered saline and fixed with 1% glutaldehyde in Sorenson's phosphate buffer (0.1 M sodium phosphate, pH 7.2) for 3 h at 4°C. The fixed cell pellet was then postfixed in osmium tetroxide and rapidly dehydrated in graded ethanols (30–100%), infiltrated overnight with propyline oxide, embedded, and mounted in Epon 812. Thin sections were cut with a Cambridge Ultramicrotome (Cambridge Instrument, Monsey, N. Y.). They were stained with uranyl acetate and lead citrate as described by Rifkind et al. (44), and examined in a Elmiskop 1A Siemens electron microscope.

RESULTS

The Distribution of Polyadenylated RNA in the Cytoplasm of Vero Cells

To determine whether all of the polyadenylated RNA in the cytoplasm is engaged in protein synthesis, its distribution in sucrose gradient sedimentation profiles was measured and is represented in Fig. 1. In exponential phase cells (Fig. 1*a*) the majority of the cytoplasmic polyadenylated RNA sediments in the region of the polysomes, while in stationary phase cells (Fig. 1*b*) it

sediments in the nonpolysomal region. The quantitation of this determination is reported in Table I.

Since the reduced levels of polysome-associated polyadenylated RNA as well as ribosomes in stationary phase cells could have resulted from the increased sensitivity of stationary phase cells to the preparation procedures (although precautions were taken to minimize cell breakage and ribonuclease activity), the state of the ribosomes within the cells was determined by electron microscopy. Thin sections of exponential phase cells and stationary phase cells are shown in Fig. 2. Although clear differences can be seen in the proportion of ribosomes associated in polysome-like structures in these sections, precise quantitation is difficult with this approach (15, 45). From repeated determinations, we estimated that $\sim^3/_4$ of the ribosomes in exponential phase cells are in polysome-like structure while only $^1/_4$ are in polysome-like structures in stationary phase cells.

From the relative sedimentation value of these polyadenylated RNA-containing structures (see Fig. 1), it is possible that some are associated with ribosomes as small functioning polysomes, as has been reported in resting lymphocytes (10). To test this, cells were pulse labeled with [^{35}S]methionine for 2.5 min, and the distribution of radioactivity was analyzed on a sucrose gradient. As can be seen in Fig. 3, no radioactivity is accumulated in the monosome regions. In fact, the ratio of peptide to polyadenylated RNA in this region is $<^1/_{40}$ that at the maximum point of polysomal polyadenylated RNA. Similar results were obtained with [^{3}H]leucine (data not shown). Thus, we conclude that most of the nonpolysomal polyadenylated RNA in stationary phase cells is not being translated by single ribosomes.

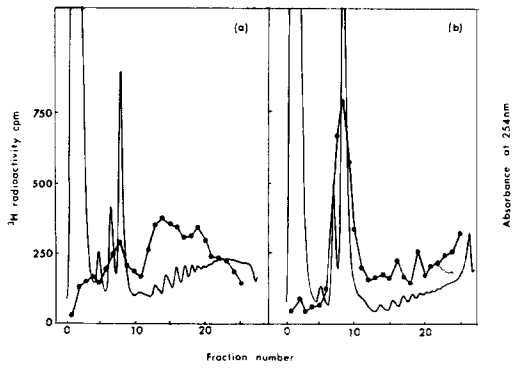

FIGURE 1 The distribution of polyadenylated RNA in the cytoplasm of Vero cells. Cytoplasmic extract was prepared as described in Materials and Methods. 0.25 ml of this cytoplasmic extract (approximately equivalent to 1×10^7 cells) was layered on 11.2 ml of 15–50% (wt/vol) sucrose gradient with 0.5 ml of 70% sucrose cushion. Centrifugation was carried out at 35,000 rpm for 150 min, in a SW41 rotor at 4°C. 0.5-ml fractions were collected into tubes containing 10% SDS. 0.15-ml aliquots of each fraction were taken for the analysis of poly(A) content, with the [^{3}H]poly(U) hybridization assay. Sedimentation is from left to right. — = A_{254}; ●–● = [^{3}H]poly(U) hybridized. (*a*) Exponential phase cells. (*b*) Stationary phase cells.

TABLE I

The Distribution of Polyadenylated RNA in the Cytoplasm of Vero cells

	% of total cytoplasmic poly(A)	
Cells	Polysomal	Nonpolysomal
Exponential phase	77 ± 4.8	23
Stationary phase	44 ± 7.3	56

The determination of the subcellular distribution of polyadenylated RNA was performed as described in Fig. 1. Polyadenylated RNA detected in the region of 40–90S is considered as nonpolysomal; that detected in the region greater than 90S is considered as polysomal. These percentages are the average of five separate determinations. The standard deviation is included.

