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Changes in RNA in Relation to Growth of the Fibroblast. I. Amounts of mRNA, rRNA, and tRNA in Resting and Growing Cells

L. F. Johnson, H. T. Abelson, H. Green and S. Penman
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The established cell lines 3T6 and 3T3 contain more of both rRNA and mRNA when they are growing than when they are resting, but mRNA is increased more than rRNA. During conversion of 3T6 cultures from resting to growing state, mRNA, rRNA, and tRNA accumulate long before DNA synthesis begins. The increases in rRNA and tRNA are coordinate, but mRNA accumulates earlier and to a greater extent than the others. The rate of protein synthesis in cultures in transition from resting to growing state increases faster than their content of rRNA and appears proportional to the amount of mRNA rather than to the number of ribosomes. The doubling of mRNA content that takes place before any cells begin to synthesize DNA should be considered in relation to the absence of change in the overall rate of synthesis of HnRNA during the same period.

Introduction

The association of the growing state of eukaryotic cells with increased RNA content was first demonstrated by Caspersson and by Brachet (see Brachet, 1950). Since about 80% of cell RNA is ribosomal, an increased amount of RNA in growing cells is actually a reflection of an increased number of ribosomes (Becker, Stanners, and Kudlow, 1971). In bacteria there is a correlation over a broad range between number of ribosomes and growth rate (Maaloe and Kjeldgaard, 1964).

Little is known about the amounts of other classes of RNA in mammalian cells under varying conditions, though the question of mRNA content has been raised in studies of resting and growing fibroblasts (Stanners and Becker, 1971). We have examined the relation of the major classes of RNA to growth in two ways: (1) by comparing the amounts of each class in resting and exponentially growing 3T3 and 3T6 cells, and (2) by following the increase in the amounts of each class in 3T6 cells during their transition from resting to growing state. Both methods show that in the growing state there is more of all classes of RNA but especially of mRNA.

Results

Determination of RNA

Figure 1B illustrates a typical sedimentation pattern of the RNA which did not bind to oligo-dT cellulose (rRNA and tRNA). The amounts of 28S and 18S RNA were estimated from the ^{32}P appearing above the hatched lines; that below the hatched lines probably represents "cytoplasmic heterogeneous RNA" (Penman, Vesco, and Penman, 1968). All the ^{32}P appearing in the 4S region was taken to be in tRNA, as only a very small contamination of 5S RNA or degraded higher molecular weight RNA could have been present. This assumption was supported by analysis of the 4S region by electrophoresis in polyacrylamide gels. Figure 1A shows the sedimentation pattern of the RNA eluted from oligo-dT cellulose. When pulse-labeled RNA is separated in this way, contaminating radioactive rRNA is usually undetectable, but when the rRNA is labeled to equilibrium, its presence in the eluted mRNA is more obvious. This contamination, amounting to less than 10% of mRNA radioactivity in all cases, was exclud-

