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# Regulation of Ribosomal Protein mRNA Content and Translation in Growth-Stimulated Mouse Fibroblasts

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When resting ($G_0$) mouse 3T6 fibroblasts are serum stimulated to reenter the cell cycle, the rates of synthesis of rRNA and ribosomal proteins increase, resulting in an increase in ribosome content beginning about 6 h after stimulation. In this study, we monitored the content, metabolism, and translation of ribosomal protein mRNA (rp mRNA) in resting, exponentially growing, and serum-stimulated 3T6 cells. Cloned cDNAs for seven rp mRNAs were used in DNA-excess filter hybridization studies to assay rp mRNA. We found that about 85% of rp mRNA is polyadenylated under all growth conditions. The rate of labeling of rp mRNA relative to total polyadenylated mRNA changed very little after stimulation. The half-life of rp mRNA was about 11 h in resting cells and about 8 h in exponentially growing cells, values which are similar to the half-lives of total mRNA in resting and growing cells (about 9 h). The content of rp mRNA relative to total mRNA was about the same in resting and growing 3T6 cells. Furthermore, the total amount of rp mRNA did not begin to increase until about 6 h after stimulation. Since an increase in rp mRNA content did not appear to be responsible for the increase in ribosomal protein synthesis, we determined the efficiency of translation of rp mRNA under different conditions. We found that about 85% of pulse-labeled rp mRNA was associated with polysomes in exponentially growing cells. In resting cells, however, only about half was associated with polysomes, and about 30% was found in the monosomal fraction. The distribution shifted to that found in growing cells within 3 h after serum stimulation. Similar results were obtained when cells were labeled for 10.5 h. About 70% of total polyadenylated mRNA was in the polysome fraction in all growth states regardless of labeling time, indicating that the shift in mRNA distribution was species specific. These results indicate that the content and metabolism of rp mRNA do not change significantly after growth stimulation. The rate of ribosomal protein synthesis appears to be controlled during the resting-growing transition by an alteration of the efficiency of translation of rp mRNA, possibly at the level of protein synthesis initiation.

The eucaryotic ribosome is a complicated structure consisting of more than 70 different proteins as well as the various species of rRNA. The genes coding for the ribosomal components are located on many different chromosomes, are present in multiple copies, and are transcribed by three different RNA polymerases (23, 24). The expression of this diverse set of genes must be controlled and coordinated in a precise manner to assure that the proper stoichiometry of the ribosomal components is maintained.

The mechanisms for regulating the expression of the genes for ribosomal proteins have been studied in some detail in procaryotes. These genes are regulated over a very wide range under different growth conditions. They are organized in several different operons that are controlled autogeneously at both the transcriptional and translational levels (7, 19, 25, 26). In contrast, very little is known about the mechanism(s) responsible for controlling the expression of the ribosomal protein (rp) genes in mammalian cells. However, the recent isolation of recombinant DNA plasmids that contain DNA sequences corresponding to several mouse rp mRNAs (15) has provided a new experimental tool for examining the structure of rp genes and the regulation of their expression. Studies with these plasmids have shown that the mouse rp genes are present in multiple copies, are dispersed throughout the genome, and are not linked with the rRNA genes (6, 16).

We used these plasmids to study the content,

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metabolism, and translation of rp mRNA in 3T6 cells in different growth states. Previous studies have shown that the content of ribosomes (measured as rRNA) is two to three times lower in resting ($G_0$) 3T6 cells than in exponentially growing cells (11). When the resting cells are serum stimulated to reenter the cell cycle, the rate of synthesis of rRNA increases twofold within the first few hours after stimulation (14), leading to an increase in ribosome (rRNA) content beginning about 6 h after stimulation (11). Other investigators have shown that the rate of synthesis of ribosomal proteins increases about threefold in 3T3 cells shortly after the cells are stimulated to proliferate (23; R. J. Tushinski and J. R. Warner, J. Cell Physiol., in press). Our results indicate that there were only minor changes in the content or metabolism of rp mRNA relative to total polyadenylated [poly(A)$^+$] mRNA in serum-stimulated 3T6 cells. However, the polysome distribution of rp mRNA changed significantly during the first few hours after serum stimulation, indicating that the control of rp mRNA translation is responsible (at least in part) for regulating rp gene expression in growth-stimulated 3T6 cells.

## MATERIALS AND METHODS

**Cell cultures.** Cultures of mouse 3T6 fibroblasts (22) were maintained on 100-mm plastic petri dishes in the Dulbecco-Vogt modification of Eagle medium (GIBCO Laboratories) supplemented with 10% calf serum (Colorado Serum). Exponentially growing cultures were seeded at low density and fed daily with fresh medium before use. The cells were about half confluent at the time of experiment. Cultures of resting cells were prepared by seeding $7 \times 10^4$ cells per cm$^2$ in medium containing 0.5% serum. These cultures were fed on days 2 and 4 and used for an experiment on day 7 after seeding. Resting cultures were serum stimulated by feeding them with fresh medium containing 10% serum (11).