In Vitro Translation of Vero Cell Cytoplasmic Polyadenylated RNA in a Wheat Germ Extract

mRNA content can also be measured by its capacity to promote protein synthesis when added to a cell-free translation system. We chose a system derived from fresh wheat germ. In this system, the amount of amino acid incorporation is directly proportional to the amount of exogenous RNA added up to 1.5 μg per 50-μl reaction, and 1 μg of cytoplasmic RNA from Vero cells (either exponential or stationary phase) leads to the incorporation of no less than 4 pmol of leucine. The template activity of nonpolysomal and polysomal RNA from exponential and stationary phase cells was determined (Table II). No significant differences were found when the nonpolysomal RNA from exponential phase cells and stationary phase cells was compared or when the polysomal RNA from these same cells was compared. However, the nonpolysomal RNA was slightly less active than the polysomal RNA, regardless of the growth state of the cells. The percentage of the total template activity present in these two fractions is also presented in Table II. It was found that, in

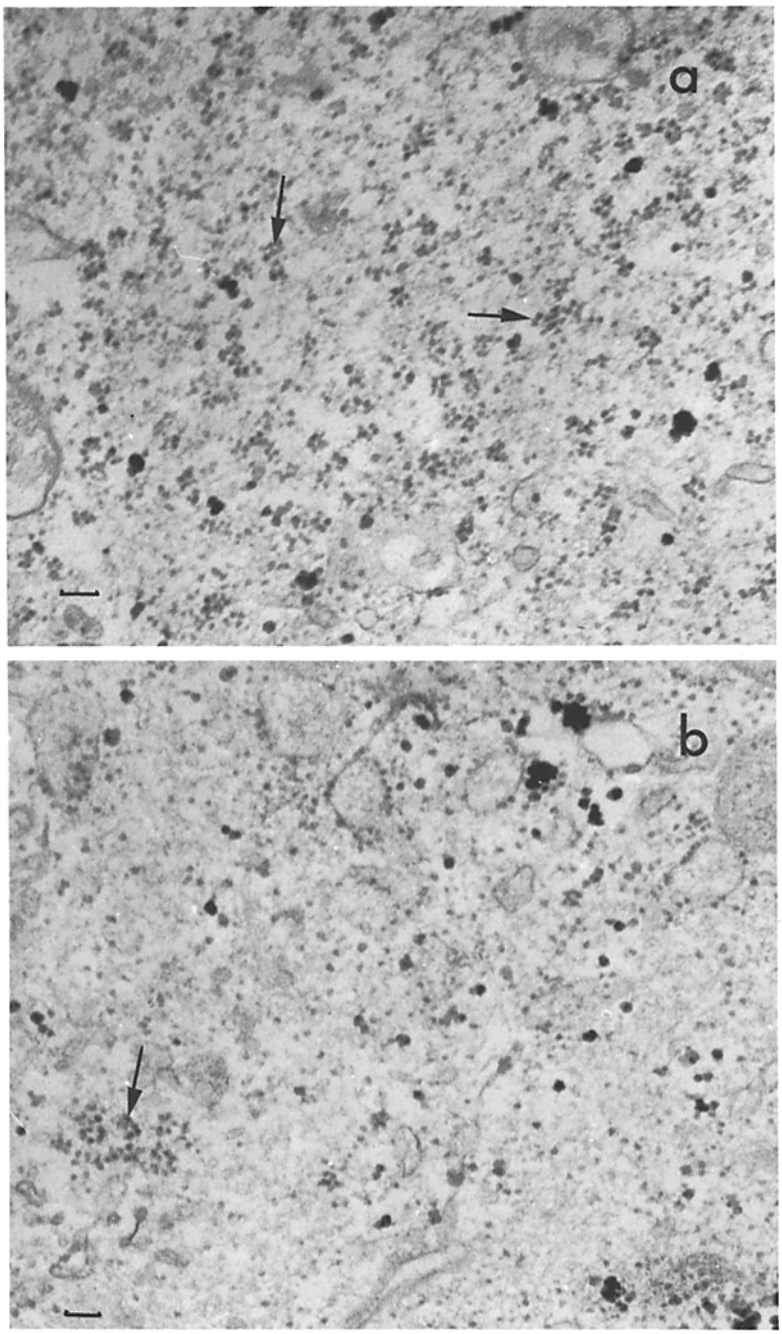

FIGURE 2 Electron micrographs of sectioned Vero cells. Arrows indicate the polysomelike structures. (*a*) The exponential phase cell. (*b*) The stationary phase cell. Bar, 1,000 Å.

exponential phase cells, 75% of the total template activity is present in polysomes, while in stationary phase cells over 60% is in nonpolysomal fractions. Therefore, there is an accumulation of mRNA, by this criterion, in the nonpolysomal fraction as cells enter the stationary phase. A similar conclusion was obtained with the use of preparations of polyadenylated RNA isolated by poly(U) affinity chromatography (Materials and Methods).

The products of in vitro translation were also

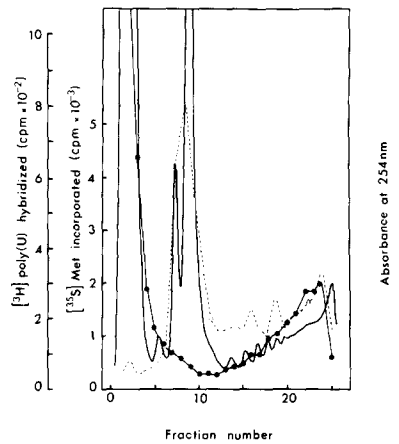

FIGURE 3 The distribution of nascent peptide chains in stationary phase Vero cells. Vero cells in stationary phase were labeled with [^{35}S]methionine for 2.5 min, as described in Materials and Methods, and processed for sucrose gradient analysis as described in the legend to Fig. 1. Trachloracetic acid-precipitable radioactivity was determined by scintillation counting. The cytoplasmic extract prepared from an unlabeled culture was analyzed on a parallel gradient, and the poly(A) content in each fraction was quantitated with [^{3}H]poly(U). $-=$ A_{254}; $\triangle-\triangle$ = [^{3}H]poly(U) hybridized; $\bullet-\bullet$ = [^{3}H]methionine incorporated.