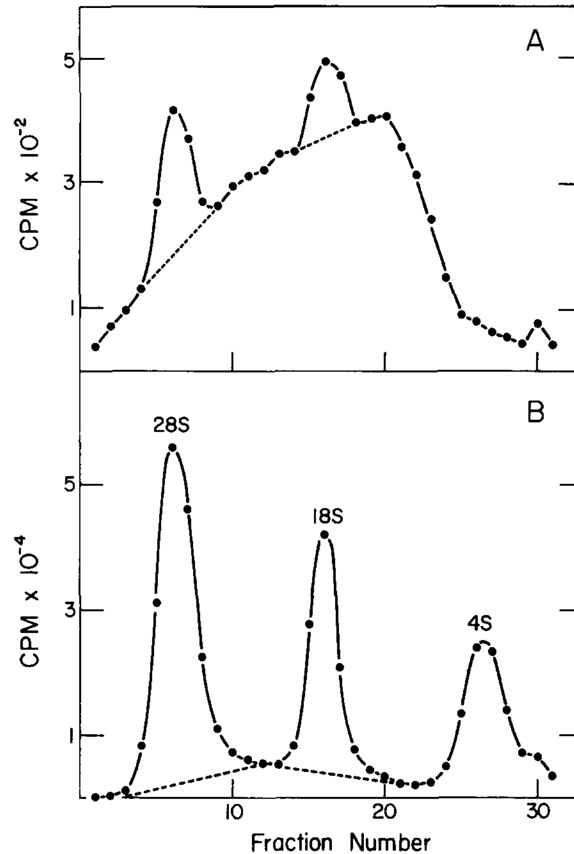

Figure 1. Separation of Major Classes of RNA by Sedimentation in Sucrose Density Gradients: Estimation of Content of Each Class
(A) Sedimentation Pattern of mRNA Adsorbed to and Eluted from Oligo-dT Cellulose
The RNA above the hatched line is contaminating ribosomal RNA and is not included in the estimate of mRNA content.
(B) Sedimentation Pattern of the RNA that Did Not Adsorb to Oligo-dT Cellulose
The region below the hatched line is not included in the estimate of 28S or 18S RNA.

Table 1. Ribosomal RNA and mRNA Content of Resting and of Exponentially Growing Cells

		Content of RNA (cpm x 10^{-2})			DNA	
Expt.	Cell type	28S	18S	mRNA	(arbitrary units)	mRNA x 100 / total rRNA
1	3T3 R*	1420	639	19.0	0.420	0.92
	3T3 G*	2620	1130	50.5	0.222	1.35
2	3T3 R	5870	2620	119.8	2.000	1.41
	3T3 G	681	285	18.6	0.080	1.93
3	3T3 R	1710	841	30.0	0.658	1.18
	3T3 R	1690	829	27.3	0.686	1.08
	3T3 G	547	238	13.0	0.080	1.66
	3T3 G	810	366	18.7	0.130	1.59
4	3T6 R	6470	3010	193.4	0.706	2.04
	3T6 R	5680	2650	148.6	0.696	1.78
	3T6 G	1580	739	58.8	0.113	2.54
	3T6 G	1420	668	58.2	0.105	2.79
	3T3 R		Means for all Experiments			1.14
	3T3 G					1.63
	3T6 R					1.91
	3T6 G					2.66

* R = Resting
* G = Growing

ed in calculating mRNA content, but if these corrections had not been made the results of the experiments would be essentially unchanged.

Amounts of the Principal Classes of RNA in Unsynchronized Growing and Resting Cells

In these experiments we labeled all nucleic acid phosphorus to equilibrium by growing the cells through a number of generations in the presence of ^{32}P-labeled phosphate of constant specific activity. The amount of label in any class of RNA is therefore a measure of the amount of that class of RNA and is independent of its rate of synthesis. Since only those mRNA molecules containing poly(A) were recovered, our estimates do not include the fraction of cellular mRNA which does not contain poly(A) (Greenberg and Perry, 1972; Adesnik and Darnell, 1972; Perry and Kelley, 1973).

In Table 1 are the results of four experiments comparing resting and growing cultures of 3T3 and 3T6. The values for the ratio mRNA/rRNA, which are independent of cell number or DNA content, are shown in the last column. These ratios were between 1% and 3% for all cultures but were about 40% higher for growing than for resting cultures. In both growing and resting cultures, the ratio was higher for 3T6 cells than for 3T3.

To compare the amounts of mRNA and of rRNA per unit of DNA, the results of the experiments described in Table 1 were normalized on the basis of DNA content. The averages for growing and resting cells are expressed as ratios in Table 2. Ribosomal RNA per unit of DNA was higher in growing cells by a factor of 2.8 for 3T3 and 1.6 for 3T6. Messenger RNA per unit of DNA was higher in growing cells by factors of 4.0 for 3T3 and 2.3 for 3T6.

The results have also been expressed as amounts of RNA per cell, on the assumption that the average DNA content per cell will be 40% higher in growing populations than in resting populations. This value was calculated for a Td of 16 hr, an S period of 7 hr, and a G2 period of 3 hr, and is only approximate. The rRNA content per cell is higher during growth by a factor of about 3.9 for 3T3 and 2.2 for 3T6, while mRNA content per cell is higher by 5.6 for 3T3 and 3.2 for 3T6.

The most important conclusions from these experiments are:

(1) The amounts of both mRNA and rRNA are greater in growing than in resting cells.