**RNA isolation.** Cultures were rinsed several times with ice-cold phosphate-buffered saline. The monolayer was then scraped into 2 ml of 0.01 M NaCl, 3 mM $MgCl_2$, 0.01 M Tris-hydrochloride (pH 8.4), and 0.5% Nonidet P-40. The cell suspension was transferred to a conical tube, blended in a Vortex mixer, and centrifuged at 800 × *g* for 2 min to remove nuclei and cell debris. Cytoplasmic RNA was purified by phenol-chloroform extraction at room temperature (20). Poly(A)$^+$ RNA was isolated by chromatography on oligodeoxythymidylic acid-cellulose (2). The RNA was bound and eluted twice from the affinity column to minimize contamination with rRNA.

**Polysome isolation.** Cell extracts were prepared as described above, except that the volume was reduced to 0.2 ml. After removal of nuclei, heparin (final concentration, 1.5 mg/ml) was added to inhibit nuclease activity. The extracts were layered on 15 to 40% linear sucrose gradients in 0.01 M NaCl, 1.5 mM $MgCl_2$, and 0.01 M Tris-hydrochloride (pH 8.4), which were subjected to centrifugation at 32,500 rpm for 120 min at 4°C in a Beckman SW-41 rotor. Gradients were collected through a UV monitor (Gilson) and separated into polysomal, monosomal, and submonosomal fractions as described previously (21). Carrier tRNA and 2 volumes of ethanol were added to each fraction. After at least 2 h at −20°C, precipitates were collected by centrifugation and dissolved in 2.5 ml of 0.1 M NaCl, 0.01 M Tris-hydrochloride, 0.001 M EDTA, and 0.5% sodium dodecyl sulfate. Poly(A)$^+$ RNA was isolated from each fraction as described above. In some experiments, the samples were digested with pronase before phenol-chloroform extraction.

**Filter hybridization.** The recombinant DNA plasmids used in these experiments were isolated by Meyuhas and Perry (15) and grown in HB-101. Plasmids p9, p24, p49, p50, p53, p57, and p60 are derivatives of pMB-9 and contain cDNA sequences for the ribosomal proteins S16, L32/33, L7, L30, L19, L13, and L18, respectively. The cDNA sequences contained in the recombinant plasmids are about one-third to one-half the size of the corresponding mRNAs (15). Plasmid-containing cells were grown in L broth supplemented with tetracycline (50 μg/ml) until they reached an absorbance at 260 nm of 0.6. At this time, chloramphenicol (200 μg/ml) was added, and the plasmid was allowed to amplify for about 18 h. Cells were collected, washed, and lysed, and plasmid was isolated according to the alkaline-sodium dodecyl sulfate procedure described previously (3), which we modified for larger quantities of cells. The partially purified plasmid was dissolved in 0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4), and 0.001 M EDTA (10 ml/liter of original culture), digested first with RNase A (0.1 mg/ml) and then with pronase (1.0 mg/ml), and was then subjected to phenol-chloroform extraction and gel filtration on Sepharose 6B. The purity of the plasmid was determined by electrophoresis on 1% agarose gels before and after restriction with *Hin*dIII and was estimated to be greater than 95% in each case. All work with recombinant DNA was conducted according to the National Institutes of Health guidelines for recombinant DNA research in effect at the time of the experiments.

Ten micrograms of plasmid DNA (either pMB-9 or equal amounts of the seven rp plasmids) was denatured in alkali and immobilized on a 13-mm nitrocellulose filter (Schleicher and Schuell) (9). The RNA that was to be hybridized was dissolved in 300 μl of 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) containing 150 μg of poly(A). This was placed in a 14-mm siliconized vial to which an rp filter and a control pMB-9 filter were added. Mineral oil was added to prevent evaporation. The vials were capped and incubated at 65°C for 45 h. After hybridization, filters were washed batchwise in 2× SSC for 1.5 h at 65°C, in 2× SSC plus boiled RNase A (20 μg/ml) for 1.5 h at room temperature, and finally overnight in 2× SSC at room temperature. Filters were then washed individually by suction filtration, dried, and counted in a toluene-based scintillation fluid. The amount of radioactivity bound to the control (pMB-9) filter was subtracted from that bound to the rp filter (usually 5 to 10 times greater than the control filter) to determine the amount of radioactivity corresponding to rp mRNA. The extensive washing and RNase treatment of the filters were necessary to minimize the amount of nonspecific binding of labeled RNA to the filters. To determine the efficiency of hybridization of the rp

mRNA sequences during the hybridization period, we added a fresh rp filter to a solution of RNA that had been incubated as described above. We found that more than 90% of the rp mRNA had hybridized during the initial 45-h incubation period.