TABLE II

The Template Activity of RNA Isolated from Cytoplasmic Fractions

RNA	Template activity (pmol leucine/ng of poly(A))		Total template activity
			%
Exponential phase	Nonpolysomal	5.2	26.2
	Polysomal	5.9	73.7
Stationary phase	Nonpolysomal	6.4	62.5
	Polysomal	7.3	37.5

Cytoplasmic RNA was fractionated, extracted, and translated under conditions which were optimized for the translation of Vero mRNA in a wheat germ extract as described in Materials and Methods. The translation was carried out at 29°C for 45 min. 1 μCi of [^{3}H]Leu at a final concentration of 8 μM was included in 50 μl reaction translation mixture. The template activity of each RNA preparation was then determined from the slope of the stimulation within the linear range of RNA concentrations. The percentage of total template contributed by each fraction was obtained from the product of the template activity times the amount of RNA of each fraction.

analysed by SDS-polyacrylamide gel electrophoresis (Materials and Methods) and found to be of comparable size. Detailed analysis of these data will be reported in a subsequent publication.

The Structural Characteristics of Polyadenylated RNA

The size of polyadenylated RNA from the nonpolysomal and polysomal fractions of exponential and stationary phase cells was determined by polyacrylamide gel electrophoresis and is shown in Fig. 4. The size of polysomal polyadenylated RNA in cells at the two growth states is indistinguishable, as is the size of nonpolysomal polyadenylated RNA. However, when the nonpolysomal and polysomal polyadenylated RNA are compared, the former has a slightly smaller modal size regardless of the growth state of the cell. This observation presents the possibility that the nonpolysomal polyadenylated RNA is partially degraded. To pursue this possibility the size of the poly(A) fragment on the 3′-terminus was measured and, then, the level at which the molecules with a 3′-poly(A) tract have the characteristic 5′-cap structure was determined. The ratio of cap structures to poly(A) fragments was used as a measure of the relative intactness of these molecules.

The length of poly(A) fragments at the 3′-terminus of the polyadenylated RNA was determined by polyacrylamide gel electrophoresis after digestion with ribonucleases A and T_1 (Materials

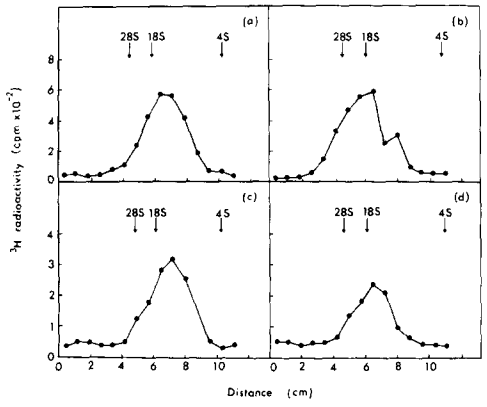

FIGURE 4 The size determination of polyadenylated RNA. RNA was subjected to electrophoresis in 2.4% polyacrylamide-0.5% agarose gels as described in Materials and Methods. The absorbance profile at 260 nm was determined to locate the positions of 28, 18, and 4S. The RNA in gel slices was eluted with hybridization buffer. The amount of poly(A) in each fraction was quantitated with [^{3}H]poly(U). (*a*) Nonpolysomal RNA from exponential phase cells, (*b*) Polysomal RNA from exponential phase cells, (*c*) Nonpolysomal RNA from stationary phase cells, (*d*) Polysomal RNA from stationary phase cells.

and Methods). The results are shown in Fig. 5. As can be seen, little, if any, size difference exists in any of the RNA preparations.

To determine the extent of capping at the 5'-terminus of the polyadenylated RNA, cells were grown in the presence of $^{32}PO_4$ for 24 h to label the majority of the cytoplasmic RNA. The polyadenylated RNA was then isolated and subjected to extensive digestion with ribonucleases (Materials and Methods). The amount of radioactivity in the cap structure was then determined by DEAE-Sephadex chromatography. The elution profiles of RNA digests prepared from the nonpolysomal and polysomal regions of exponential and stationary phase cells were similar, if not identical. A representative pattern is shown in Fig. 6. The results of this determination are reported in Table III. As can be seen, for every 100 nucleotide phosphates in poly(A), there are ~2.5 nucleotide phosphates in cap structures. No significant difference exists between nonpolysomal and polysomal polyadenylated RNA in this respect, regardless of whether they are in exponential or stationary phase cells. Therefore, we conclude that nonpolysomal polyadenylated RNA is intact relative to polysomal polyadenylated RNA.

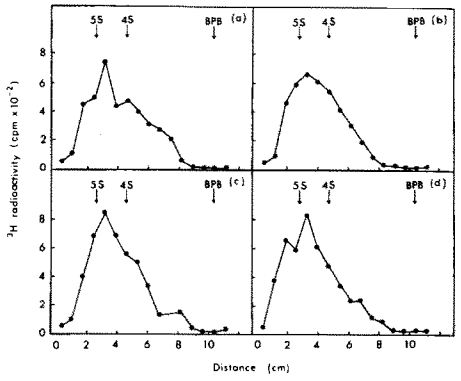

FIGURE 5 The length determination of poly(A) fragments. Poly(A) fragments prepared from various RNA samples were subjected to electrophoresis in 12.5% polyacrylamide gels as described in Materials and Methods. An RNA sample containing 4 and 5S RNA was loaded on a parallel gel to serve as size markers. Electrophoresis was carried out at 90 V until the bromphenol blue reached 1.5 cm from the bottom. The poly(A) fragments were eluted from the gel slices and then hybridized to [^{3}H]poly(U). (*a*) Nonpolysomal RNA from exponential phase cells, (*b*) Polysomal RNA from exponential phase cells, (*c*) Nonpolysomal RNA from stationary phase cells, (*d*) Polysomal RNA from stationary phase cells.