(2) Messenger RNA is increased more than rRNA.

Amounts of the Principal Classes of RNA during the Transition from Resting to Growing State

In cultures of 3T6 cells resting in 0.5% serum, usually fewer than 0.5% of the cells are engaged in DNA synthesis. When serum, which contains fibroblast growth factors, is added to a concentration of 10%, the cells enter a division cycle in quasi-synchronous fashion. After 11 hr, the first cells enter DNA synthe-

Table 2. RNA Content by ^{32}P Corrected for DNA Content or for Cell Number and Compared in Growing and Resting States

	Per unit of DNA		Per cell	
Cell type	rRNA	mRNA	rRNA	mRNA
3T3 (G/R)	2.8	4.0	3.9	5.6
3T6 (G/R)	1.6	2.3	2.2	3.2

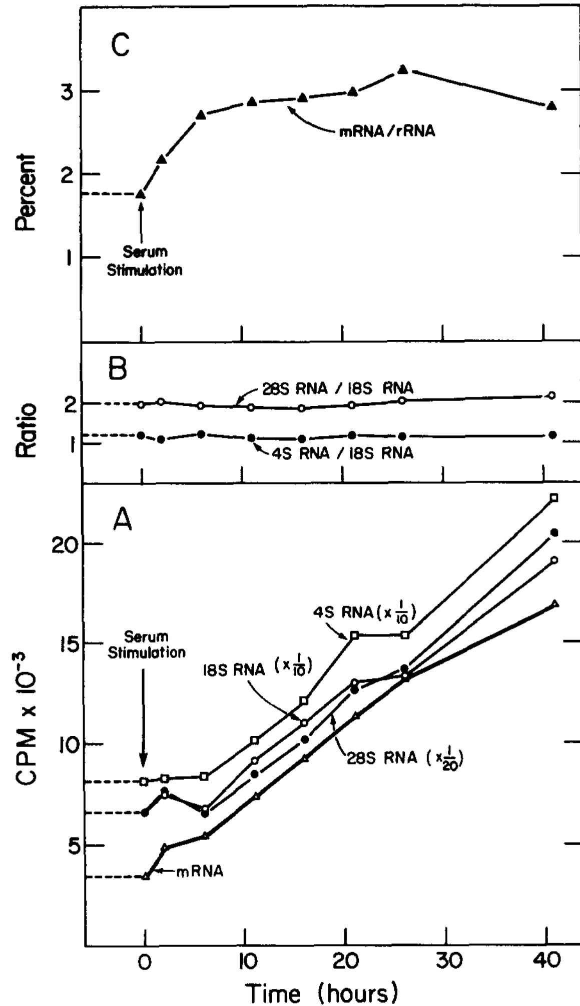

Figure 2. Increase in RNA Content following Serum Stimulation

Examination of similar cultures by radioautography following brief exposure to tritiated thymidine showed that DNA synthesis began after 10–11 hr. Total DNA content doubled in about 30 hr.

sis, and at about 24 hr, the first cell divisions take place.

Cultures of 3T6, equilibrated with ^{32}P and resting in 0.5% serum, were stimulated to enter a division cycle by the addition of fresh medium containing 10% calf serum and ^{32}P of the same specific activity as before. At intervals, cultures were harvested and analyzed for their content of the different classes of RNA (figure 2A).

No significant increase in ribosomal and 4S RNA was observed until 11 hr after stimulation. Thereafter, these two classes of RNA accumulated for the 41-hr period of the experiment. The increases of rRNA and of 4S RNA were strictly coordinate; Figure 2B shows that there was virtually no change in ratio of 28S/18S or 4S/18S throughout the experiment.

The behavior of mRNA was distinctly different, for it began to increase earlier than the other classes of RNA (Figure 2A). The amount of mRNA continued to increase linearly throughout the experiment, except for the last time examined (41 hr), when cell number would have doubled, and the population would be reverting to a resting condition.

The more rapid increase in mRNA content is better illustrated in Figure 2C. The ratio of mRNA to rRNA increased approximately 50% in the first 6 hr after serum stimulation and ultimately doubled. The ratio may have risen significantly higher than that of unsynchronized growing cells (Table 1).