To determine the amount of labeled RNA hybridizing to individual plasmids, the "dot blot" hybridization procedure of Kafatos et al. (12) was used. Individual plasmids (1.5 μg) were denatured and spotted onto a 13-mm nitrocellulose filter with a 1.6-mm siliconized capillary tube to ensure a constant diameter of each spot. Hybridizations were performed as described above. Filters were then exposed to X-ray film (Kodak X-Omat AR) with a Dupont Lightning Plus intensifying screen. Intensities of individual dots were determined by a laser densitometer. These were compared with the intensities of dots with a known amount of radioactivity to determine the amount of radioactive RNA hybridized to each dot.

## RESULTS

**Content of rp mRNA.** rp mRNA was assayed with the technique of DNA-excess filter hybridization. In most experiments, the hybridization probe was a mixture of equal amounts of seven different plasmids containing rp cDNA sequences. It was necessary to use the combination probe to obtain an adequate amount of radioactive RNA hybridized to the filter. The content of rp mRNA was determined in resting and exponentially growing cells by labeling the cells to equilibrium with $^{32}PO_4$ at low specific activity, isolating total poly(A)$^+$ mRNA, and determining the amount of labeled rp mRNA. To determine the time necessary to label the cells to equilibrium, cultures of resting or exponentially growing cells were fed with medium containing $^{32}PO_4$; at various times, cultures were harvested and the amount of radioactivity in rp mRNA and total mRNA was determined. We found that in growing cells, the ratio of labeled rp mRNA to total labeled poly(A)$^+$ mRNA increased from a value of about 0.3% after 4 h of labeling to about 0.5% after 24 h of labeling and remained at this value at later times. In resting cells, the ratio was about 0.15% after 4 h of labeling, but was still increasing even after 2 days of continuous labeling (data not shown). Therefore, to be certain that the resting cells were labeled to equilibrium, we labeled the resting cultures from the time they were first plated until the time of stimulation, a total of 7 days. We found that about 0.6 to 0.65% of total poly(A)$^+$ mRNA hybridized to the immobilized rp cDNA in resting 3T6 cells, which was slightly greater than the value of 0.5% found in exponentially growing cells. The rp mRNA in the poly(A)$^+$ mRNA fraction represents essentially all of the rp mRNA of the cell since in resting, exponentially growing, and serum-stimulated cells at least 85% of the rp mRNA sequences are retained by oligodeoxythymidylic acid-cellulose (data not shown).

Previous studies have shown that the content of total poly(A)$^+$ mRNA per cell is about three times greater in exponentially growing 3T6 cells than in resting cells (11). Thus, even though the relative content is somewhat lower in exponentially growing cells, the absolute content of rp mRNA is about 2 to 2.5 times greater in growing than in resting cells. Since the immobilized cDNA sequences represent only one-third to one-half of seven different rp mRNAs and since there are a total of about 70 ribosomal proteins, we estimated that 10 to 15% of total poly(A)$^+$ mRNA corresponds to rp mRNA in resting or growing 3T6 cells.

We used the technique of dot hybridization to determine whether the relative content of the individual rp mRNAs was the same in resting and growing cells. A typical result is shown in Fig. 1. The amount of radioactivity in the individual spots was determined by densitometry and summarized in Table 1. We found that the relative amounts of the individual rp mRNAs were the same (with the possible exception of the mRNA coding for L18) in resting and exponentially growing 3T6 cells. Minor differences in relative distribution would be difficult to detect because of the rather large error of this technique.

To determine rp mRNA content in serum-stimulated cells, resting cells were labeled for 7 days with $^{32}PO_4$ as described above and then stimulated with fresh medium containing 10% serum and $^{32}PO_4$ at the same initial specific activity. Figure 2 shows the results of a typical experiment. The content of rp mRNA remained constant for the first 6 h after serum stimulation and then began to increase in a linear manner and doubled by about 20 h after stimulation (Fig. 2b). Total poly(A)$^+$ mRNA content increased linearly from the time of stimulation and doubled by about 12 h after stimulation (Fig. 2a) as observed previously (11). Thus, the ratio of rp mRNA to total poly(A)$^+$ mRNA decreased from about 0.65% in resting cells to about 0.4 to 0.45% (approximately the same value observed in growing cells) within the first 6 h after stimulation and remained at this value at later times (Fig. 2c). Similar results were obtained with cultures that had been labeled for 2 days before stimulation (data not shown).

**Stability of rp mRNA.** To determine the half-life of rp mRNA, cultures were labeled with [$^3$H]uridine for 4 h. The medium was then replaced with "chase" medium containing unlabeled uridine and cytidine. Cultures were harvested at various times thereafter, and the amount of radioactivity in poly(A)$^+$ rp mRNA and total mRNA was determined. It should be