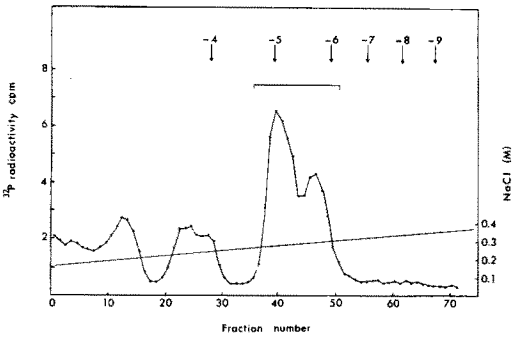

FIGURE 6 Chromatography on DEAE-sephadex column of enzymatic digests of polyadenylated RNA. ^{32}P-labeled polyadenylated RNA was digested with ribonucleases A, T_1, and T_2 as described in Materials and Methods, then loaded with oligo(A) of various lengths onto a DEAE-Sephadex column preequilibrated with 20 mM Tris-HCl, pH 7.6, and 7 M urea. The column was then washed with 40 ml of 0.135 M NaCl in Tris-urea buffer. The oligo nucleotides were then eluted with an 85-ml linear gradient of 0.14–0.4 M NaCl in Tris-urea buffer. The radioactivity in each fraction was determined by scintillation counting.

TABLE III

The Determination of 5'-Cap Structures in Polyadenylated RNA

Cells	Experiment	No. of nucleotide phosphates in cap structures per 100 phosphates in poly(A)	
		Nonpolysomal	Polysomal
Exponential phase	I	2.7	2.2
	II	2.3	2.1
Stationary phase	I	2.4	2.1
	II	3.1	2.5
Stationary phase + Cycloheximide	I	3.1	2.9

Cells were labeled for 24 h with $^{32}PO_4$ at 50 μCi/ml in DME plus 10% calf serum with 1% of regular level of phosphate. The polyadenylated RNA was isolated by poly(U)-GF/C column. The amount of radioactivity in cap structures was quantitated by DEAE-sephadex chromatography, and was compared to that in poly(A) fragments.

The Effect of Cycloheximide on the Translation of Polysomal Polyadenylated RNA

While the nonpolysomal polyadenylated RNA may be largely intact in both exponential and stationary phase cells, some subtle alteration may have rendered it incapable of initiating translation in vivo. Cycloheximide at a concentration that

partially inhibits protein synthesis has been shown to cause the accumulation of large polyribosomes in the cells (16, 58). The effect of 0.2 μg/ml of cycloheximide on the subcellular distribution of polyadenylated RNA in stationary phase cells is shown on Fig. 7. As can be seen under this condition, stationary phase cells resumed a distribution pattern almost identical to that of untreated exponential phase cells (Fig. 1*a*). The proportion of nonpolysomal polyadenylated RNA in the treated stationary phase cells was even slightly lower than that of untreated exponential phase cells. Thus, most of the nonpolysomal polyadenylated RNA in the stationary phase cells can function as a template for translation in vivo in the presence of cycloheximide.

Again, the intactness of the polyadenylated RNA present in the nonpolysomal fraction after cycloheximide treatment was tested, as described above, with respect to the length of poly(A) fragments and the extent of 5′-capping. This determination, presented in Table III, shows that the small portion (<10%) of polyadenylated RNA remaining in the nonpolysomal fraction is structurally intact by these criteria, and thus probably represents the basal level of cytoplasmic polyadenylated RNA that is not associated with polysomes.

The Level of Cytoplasmic Polyadenylated RNA in Exponential and Stationary Phase Cells

The proportion of poly(A) tracts in RNA isolated from the cytoplasmic extract sap by phenol-choroform-isoamyl alcohol extraction (Materials and Methods) from exponential and stationary phase cells was determined by saturation hybridization with [^{3}H]poly(U). For exponential cells it is 0.10 ± 0.024%, and for stationary phase cells it is 0.098 ± 0.019%. (These numbers are the average and standard deviation from four independent determinations.) Thus, no difference has been found between cells in these two growth states. Since the size of the poly(A) fragments is the same (Fig. 5) and the majority, if not all, of the poly(A) fragments are covalently linked to large RNA molecules (Fig. 4), the ratio of cytoplasmic polyadenylated RNA to ribosomes is constant in cells in different states of growth. Previous experiments in this laboratory have demonstrated a decreased level (to 21%) of ribosomes in stationary phase Vero cells (15). Therefore, we conclude that there is also a parallel decline in the amount of mRNA in stationary phase cells.