The Rate of Protein Synthesis during Transition from Resting to Growing State

The rate of leucine incorporation was determined at intervals after serum stimulation of resting cultures of 3T6. The results shown in Figure 3 are normalized to the zero times values. It is clear that the rate of cell protein synthesis rose faster than the cellular content of rRNA and followed quite closely the increasing content of mRNA. Similarly, when resting 3T3 cells were stimulated to enter a division cycle by the addition of medium containing 50% serum, the increase in rate of protein synthesis exceeded the increase in rRNA content (data not shown).

Discussion

Ribosomal RNA in Relation to Growth

In all experiments, the amount of rRNA was greater in growing than in resting cells. The difference of approximately 1.6-fold per unit of DNA for 3T6 is in close agreement with the values obtained earlier for hamster embryo cells (Becker, Stanners, and

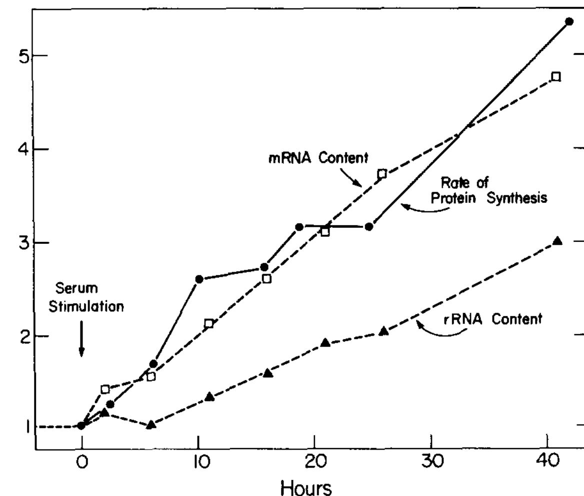

Figure 3. Protein Synthesis during Transition from Resting to Growing State

The rate of protein synthesis was determined as the amount of tritiated leucine incorporated by intact cells in a 30-min period. Values for mRNA content and rRNA content were normalized from the data of Figure 2. Ordinate gives all values relative to those of unstimulated cultures (time zero).

Kudlow, 1971). The difference was considerably larger for 3T3 cells (2.8-fold per unit of DNA).

Following serum stimulation of resting cultures of 3T6, the amounts of rRNA and tRNA increased quite slowly at first. Though the curves of Figure 2 suggest a lag of as much as 6 hr, earlier measurements of incorporation of nucleotide triphosphates by permeable "ghost monolayers" showed that the rate of transcription of rRNA increased within 10 min after serum stimulation (Mauck and Green, 1973). Increased synthesis is simply not reflected in an increase in total cytoplasmic content for a considerably longer period.

The subsequent increases in amount of rRNA and of tRNA were strictly coordinate. This is in marked contrast to the situation in bacteria (which will be discussed later) and the question that arises is how the accumulation of the two classes is controlled coordinately, given that one is made in the nucleolus and the other in the nucleoplasm. Since the stability of the two classes of RNA is quite different in both resting and growing cells (Abelson, Johnson, Penman, and Green, in preparation), coordinate accumulation does not necessarily imply coordinate synthesis.

Messenger RNA in Relation to Growth

The cytoplasmic content of poly(A) (+) mRNA changes in an extremely interesting way during transition from resting to growing state. First, it increases more rapidly than either tRNA or rRNA. Since mRNA is a relatively short-lived class of RNA (Singer and Penman, 1973; Greenberg and Perry, 1972), a change in its rate of formation should lead to a perceptible change in amount more quickly than in the case of tRNA or rRNA. The relative rate of accumulation of mRNA continues to be faster than that of rRNA or tRNA for many hours, so that the ratio of mRNA to the other classes of RNA becomes progressively elevated.

The second point of interest is that though the mRNA content doubles before DNA synthesis begins, the overall rate of transcription of HnRNA, the precursor of mRNA (Darnell, Wall, and Tushinski, 1971; Edmonds, Vaughan, and Nakazato, 1971; Lee, Mendecki, and Brawerman, 1971), is not increased prior to DNA synthesis (Mauck and Green, 1973). The increase in cytoplasmic content of mRNA is therefore not brought about by an overall increase in the transcription of nucleoplasmic genes. Accumulation of mRNA might result from increased conversion of HnRNA to mRNA. The observation that the nuclear poly (A)(+) HnRNA molecules are smaller in growing than in resting lymphocytes (Cooper, 1974) may be related to such a change.