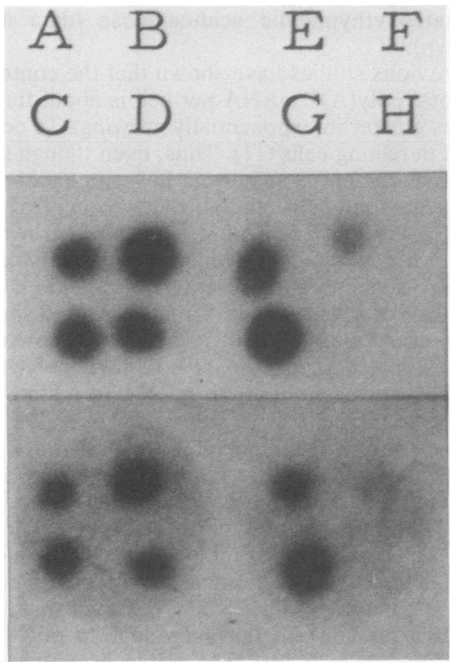


FIG. 1. Relative content of rp mRNA species. Individual plasmids containing rp cDNA were immobilized on nitrocellulose disks as described in the text. The disks were incubated with equilibrium-labeled poly(A)$^+$ mRNA from resting (bottom half of figure) and exponentially growing (top half of figure) cells, and the amount of rp mRNA hybridizing to each spot was determined by autoradiography. A, p9; B, p24; C, p49; D, p50; E, p53; F, p57; G, p60; H, pMB-9 (control).

noted that it is not possible to detect mRNA with an extremely short half-life (1 to 2 h) in this type of experiment. Figure 3 shows that the half-life of total mRNA was about 9 h in either resting or growing cells, which is in agreement with previous observations (1). The half-life of rp mRNA was found to be about 11 h in resting 3T6 cells and 8 h in growing cells.

**Rate of synthesis of rp mRNA.** We determined the rate of synthesis of rp mRNA relative to total poly(A)$^+$ mRNA so that we would not have to make corrections for the specific activity of the nucleotide pool, which changes rapidly after serum stimulation (4). Cultures of resting, exponentially growing, or serum-stimulated cells were labeled for 2 h with [$^3$H]uridine. The amount of labeled rp mRNA in the poly(A)$^+$ and poly(A)$^-$ RNA fractions was determined and normalized to the radioactivity in total poly(A)$^+$ mRNA. Again, more than 85% of the rp mRNA sequences were found to be in the poly(A)$^+$ RNA fraction (data not shown). We found that the percentage of total poly(A)$^+$ mRNA corresponding to rp mRNA was 0.10 ± 0.02% (four determinations) in resting cells and 0.16 ± 0.03% (six determinations) in exponentially growing cells. Figure 4 shows that the relative rate of synthesis of rp mRNA appeared to increase by 40 to 50% within the first 6 h after serum stimulation and then remained constant until at least 24 h after stimulation. Similar results were obtained in six separate experiments, although the magnitude of the increase varied somewhat from experiment to experiment.

**Polysomal distribution of rp mRNA.** In our initial experiments, cultures were labeled with [$^3$H]uridine or $^{32}PO_4$ for 2 to 3 h. Cytoplasmic extracts were separated into subpolysomal (monosomal plus submonosomal) and polysomal fractions by sucrose gradient sedimentation (Fig. 5). Poly(A)$^+$ mRNA was purified from both fractions, and the amount of rp mRNA was determined. Figure 6 shows that the percentage of total poly(A)$^+$ mRNA present in the polysomal fraction was the same (approximately 70%) in resting, exponentially growing, and serum-stimulated cells, in agreement with previous observations (21). In contrast, there was a significant shift in the distribution of rp mRNA. In exponentially growing cells, about 85% of the rp mRNA was found in the polysome fraction. However, in resting cells, only about 55% of the rp mRNA was in the polysome fraction. This

TABLE 1. Relative content of individual rp mRNAs in resting and growing cells[a]

| Plasmid | Ribosomal protein | Percentage of total mRNA hybridized | |
|---|---|---|---|
| | | Growing 3T6 | Resting 3T6 |
| p9 | S16 | 13 | 6 |
| p24 | L32/33 | 30 | 25 |
| p49 | L7 | 11 | 9 |
| p50 | L30 | 14 | 9 |
| p53 | L19 | 10 | 8 |
| p57 | L13 | 3 | 4 |
| p60 | L18 | 20 | 39 |

[a] Cultures of resting cells were labeled for 7 days in medium containing 20 μCi of $^{32}PO_4$ per ml. Growing cultures were labeled for 2 days in medium containing 5 μCi of $^{32}PO_4$ per ml. Cultures were harvested, and cytoplasmic poly(A)$^+$ mRNA was isolated and hybridized to filters containing individual dots of each rp cDNA plasmid as in Fig. 1. After hybridization, the filters were exposed to X-ray film for 3 to 16 days. The intensities of the individual spots were determined with a densitometer. Each value represents the mean of the results obtained in three or four separate experiments. The standard deviation was usually about 30%. Since the plasmids do not contain full-length cDNA corresponding to each message, the percentages given in the table must be corrected by the size of the cDNA insert (15) to give the actual distribution (by mass) of the rp mRNA species.