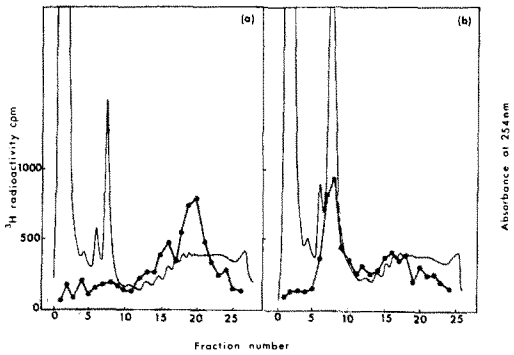

FIGURE 7 The effect of cycloheximide on the distribution of polyadenylated RNA in stationary phase cells. Cells that have reached stationary phase were treated with cycloheximide at 0.2 μg/ml for 45 min. Cytoplasmic extract was prepared, and the distribution of polyadenylated RNA was determined as described in Fig. 1. – = A_{254}; ●–● = [^{3}H]poly(U) hybridized. (*a*) Cells treated with cycloheximide, (*b*) Untreated controls.

DISCUSSION

Vero cells are derived from an African green monkey kidney (67). They grow with a typical epithelial-like pattern (17). They are aneuploid with a modal chromosome number of 55. Their growth requires serum, and is anchorage dependent and density inhibited.

The Existence of Nonpolysomal mRNA

In this study, the steady-state distribution of cytoplasmic polyadenylated RNA was measured. In the cytoplasmic extracts prepared from stationary phase cells, large amounts of polyadenylated RNA were present in the nonpolysomal region of the sucrose gradients (Fig. 1). The presence of this RNA could have been brought about by the lysis of nuclei or by the activation of a ribonuclease during the sample preparation. The first possibility was ruled out by the cycloheximide experiment (Fig. 7), since after treatment with a low dose the level of nonpolysomal polyadenylated RNA was significantly lowered. Nuclear polyadenylated RNA would not be expected to respond to this treatment. The second possibility, the effect of ribonuclease, was ruled out by the electron microscope study. Direct examination of thin sections of stationary phase cells revealed that the majority of the ribosomes were not in polysomal structures in the intact cells. The activation of a ribonuclease during extract preparation cannot explain this observation. Therefore, we conclude that this

nonpolysomal polyadenylated RNA is in fact present in the cytoplasm of the intact stationary phase cells.

The majority of the nonpolysomal polyadenylated RNA is present in structures with sedimentation values of 60–90S. This observation suggested that at least a portion of this nonpolysomal polyadenylated RNA may be translated by single ribosomes. However, the lack of detectable amounts of nascent polypeptide chains in this region (Fig. 3) excludes this possibility at least for the majority of these polyadenylated RNA-containing structures.

Untranslated cytoplasmic mRNA has been found in a wide range of cell types, for example, cells in the liver of fasting rats (22, 68), unfertilized sea urchin eggs (56), cultured cells under conditions where protein synthesis was interrupted (51, 64), and cells in the quiescent state of growth (2, 41, 49, 50). In this work, while the level of nonpolysomal mRNA was higher in stationary phase cells, the proportion of nonfunctioning ribosomes (0.5 M NaCl-dissociable 80S ribosomes as described by Kay et al. [29] and Cooper et al. [10]) was also higher (Lee, unpublished results). In fact, the ratio of polyadenylated RNA to ribosomes was the same in exponential phase and stationary phase cells, indicating that neither the supply of ribosomes nor the supply of mRNA is the limiting factor in the protein synthetic machinery. These observations suggest that the accumulation of nonpolysomal mRNA in the cytoplasm may represent a strategy of the cell to respond immediately to the elevated demand of protein synthesis during metabolic step up.

The Structural Characteristics of Nonpolysomal mRNA

The nonpolysomal mRNA observed in this work migrated slightly faster than the corresponding polysomal mRNA during electrophoresis (Fig. 4). At least two explanations for this are possible. Either this species of RNA has been degraded (either in the cell or as an artifact of isolation) or a subfraction of this RNA preparation is in fact naturally smaller. In this work, we have concerned ourselves particularly with the first possibility.

When the length of poly(A) fragments was analyzed by polyacrylamide gel electrophoresis, it was found to be comparable for nonpolysomal and polysomal polyadenylated RNA (Fig. 5). The extent of the cap modification of the polyadenylated RNA was also determined. Since any nucleolytic cleavage of the polyadenylated RNA molecule will affect the ratio of the nucleotide phosphate in caps to that in 3′-poly(A) fragments, this ratio was used as a measure of the relative intactness of the various polyadenylated RNA species. With this measurement, the nonpolysomal polyadenylated RNA was shown to be as intact as polysomal polyadenylated RNA. Several lines of evidence have suggested that for certain messenger RNA species the capped messenger molecules are selectively translated over the uncapped (30, 42, 47). The demonstration that nonpolysomal polyadenylated RNA is translated with similar efficiency in vitro (Table II), as well as this measure of the relative intactness of the molecules excludes the possibility that the utilization of mRNA is regulated by the enzymatic activity that blocks the 5′-terminus of mRNA in cells at different states of growth.

The finding that the cytoplasmic polyadenylated RNAs shift from the nonpolysomal region into the polysomal when the rate of peptide chain elongation is reduced with cycloheximide suggests that the nonpolysomal RNA can be translated in vivo. Thus, there are two populations of mRNA in stationary phase cells; one is translated at a higher frequency than the other. The difference between these two mRNA populations may reside in subtle structural modifications, such as methylation (6, 42), or in the presence of specific proteins bound to one or the other fraction, thus impeding translation (9, 63). It is also possible that these two populations of mRNA contain sequences that code for different peptides. Subsequent data from this laboratory support this latter suggestion (Lee and Engelhardt, manuscript in preparation).