Finally, the rate of protein synthesis in 3T6 cultures in transition from resting to growing state increases faster than the rRNA content (Figure 3) and appears closely proportional to mRNA content. It has been shown previously that the difference between the rates of protein synthesis in resting and growing cells cannot be accounted for entirely by differences in ribosome content, and that the rate at which the ribosomes initiate translation is higher in growing than in resting cells (Stanners and Becker, 1971). If mRNA is a rate-determining component in protein synthesis, a higher mRNA/rRNA ratio in the growing state should increase the rate of protein synthesis per ribosome. The curves of Figure 3 show that the rate of protein synthesis increased by a factor of about 4-fold within 30 hours after serum stimulation, an increase equal to that of the mRNA content. Since the ribosome content, as indicated by the amount of rRNA, increased by a factor of only 2 in the same period, the rate of protein synthesis per ribosome must also have increased by a factor of 2. We suggest that the increased utilization of ribosomes in growing fibroblasts may be due to the greater abundance of mRNA. The ability of phytohemagglutinin to stimulate protein synthesis in lymphocytes even when new ribosome synthesis is prevented (Kay, Levinthal, and Cooper, 1969) may also be partly due to an increase in mRNA.

The Relation between Growth and RNA Content in Mammalian Cells

The relation between growth rate and RNA content has been extensively investigated in bacteria. The results of these studies may be compared with what we have found for mammalian cells.

(1) The relation between the number of ribosomes and the amount of tRNA is not constant in bacteria growing at different rates. The tRNA content is independent of growth rate, while ribosome content is reduced at low growth rates. As a result, the relation between ribosome content and tRNA content can vary over a range of 10-fold (Maaloe and Kjeldgaard, 1966, p. 91; Rosset, Julien, and Monier, 1966). In contrast, the ratio tRNA/rRNA in the fibroblast appears to be independent of growth, since it remained constant during the transition from resting to growing state.

What appears to be coordinate regulation of content of tRNA and rRNA in the fibroblast would probably require noncoordinate synthesis. In resting cells, neither tRNA nor rRNA is stable, and the lifetimes of the two are different; whereas in growing cells, the rRNA is stable, but tRNA is not (Abelson, Johnson, Penman, and Green, in preparation). The relative rates of synthesis of the two classes of RNA may therefore vary in order to maintain constant the ratio between the amounts of the two classes during transition from resting to growing state. In bacteria also, tRNA synthesis and rRNA synthesis appear to

be regulated independently (Rosset, Julien, and Monier, 1966; Maaløe and Kjeldgaard, 1966).

(2) In bacteria growing at different rates, the rate of protein synthesis per ribosome has been claimed to be approximately constant over a 10-fold range of ribosome content (Neidhardt, 1964; Maaløe and Kjeldgaard, 1966). On the other hand, Rosset, Julien, and Monier (1966) found that at high bacterial growth rates there was an increase in the rate of protein synthesis per cellular ribosome comparable to that described here for 3T6 and that described earlier for Syrian hamster fibroblasts by Stanners and Becker (1971).

(3) In both bacteria (Lavallé and de Hauwer, 1968) and mammalian cells, the content of mRNA appears to be regulated independently of other classes of RNA. In bacteria, the control of mRNA production is at the level of transcription. In mammalian cells, the putative precursor of mRNA (HnRNA) is apparently transcribed at a constant overall rate per unit of DNA, whether the cells are resting or growing, and the possibility exists that mRNA production may be regulated at a post-transcriptional stage.

Differences in RNA Content between 3T3 and 3T6

Though differences in RNA content between growing and resting cells were in the same direction for 3T3 and 3T6, the magnitude of the changes was rather different. The increase in rRNA and mRNA in growing cells was nearly twice as great for 3T3 as for 3T6 (Table 2). On the other hand, the ratio mRNA/rRNA was higher for 3T6 than for 3T3 under both conditions (Table 1).