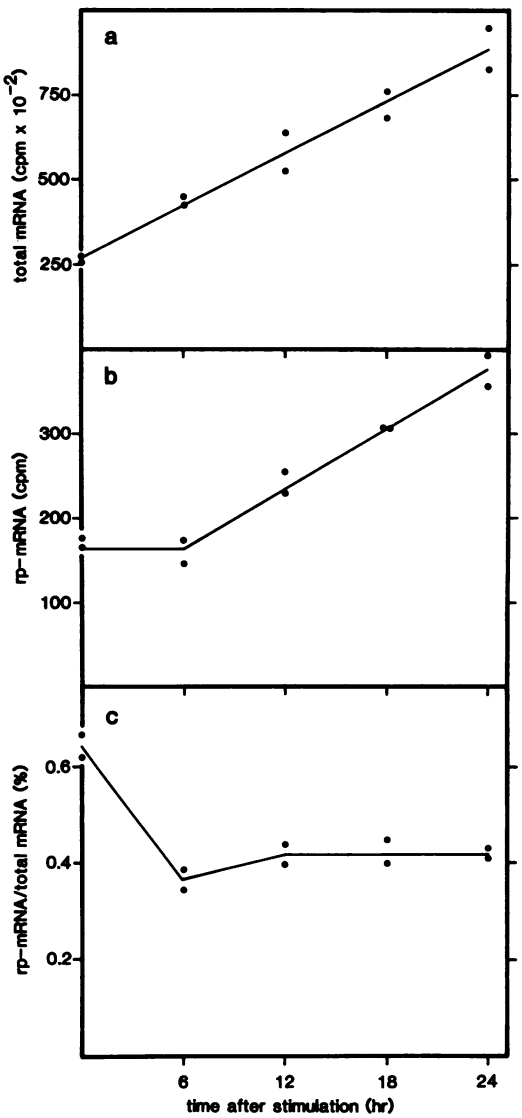


FIG. 2. Content of rp mRNA in serum-stimulated cultures. Cultures of resting 3T6 cells were seeded in medium containing 0.5% serum and 10 μCi of $^{32}PO_4$ per ml. Cultures were fed 2 and 4 days later with the same medium and used for an experiment 7 days after seeding. At time 0, the cultures were serum stimulated with fresh medium containing 10% serum and $^{32}PO_4$ at the same initial specific activity. The cultures were harvested at the indicated times and assayed for the amount of radioactivity (proportional to content) of total poly(A)$^+$ mRNA (a), rp poly(A)$^+$ mRNA (b), and the ratio of rp mRNA to total poly(A)$^+$ mRNA (c) as described in the text. In this figure and all subsequent figures, each datum point represents the value obtained from an individual culture of cells.

percentage increased to the value found in growing cells within 3 h after serum stimulation. In one experiment, growing cells labeled with $^{32}PO_4$ were mixed with resting cells labeled with [$^3$H]uridine before polysome fractionation. The distributions of total and rp mRNA were the same as in Fig. 6, confirming that the changes in distribution between resting and growing cells were not due to differences in the fractionation of gradients into polysomal and subpolysomal fractions.

We next studied in more detail the distribution of total and rp mRNA in the subpolysomal region. Cultures of resting and exponentially growing cells were labeled for 2 h, and cytoplasmic extracts were separated into submonosomal, monosomal, and polysomal regions as shown in Fig. 5. The amount of total poly(A)$^+$

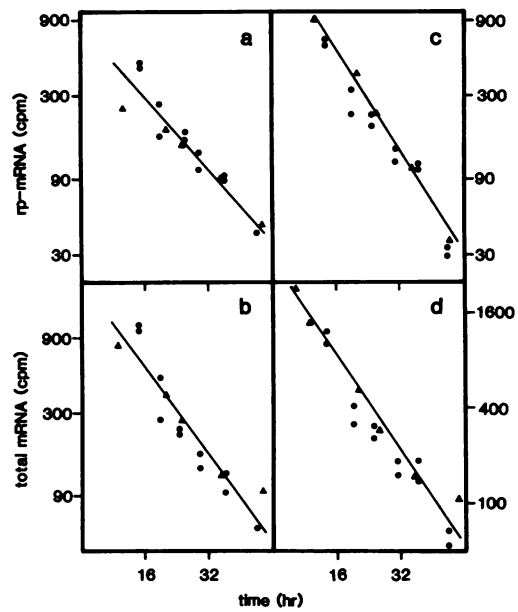


FIG. 3. Stability of rp mRNA. Cultures of resting (a and b) or growing (c and d) cells were labeled with [$^3$H]uridine (50 μCi/ml) for 4 h. At time 0, the medium from growing cultures was replaced with fresh medium containing 5 mM uridine, 2.5 mM cytidine, and 10% serum. Resting cultures were fed with conditioned medium (from replicate cultures of resting cells) containing 5 mM uridine and 2.5 mM cytidine. At various times, cultures were harvested, and the amount of radioactivity in total poly(A)$^+$ mRNA (b and d) and rp poly(A)$^+$ mRNA (a and c) was determined. These values were plotted on a logarithmic scale as a function of time. At the end of the chase period, the growing cultures were just at confluence. The different symbols represent data from two independent experiments. The values for total poly(A)$^+$ mRNA represent 1% of the total sample. The half-lives, determined by least-squares analysis of the data, are: (a) 10.7 h; (b) 8.5 h; (c) 7.6 h; (d) 8.5 h.