The Effect of Growth Conditions on Nonpolysomal RNA

In general, one of two approaches has been adopted to effect the transition of cells between growing and resting states: growth into the stationary phase, or deprivation of an essential nutrient or growth factor. These two procedures have led to different results with regard to the questions of nonpolysomal mRNA accumulation. With the first procedure, where cells were permitted to progress into the stationary phase, it has been shown that protein synthesis diminishes (13, 36, 59, 61, 65). This is true even when medium is replaced continually by perfusion (8, 32) or by daily medium change (35). In this work, we

adopted the experimental approach of replacing the growth medium daily. This procedure eliminates the possibility that the cells have entered stationary phase because the growth medium has been depleted of growth factors (21, 24, 60) or essential nutrients (Pergolizzi and Engelhardt, unpublished data). Using this procedure, we found an accumulation of mRNA that is not being translated in stationary phase cells. This observation agrees with the observations made in other stationary phase cells grown in high concentration of serum (2, 41, 49).

The second general experimental approach used to effect the transition between growing and resting cells was the manipulation of the concentration of an essential nutrient or growth factor in the growth medium. For example, lowering the serum level leads to a decrease in protein synthesis (1, 20, 23, 25, 28, 53, 57). We do not distinguish here between serum deprivation (1) and lowering the serum concentration in the growth medium (18, 62), since conventional serum deprivation procedures never completely removed serum growth factors from the medium (21).

When exponential phase Vero cells were grown in the absence of serum for 20 h, there was a decrease in the rate of protein synthesis (20), but there was no accumulation of nonpolysomal polyadenylated RNA and ribosomes as was seen with stationary phase Vero cells (data not shown). Thus, this finding is consistent with the observation that cells maintained in medium containing a low concentration of serum (with periodic changes) did not have any nonpolysomal polyadenylated RNA (26, 27, 38). In studies with 3T6 cells, during the reinitiation of growth by supplementing the medium with sufficient fresh serum, the increased rate of protein synthesis was accompanied by a rapid influx of polyadenylated RNA (26, 27).

Therefore, it is likely that the cells respond to growth cessation in stationary phase by reducing the utilization of preexisting mRNA, while they respond to the arrest of growth by limiting-levels of serum by reducing the amount of mRNA available for translation. However, further investigation is required to clarify this point.

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REFERENCES

1. Amos, H. 1964. Effect of actinomycin D and chloramphenicol on protein synthesis in chick fibroblasts. *Biochim. Biophys. Acta.* **80**:269–278.
2. Bandman, E., and T. Gurney. 1975. Differences in the cytoplasmic distribution of newly synthesized poly(A) in serum-stimulated and resting cultures of Balb/C-3R3 cells. *Exp. Cell Res.* **90**:159–168.
3. Becker, H., C. P. Stanners, and J. E. Kudlow. 1971. Control of macromolecular synthesis in proliferating and resting syrian hamster cells in monolayer culture. II. Ribosome complement in resting and early G1 cells. *J. Cell. Physiol.* **77**:43–50.
4. Bishop, D. H. L., J. R. Claybrook, and S. Spiegelman. 1967. Electrophoretic separation of viral nucleic acids on polyacrylamide gels. *J. Mol. Biol.* **26**:373–387.
5. Bishop, J. O., Rosbash, M., and D. Evans. 1974. Polynucleotide sequences in eukaryotic DNA and RNA that form ribonuclease-resistant complex with polyuridylic acid. *J. Mol. Biol.* **85**:75–88.
6. Both, G. W., A. K. Banerjee, and A. J. Shatkin. 1975. Methylation dependent translation of viral mRNA *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1189–1193.
7. Bucher, N. L. R. 1967. Experimental aspects of hepatic regeneration. *N. Engl. J. Med.* **277**:686–696.
8. Castor, L. N. 1977. Response of protein synthesis and degradation in growth control of WI-38 cells. *J. Cell. Physiol.* **92**:457–468.
9. Civelli, O., A. Vincent, J. E. Buri, and K. Sherrer. 1976. Evidence for a translational inhibitor linked to globin mRNA in untranslated free cytoplasmic messenger ribonucleic protein complexes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **72**:71–76.
10. Cooper, H. L., S. L. Berger, and R. Braverman. 1976. Free ribosomes in physiologically nondividing cells. Human peripheral lymphocytes. *J. Biol. Chem.* **251**:4891–4900.
11. Cowdry, E. V. 1950. Textbook of Histology. Lea and Febiger. Philadelphia, Pa. 640 pp.
12. Emerson, C. P. 1971. Regulation of the synthesis and stability of ribosomal RNA during contact inhibition of growth. *Nat. New Biol.* **232**:101–106.
13. Engelhardt, D. L. 1971. An inhibitor of protein synthesis in cytoplasmic extracts of density-inhibited cells. *J. Cell. Physiol.* **78**:333–344.
14. Engelhardt, D. L., and J. Sarnoski. 1975. Variations in the cell-free translating apparatus of cultured animal cells as a function of time during cell growth. *J. Cell. Physiol.* **86**:15–30.
15. Evans, R. B., V. Morhenn, A. L. Jones, and G. M. Tomkins. 1974. Concomitant effects of insulin on surface membrane conformation and polysome profile of serum-starved Balb/C 3T3 fibroblasts. *J. Cell Biol.* **61**:95–106.
16. Fan, H., and S. Penman. 1970. Regulation of protein synthesis in mammalian cells. II. Inhibition of protein synthesis at the level of initiation during mitosis. *J. Mol. Biol.* **50**:655–670.
17. Federoff, S. J. 1967. Proposed usage of animal tissue culture terms. *J. Natl. Cancer Inst.* **38**:607–611.
18. Gospodarowicz, D., and J. Moran. 1975. Optimal conditions for the study of growth control in Balb/C 3T3 fibroblasts. *Exp. Cell Res.* **90**:279–284.
19. Grisham, J. W. 1962. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H^3. *Cancer Res.* **22**:842–849.
20. Hassel, J. A., and D. L. Engelhardt, 1973. Translational inhibition in extracts from serum-deprived animal cells. *Biochim. Biophys. Acta.* **325**:545–553.
21. Hassell, J. A., and D. L. Engelhardt. 1977. Factors regulating the multiplication of animal cells in culture. *Exp. Cell Res.* **107**:159–167.
22. Henshaw, E. C., and J. K. Loebenstein. 1970. Rapidly labelled polydisperse RNA in rat-liver cytoplasm – evidence that it is contained in ribonucleoprotein particles of heterogeneous size. *Biochim. Biophys. Acta.* **199**:405–420.
23. Hershko, A., P. Mamont, R. Shields, and G. M. Tomkins. 1971. The pleiotypic response. *Nat. New Biol.* **232**:206–211.
24. Holley, R. W., and J. A. Kiernan. 1968. Contact inhibition of cell division in 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.* **60**:300–304.
25. Johnson, L. F., H. T. Abelson, H. Green, and S. Penman. 1974. Changes in RNA in relation to growth of the fibroblast. I. Amounts of mRNA, rRNA, and tRNA in resting and growing cells. *Cell.* **1**:95–100.