In the same serum concentration, the saturation density of 3T6 cells is 10-fold higher than that of 3T3 (Todaro et al., 1967), indicating that it has a much lower serum requirement for growth (lower sensitivity to contact inhibition). The ability of a cell line to grow to high density in culture (one of the prominent features of the "transformed" phenotype) may be correlated with a relatively fixed rRNA content and a relatively high mRNA/rRNA ratio.

Experimental Procedures

Cell Culture

Stocks of mouse fibroblast lines 3T3 and 3T6 (Todaro and Green, 1963) were maintained in the Dulbecco-Vogt modification of Eagle's medium supplemented with 10% calf serum. For experiments on growing cultures the cell density was kept below $10^4/cm^2$ for 3T3 and below $10^5/cm^2$ for 3T6. Under these conditions, 3T3 doubles in about 19 hr, and 3T6 in about 15 hr.

To prepare resting cultures of 3T3, the cells were allowed to grow to confluence; after 2 additional medium changes at 2-day intervals, the cultures were ready for use 3 days after the last medium change. Fewer than 0.1% of the cells were synthesizing DNA, as determined by autoradiography following a brief exposure to tritiated thymidine.

Essentially, resting cultures of 3T6 can be obtained in medium containing 10% serum, but because of the high saturation density of this line (10 times higher than 3T3), the medium becomes very acidic, and there is a risk of nutritional limitation for small molecules. By reducing the content of serum in the medium to 0.5%, the saturation density of 3T6 may be reduced to near that of 3T3 in 10% serum (5×10^4 cells/cm^2). 5×10^6 cells of stock cultures of 3T6 were inoculated into 85-mm petri dishes and fed with medium containing 0.5% calf serum. After 2 changes of medium containing 0.5% serum, at 2-day intervals, the cells were in the resting state 3 days after the last change of medium. Fewer than 0.5% of the cells were synthesizing DNA.

Studies on the transition from resting to growing state were carried out by the addition of fresh medium containing 10% serum to cultures of resting 3T6 cells. DNA synthesis began 10–12 hr later, and cell divisions began by about 25 hr. In these experiments the cultures were followed for between 1 and 2 doublings, after which a new saturation density was approached. The entrance of the cells into the first division cycle is partly synchronized, but the division cycle does not take place with the same rapidity as in sparse exponentially growing cultures.

Labeling of RNA with ^{32}P

Cells were first grown in medium containing $^{32}PO_4$ at the same initial specific activity (5.6 μCi/μmole, 5 μCi/ml) for at least 5 doublings. Even assuming all the original RNA was stable, the specific activity of the phosphate in cell nucleic acid would be at least 97% of that in the medium; actually, because of turnover and further labeling described below, the equilibrium is probably more complete. The cultures were then trypsinized and grown for 3 more doublings in the labeled medium. The amount of radioactivity in the RNA of the growing cells was then determined.

To prepare ^{32}P-labeled resting cultures, cells were labeled while growing and then kept in labeled medium containing 0.5% serum until they reached the resting state. For study of the transition from resting to growing state, these cultures were then stimulated to enter a division cycle with medium containing 10% serum and the same amount of $^{32}PO_4$. The serum was dialyzed against 10 volumes of medium prior to use to ensure a constant phosphate concentration in media with different serum content.

In order to be sure that the viability of cells was not affected by their ^{32}P content, we compared the growth of labeled and unlabeled cells. After growth for 5 days in medium containing ^{32}P at 3 times higher specific activity than that used in the experiments, 3T3 cultures were at the same cell density as unlabeled control cultures and appeared equally healthy. After reaching confluence, the labeled cells were replated at high dilution and were found to form colonies with about the same efficiency as unlabeled cells.