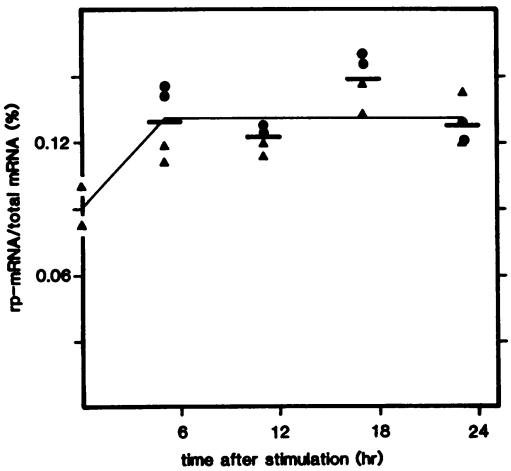


FIG. 4. Rate of production of rp mRNA after serum stimulation. Cultures of resting 3T6 cells were serum stimulated at time 0. At various times, cultures were labeled for 120 min with either (●) $^{32}PO_4$ (50 μCi/ml) in medium containing 2% of the normal amount of phosphate or (▲) [$^3$H]uridine (150 μCi/ml). Resting cultures were labeled in conditioned medium containing 0.5% serum. Total poly(A)$^+$ mRNA was isolated, and the amount of radioactivity in rp mRNA was determined by hybridization and normalized to the radioactivity in total mRNA. Horizontal bars represent the labeling interval.

mRNA and rp mRNA in each region was determined as described above. Table 2 shows that about 30% of the cytoplasmic rp mRNA was isolated from the monosomal fraction in resting cells. In contrast, only about 5% was in this fraction in growing cells. About 15% of total poly(A)$^+$ mRNA was in this fraction in either resting or growing cells. It remains to be determined whether the rp mRNA isolated from the monosomal fraction is translationally active.

To determine whether the polysomal distribution of total rp mRNA was the same as the distribution of pulse-labeled rp mRNA, we exposed the cells to [$^3$H]uridine for 10.5 h (approximately one half-life) before fractionation. Table 2 shows that the distribution of the long-term labeled rp mRNA as well as total poly(A)$^+$ mRNA was generally the same as that of the pulse-labeled mRNA. We did find that there was a slight decrease in the amount of total and rp mRNA in the polysomal fraction in the long-term labeled cells. We are not certain whether this is due to a small amount of radiation damage to the cells or to a slight decrease in the efficiency of translation of older mRNA.

## DISCUSSION

Our results show that the content, the rate of synthesis, and the stability of rp mRNA relative to total poly(A)$^+$ mRNA change very little in mouse 3T6 fibroblasts during a serum-induced transition from the resting to the growing state. We found that the content of rp mRNA relative to total mRNA is, in fact, slightly lower in growing than in resting cells. However, since total mRNA content is greater in growing cells than in resting cells, the growing cells contain at least twice as much rp mRNA per cell as do resting cells.

Previous studies in both eucaryotes and procaryotes have shown that the content and metabolism of the individual ribosomal proteins appear to be regulated in a highly coordinated manner (8, 17, 18, 23). We found that the relative amounts of the seven rp mRNAs we studied also remained approximately the same in resting and growing cells. It remains to be determined whether the balance in rp mRNA content is maintained by coordinating the transcription of the unlinked rp genes (6), by coordinating the processing or export (or both) of the transcription products, or by some other mechanism.

When resting cells were serum stimulated, the content of rp mRNA remained constant for the first 6 h and then began increasing linearly. The content of rRNA behaves in a very similar man-

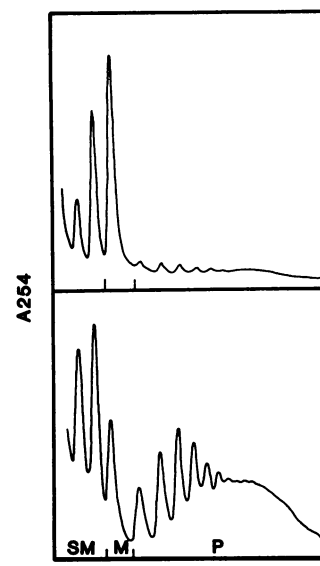


FIG. 5. Polysome profile of resting and growing 3T6 cells. Cultures of resting or exponentially growing cells were harvested, and their cytoplasmic compartments were subjected to fractionation on linear 15 to 40% sucrose gradients. Sedimentation was from left to right. Absorbance at 254 nm was monitored continuously as the gradient was collected. The vertical bars on the abscissa indicate the boundaries of the submonosomal (SM), monosomal (M), and polysomal (P) fractions.