26. JOHNSON, L. F., R. LEVIS, H. T. ABELSON, H. GREEN, and S. PENMAN. 1976. Changes in RNA in relation to growth of the fibroblast. IV. Alterations in the production and processing of mRNA and rRNA in resting and growing cells. *J. Cell Biol.* **71**:939–949.
27. JOHNSON, L. F., J. G. WILLIAMS, H. T. ABELSON, H. GREEN, and S. PENMAN. 1975. Changes in RNA in relation to growth of the fibroblast. III. Posttranscriptional regulation of mRNA formation in resting and growing cells. *Cell.* **4**:69–75.
28. KAMINSKAS, E. 1972. Serum-mediated stimulation of protein synthesis in Ehrlich ascites tumor cells. *J. Biol. Chem.* **247**:5470–5476.
29. KAY, J. E., T. AHERN, and M. ATKINS. 1971. Control of protein synthesis during the activation of lymphocytes by phytohaemagglutinin. *Biochim. Biophys. Acta.* **247**:322–334.
30. KEMPER, B. 1976. Inactivation of parathyroid hormone mRNA by treatment with periodate and aniline. *Nature (Lond.).* **262**:321–323.
31. KRUSE, P. F., JR. 1972. Use of perfusion systems for growth of cell and tissue cultures. *In* Growth Nutrition and Metabolism of Cells in Culture. editors. G. H. Rothblat and V. J. Cristofeld. vol. II. Academic Press, Inc., New York. 445 pp.
32. KRUSE, P. F., JR., E. MIEDEMA, and H. C. CARTER. 1967. Amino acid utilizations and protein synthesis at various proliferation rates, population densities, and protein contents of perfused animal cell and tissue cultures. *Biochemistry.* **6**:949–955.
33. KRYSTOSEK, A., M. L. CAWTHON, and D. KABAT. 1975. Improved methods for purification and assay of eukaryotic messenger ribonucleic acid and ribosomes. Quantitative analysis of their interaction in a fractionated reticylocyte cell-free system. *J. Biol. Chem.* **250**:6077–6084.
34. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227**:680–685.
35. LEE, G. T-Y., and D. L. ENGELHARDT. 1977. Protein metabolism during growth of Vero cells. *J. Cell. Physiol.* **92**:293–302.
36. LEVINE, E. M., Y. BECKER, C. W. BOONE, and H. EAGLE. 1965. Contact inhibition, macromolecular synthesis and polyribosomes in cultured human diploid fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **53**:350–356.
37. LEVINE, E. M., D. Y. JENG, and Y. CHANG. 1974. Contact inhibition polyribosomes and cell surface membranes in cultured animal cells. *J. Cell. Physiol.* **84**:349–364.
38. LEVIS, R., L. MCREYNOLDS, and S. PENMAN. 1977. Coordinate regulation of protein synthesis and messenger RNA content during growth arrest of suspension Chinese hamster ovary cells. *J. Cell. Physiol.* **90**:485–502.
39. MALT, R. A., and S. K. STODDARD. 1966. Synthesis of ribosomal RNA and of microsomal membranes in the renoprival kidney. *Biochim. Biophys. Acta.* **119**:207–210.
40. MARCU, K., and B. DUDDOCK. 1974. Characterization of a highly efficient protein synthesizing system derived from commercial wheat germ. *Nucleic Acids Research.* **1**:1385–1397.
41. MEEDEL, R. H., and E. M. LEVINE. 1977. Messenger RNA regulation in human diploid fibroblasts. *J. Cell. Physiol.* **90**:211–224.
42. MUTHUKRISHNAN, S., G. W. BOTH, Y. FURUICHI, and A. J. SHATKIN. 1975. 5'-terminal 7-methguanosine in eukaryotic mRNA is required for translation. *Nature (Lond.).* **255**:33–37.
43. PERRY, R. P., and D. E. KELLY. 1974. Existence of methylated messenger RNA in mouse L cells. *Cell.* **1**:37–42.
44. RIFKIND, R. A., D. CHULI, and H. EPLER. 1969. An ultrastructural study of early morphogenetic events during the establishment of fetal hepatic erythropoiesis. *J. Cell Biol.* **40**:343–365.
45. RIFKIND, R. A., L. LUZZATTO, and P. A. MARKS. 1964. Size of polysomes in intact reticulocytes. *Proc. Natl. Acad. Sci. U. S. A.* **52**:1227–1232.
46. ROSBASH, M., and P. J. FORD. 1974. Polyadenylic acid-containing RNA in *Xenopus laevis* oocytes. *J. Mol. Biol.* **85**:87–101.