Isolation of RNA

Cultures were washed 3 times with 10-ml volumes of phosphate-buffered saline (PBS) and chilled to 0°C. Two ml of RSB (0.01 M sodium chloride, 0.003 M magnesium chloride, and 0.01 M Tris, pH 8.5) were added, together with 20 μl of diethylpyrocarbonate to inhibit nucleases. NP-40 (Shell Oil Company) was then added to a final concentration of 0.5%, and the cells were scraped from the petri dish with a rubber policeman. The suspension was stirred in a Vortex mixer for 30 sec, and the nuclei were removed by centrifugation at 800 $\times$ g for 2 min and stored at −70°C for DNA assay. The supernatant, whether prepared from resting or growing cells, contained over 85% of the cellular RNA. This supernatant was brought to 0.1 M sodium chloride, 0.01 M EDTA, pH 7.4, and 0.5% sodium dodecyl sulfate (SDS) and extracted 5 times at room temperature by the phenol-chloroform method of Penman (1969) to ensure complete deproteinization. The RNA was precipitated several times with 2 volumes of cold ethanol to remove labeled nucleotides.

Isolation of poly(A) (+) mRNA was based on the presence of the poly(A) sequence (Sheldon, Jurale, and Kates, 1972); Nakazato and Edmonds, 1972; Greenberg and Perry, 1972) and its affinity for oligo-dT cellulose (Singer and Penman, 1973). We found that

this procedure permitted recovery of over 95% of the poly(A) (+) mRNA of the cytoplasm. Essentially all of this RNA sediments with the ribosomes and is released by EDTA (Singer and Penman, 1973).

Oligo-dT cellulose (Collaborative Research, Waltham, Mass.) in a packed volume of approximately 0.2 ml was placed in the bottom of a conical tube. The oligo-dT cellulose was washed extensively with elution buffer (10 mM Tris, pH 7.4, and 0.05% SDS) and then equilibrated with binding buffer (400 mM NaCl, 10 mM Tris, pH 7.4, 0.5% SDS). To prevent nonspecific adsorption of RNA to the oligo-dT cellulose, a large excess of yeast tRNA was added to the suspension. Labeled RNA dissolved in 0.2 ml of binding buffer was then added to the suspension and allowed to react for 2 min. Two ml of binding buffer were then added; the suspension was stirred in a Vortex mixer, and the cellulose was sedimented. The supernatant was removed and the washing repeated. The two washes, containing the unadsorbed RNA (tRNA and rRNA), were combined and mixed with 2 volumes of ethanol. The oligo-dT cellulose was then washed several more times with binding buffer, and the supernatants discarded. The RNA bound to the oligo-dT cellulose was then removed with two 2-ml aliquots of elution buffer. The eluate was made 0.3 M in NaCl, carrier tRNA was added, and the RNA was precipitated with 2 volumes of ethanol. The RNA precipitates were collected, dissolved in SDS buffer (0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, 0.5% SDS, pH 7.4), and analyzed in 15–30% sucrose gradients using the SW 27 (Spinco) rotor for 16 hr at 25,000 rpm and 24°C. The gradients were collected through a Gilford recording spectrophotometer, and fractions were counted by liquid scintillation. All samples of each experiment were counted within a short period to avoid correcting for ^{32}P decay.

DNA Assay

The method of Burton (1956) was employed. To each sample of pelleted nuclei, 1 mg of bovine serum albumin was added. Nucleic acids were quantitatively precipitated with 0.5 N perchloric acid at 0°C. The precipitates were collected by centrifugation, suspended in 1.0 ml of 0.5 N perchloric acid, and heated to 70°C for 15 min. After centrifugation, the supernatant was added to 2 ml of reagent (20 ml glacial acetic acid containing 0.3 g diphenylamine and 1.5 mg acetaldehyde), incubated at 30°C overnight, and assayed by absorption at 600 nm.

Protein Synthesis

The rate of protein synthesis in resting or serum-stimulated cultures was obtained from the rate of incorporation of tritiated leucine. Cultures grown on 32-mm petri dishes were assayed by removing the medium, adding 1.0 ml of fresh medium containing 5 μCi ^{3}H-leucine (final specific activity 6.25 μCi/μmole), and incubating at 37°C for 30 min. The monolayer was then rinsed twice with 2 ml of serum-free medium and dissolved with 2 ml of 0.1 M NaOH. The solution was immediately neutralized with 1 M HCl, and the proteins were precipitated by adding an equal volume of 10% trichloroacetic acid containing 1% Casamino acids. The suspensions were chilled for at least 30 min and collected and washed on Millipore filters. The filters were placed in Aquasol (New England Nuclear Corp.) and counted by liquid scintillation.

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