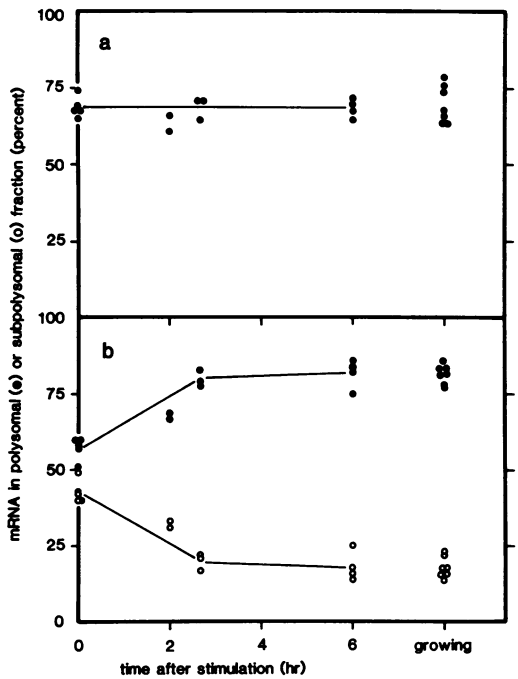


FIG. 6. Polysome distribution of rp mRNA. Cultures of resting, exponentially growing, or serum-stimulated cells were labeled for 2 or 3 h before harvest with [$^3$H]uridine (200 μCi/ml). In some experiments, growing cells were labeled for 2 h with $^{32}PO_4$ (50 μCi/ml) in medium containing 2% of the normal level of phosphate. After being labeled, the cells were harvested, and the cytoplasmic compartment was separated into polysomal and subpolysomal fractions as described in the text. The percentage of labeled total poly(A)$^+$ mRNA (a) and rp poly(A)$^+$ mRNA (b) isolated from the polysomal (●) and subpolysomal (○) fractions was determined and expressed as a function of time after stimulation.

ner in stimulated 3T6 cells (11), suggesting that the content of rp mRNAs and rRNA may be coordinated. In contrast, total mRNA content began increasing immediately after stimulation so that the ratio of rp mRNA to total mRNA actually decreased during the first few hours after stimulation before stabilizing at approximately the value found in growing cells.

Somewhat different observations were made in rat liver cells that were stimulated to grow after partial hepatectomy (6a). In this case, the content of the rp mRNA species relative to that of total mRNA increased significantly after growth stimulation. Apparently the extent of regulation of rp mRNA content varies somewhat in different cells or tissues under different physiological conditions.

The rate of production of rp mRNA relative to total mRNA appeared to increase about 40% during the first few hours after stimulation. This observation is puzzling since the relative content was decreasing during this period. There may be several explanations for this observation. First, it should be kept in mind that in the pulse-labeling experiment, we were measuring the rate of labeling of rp mRNA relative to the rate of labeling of (predominantly) short-lived mRNA molecules. However, in the content experiment, we were measuring the amount of radioactivity in rp mRNA relative to that in (predominantly) long-lived messages. It is possible that the production of short-lived mRNA decreases after stimulation, leading to an increase in the rate of labeling of rp mRNA relative to total mRNA. Another possible explanation is that the effective labeling time of mRNA may be significantly shorter in resting than in growing or stimulated cells because of the lower rate of transport of [$^3$H]uridine in resting cells (4). The ratio of labeled rp mRNA to total mRNA increases as a function of labeling time since rp mRNA has a longer half-life than do the most labile mRNA species. Therefore, if the effective labeling time

TABLE 2. Distribution of rp mRNA between submonosomal, monosomal, and polysomal fractions[a]

| Fraction | Percentage of labeled mRNA in fraction | |
|---|---|---|
| | Total mRNA | rp mRNA |
| Resting (labeled for 2 h) | | |
| Submonosomal | 21 | 30 |
| Monosomal | 14 | 27 |
| Polysomal | 65 | 43 |
| Growing (labeled for 2 h) | | |
| Submonosomal | 14 | 8 |
| Monosomal | 14 | 5 |
| Polysomal | 72 | 87 |
| Resting (labeled for 10.5 h) | | |
| Submonosomal | 21 | 32 |
| Monosomal | 17 | 34 |
| Polysomal | 62 | 34 |
| Growing (labeled for 10.5 h) | | |
| Submonosomal | 22 | 24 |
| Monosomal | 11 | 11 |
| Polysomal | 67 | 65 |

[a] Cultures of resting and growing cells were labeled for 2 h with 15 μCi of [$^3$H]uridine per ml or for 10.5 h with 200 μCi of [$^3$H]uridine per ml. Cytoplasmic extracts were separated into submonosomal, monosomal, and polysomal fractions as shown in Fig. 5. The amount of labeled total poly(A)$^+$ mRNA or rp mRNA in each fraction was determined and expressed as a percentage. Each value represents the average of at least two separate determinations.

in resting cells is shorter than in stimulated cells, this might lead to an apparent reduction in the relative rate of production of rp mRNA in resting as compared with stimulated or growing cells. Alternatively, the increase in the rate of production might be real, but is counterbalanced by a transient decrease in the stability of rp mRNA during the first few hours after stimulation. Further studies are required to distinguish among these possibilities.