47. ROSE, J. K. 1975. Heterogeneous 5'-terminal structures occur on vesicular stomatitis virus mRNAs. *J. Biol. Chem.* **250**:8098–8104.
48. RUBIN, H., and M. G. STOKER. 1967. Density-dependent inhibition of cell growth in culture. *Nature (Lond.).* **215**:171–172.
49. RUDLAND, P. S. 1974. Control of translation in cultured cells: continued synthesis and accumulation of messenger RNA in nondividing cultures. *Proc. Natl. Acad. Sci. U. S. A.* **71**:750–754.
50. RUDLAND, P. S., S. WEIL, and A. R. HUNTER. 1975. Changes in RNA metabolism and accumulation of presumptive messenger RNA during transition from growing to the quiescent state of cultured mouse fibroblasts. *J. Mol. Biol.* **96**:745–766.
51. SCHOCHETMAN, G., and R. P. PERRY. 1972. Characterization of the messenger RNA released from L cell polysomes as a result of temperature shock. *J. Mol. Biol.* **63**:577–590.
52. SCHIBLER, U., and R. P. PERRY. 1976. Characterization of the 5'-termini of hnRNA in mouse L cells–implications for processing and cap formation. *Cell.* **9**:121–130.
53. SCHWARTZ, A. G., and H. AMOS. 1968. Insulin dependence of cells in primary culture: influence on ribosome integrity. *Nature (Lond.).* **219**:1366–1367.
54. SHELDON, R., C. JURALE, and J. KATES. 1972. Detection of polyadenylic acid sequences in viral and eukaryotic RNA. *Proc. Natl. Acad. Sci. U. S. A.* **69**:417–421.
55. SILVERSTEIN, S., R. MILLETTE, P. JONES, and N. ROIZMAN. 1976. RNA synthesis in cells infected with *Herpes simplex* virus. XII. Sequence complexity and properties of RNA differing in extent of adenylation. *J. Virol.* **18**:977–991.
56. SLATER, I., D. GILLESPIE, and D. M. SLATER. 1973. Cytoplasmic adenylation and processing of maternal RNA. *Proc. Natl. Acad. Sci. U. S. A.* **70**:406–411.
57. SOEIRO, R., and H. AMOS. 1966. Arrested protein synthesis in polysomes of cultured chick embryo cells. *Science (Wash. D. C.).* **154**:662–665.
58. STANNERS, C. P. 1966. The effect of cycloheximide on polyribosomes from hamster cells. *Biochem. Biophys. Res. Commun.* **24**:758–764.
59. STANNERS, C. P., and H. BECKER. 1971. Control of macromolecular synthesis in proliferating and resting syrian hamster cells in monolayer culture. I. Ribosome function. *J. Cell. Physiol.* **77**:31–42.
60. THRASH, C. R., and D. D. CUNNINGHAM. 1975. Growth limitation of 3T3 mouse fibroblasts by available growth surface area and medium components. *J. Cell. Physiol.* **86**:301–310.
61. TODARO, G. J., J. LAZAR, and H. GREEN. 1965. The initiation of cell division in a contact inhibited mammalian cell line. *J. Cell. Comp. Physiol.* **66**:325–333.
62. TSAI, R. L., and H. GREEN. 1973. Rate of RNA synthesis in ghost monolayers obtained from fibroblasts preparing for division. *Nat. New Biol.* **243**:168–169.
63. VAN VENROOIJ, W. J., C. A. G. VAN EEKELEN, R. T. P. JANSEN, and J. M. G. PRINCEN. 1977. Specific poly-A-binding protein of 76,000 molecular weight in polyribosomes is not present on poly-A of free cytoplasmic mRNA. *Nature (Lond.).* **270**:189–191.
64. VESCO, C., and B. COLUMBO. 1970. Effect of sodium fluoride on protein synthesis in HeLa cells: inhibition of ribosome dissociation. *J. Mol. Biol.* **47**:335–352.
65. WARD, G. A., and P. G. W. PLAGEMANN. 1969. Fluctuations of DNA-dependent RNA polymerase and synthesis of macromolecules during the growth cycle of Novikoff rat hepatoma cells in suspension culture. *J. Cell. Physiol.* **73**:213–232.
66. WEBER, M. J. 1972. Ribosomal RNA turnover in contact inhibited cells. *Nat. New Biol.* **235**:58–61.
67. YASUMURA, Y., and Y. KAWAKITA. 1963. A line of cells derived from African green monkey kidney. *Nippon Rinsho.* 21:1209–1210.
68. ZAHRINGER, J., B. S. BALIGA, and H. N. MUNRO. 1976. Novel mechanism for translational control in regulation of ferritin synthesis by iron. *Proc. Natl. Acad. Sci. U. S. A.* **73**:857–861.