Although the rates of synthesis of ribosomal proteins relative to total cellular proteins have not been determined in growth-stimulated 3T6 cells, such determinations have been made in a number of other types of cells, including serum-stimulated mouse 3T3 cells (23; Tushinski and Warner, in press), insulin-treated chick fibroblasts (5), and regenerating rat liver (17). In all of these cases, growth stimulation led to a significant increase in the rate of synthesis of ribosomal proteins. In 3T3 cells, the rate increased threefold during the first 2 h after stimulation. However, since total protein synthesis also increased by a factor of two during this period, the relative rate of ribosomal protein synthesis increased by only a factor of about 50%. In 3T6 cells, the rate of synthesis of rRNA increases twofold during the first few hours after stimulation (14), leading to an increase in ribosome content beginning about 6 h after stimulation (11). In light of all of these observations, we were surprised to see that the content of rp mRNA remained constant during the first 6 h after stimulation, a time when the rate of synthesis of ribosomal proteins should be increasing. One possible explanation was that rp mRNA might be translated more efficiently in stimulated or growing cells than in resting cells. Therefore, we decided to examine this possibility directly in 3T6 cells. We found that the percentage of pulse-labeled or long-term labeled rp mRNA associated with polysomes was much lower in resting than in serum-stimulated or growing cells. This supports the idea that the rate of synthesis of ribosomal proteins was indeed controlled at the level of mRNA translation during the first 6 h after stimulation. At later times, rp mRNA content increased, permitting still greater increases in the rate of synthesis of ribosomal proteins. The translation of rp mRNA appears to be controlled in a very selective manner since the polysome distribution of total mRNA did not change after stimulation.

In resting cells, about 30% of the rp mRNA was isolated from the monosomal fraction of the polysome gradient. At present, we do not know whether this mRNA is in the form of a messenger ribonucleoprotein complex or is actually associated with a single ribosome. If the latter is true, it will be important to determine whether the ribosome is actually translating the message or is frozen at the initiation site or some other location on the message. If, in fact, the rp mRNA in the monosomal fraction is being translated, this still indicates that the translation of rp mRNA is being regulated. Let us assume that all rp mRNA in both resting and growing cells is potentially translatable, but that the initiation of rp mRNA translation occurs much less efficiently in resting than in growing cells. This would lead to a decrease in the number of ribosomes per rp mRNA in resting cells as compared with growing cells (assuming that the rate of elongation was the same in both cells). Since the coding region of the rp mRNAs is only about 450 nucleotides (15), which is about the same size as the coding region of globin mRNA, the maximum number of ribosomes that could be associated with the rp mRNA is probably about four or five (13). Therefore, a several-fold decrease in the initiation frequency of rp mRNA in resting cells would result in a significant fraction of rp mRNA being translated by a single ribosome. There might also be a significant fraction of rp mRNA which is not associated with a ribosome and which appears in the monosome fraction. Therefore, our observations are consistent with (but do not prove) the idea that rp mRNA is controlled in resting cells at the level of protein synthesis initiation.

Another indication that the synthesis of ribosomal proteins is regulated at the level of mRNA translation comes from comparing the relative rate of ribosome production in resting and growing cells with the relative content of rp mRNA. Exponentially growing cells with a doubling time of about a day contain about twice as many ribosomes per cell as do resting cells (11). In growing cells, ribosome turnover is negligible, whereas in resting cells, the turnover is about 20% per day (1). This means that the rate of ribosome production, and consequently the rate of ribosomal protein synthesis, is about 10-fold greater in growing cells than it is in resting cells. Yet, the amount of rp mRNA per cell is only about 2- to 2.5-fold greater in growing cells. This suggests a four- to fivefold increase in the rate of ribosomal protein synthesis per rp mRNA molecule. A four- to fivefold increase in the number of ribosomes associated with each rp mRNA molecule would account for this increase. The overall increase in translational efficiency of total poly(A)$^+$ mRNA is not very great since the rate of total protein synthesis is roughly proportional to the increase in poly(A)$^+$ mRNA content (11). Thus, the increase in translational efficiency postulated for rp mRNAs appears to represent a species-specific phenomenon. Recent studies of the rate of synthesis of ribosomal proteins relative to other cellular proteins in

resting chicken embryo fibroblasts suggest that the initiation of rp mRNA translation does occur much less efficiently than that of other mRNAs (10). It will be interesting to determine the mechanism by which the translation of rp mRNA is regulated and, in particular, whether it bears any relationship to the autogenous regulation of rp mRNA translation that has been found to occur in *Escherichia coli* (19).

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