

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

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ABSTRACT

In previous reports, it was shown that both the concentration and rate of production of rRNA and mRNA were greater in growing than in resting 3T6 fibroblasts. Studies on isolated nuclei indicated that ribosomal RNA production is apparently controlled at the level of transcription. In contrast, hnRNA, the putative precursor of mRNA, appeared to be synthesized at the same rate in resting and growing cells. This finding was unexpected and has been tested in several ways. In this report, we show by an independent method that the relative rate of production of mRNA compared to hnRNA is several-fold higher in growing than in resting cells. However, the kinetics of processing of mRNA appear unchanged. This result suggests either that mRNA arises from a small subfraction of hnRNA or that the efficiency of processing of the hnRNA precursor is an important control mechanism which determines mRNA production in growing and resting states.

Comparison of the initial rates of labeling of hnRNA and cytoplasmic message gives the efficiency with which the cytoplasmic mRNA is produced from nucleoplasmic RNA. The very low efficiency (3–4% in growing and 1–2% in resting cells) suggests that not every hnRNA molecule gives rise to a cytoplasmic message.

In contrast to the similar kinetics of mRNA production in resting and growing states, processing of ribosomal RNA is much slower in the resting state and the emergence time for 28S RNA from nucleolus is greatly lengthened.

The regulation of RNA content appears to be an important aspect of the control of cell growth. It has been long known that growing cells contain significantly more ribosomes than do resting cells

(3). In our own studies on mouse fibroblast lines 3T6 and 3T3, we found that mRNA is increased in growing cells even more than the ribosomes. For example, per unit of DNA in 3T6 cells, the

difference was about 1.6-fold for rRNA and 2.3-fold for poly A(+) mRNA (5). When expressed as amounts per cell, the differences are even greater.

The increase in both rRNA and tRNA appears due largely to increased transcription (7, 8). The increased stability of both ribosomal and transfer RNA also contributes a small amount to the increase in the cellular content of these molecules (1).

The situation with regard to messenger RNA is far less clear. Measurements on isolated nuclei indicate that the rate of transcription of hnRNA, the putative precursor to mRNA, does not change in going from the resting to growing state in 3T6 cells (7). This rather surprising finding can be interpreted in two ways. Either mRNA is derived from a very small subfraction of hnRNA whose changes in rates of synthesis cannot be detected in measurements or, conversely, a significant fraction of hnRNA serves as precursor to cytoplasmic message and the rate of mRNA production is controlled at a post-transcriptional level. Evidence supporting but not proving the latter mechanism was obtained in experiments which showed that while the ratio of poly A to hnRNA synthesis in the nucleus was similar in resting and growing cells, the fraction of poly A exported to the cytoplasm was greater in the growing than in the resting state (6).

The previous experiments all imply that the relative rates of production of mRNA and hnRNA are different in growing and resting cells. In these experiments we confirm these differences by a completely different method. This was done by adding a small amount of labeled nucleosides to cultures and comparing the initial slope of the labeling of hnRNA to the initial slope of the labeling of the mRNA which later emerges into the cytoplasm. Since, in contrast to ribosomal RNA, the kinetics of mRNA formation appear unaltered in the resting and growing state, a comparison can be made between the resting and growing states. In particular, the method is insensitive to the efficiency with which radioactive precursor is transported into the cell, since only the ratio of the initial slopes of labeling of the two species is compared. Since the initial cytoplasmic mRNA must be derived from the initial labeling of the nuclear RNA, later nucleotide pool behavior becomes inconsequential. However, it is interesting to note that the data presented indicate there is no great difference in pool behavior in resting and growing

cells apart from differences in the amount of total uptake.

MATERIALS AND METHODS

Cell Culture

Stocks of the mouse fibroblast line 3T6 (12) were maintained in the Dulbecco-Vogt modification of Eagle's medium supplemented with 10% calf serum. Experiments with growing cultures were conducted in 50-mm dishes at cell densities of less than $5 \times 10^4/\text{cm}^2$, i.e., at less than $1/10$ of saturation density in 10% serum. Cultures of resting 3T6 cells were prepared by inoculating 50-mm petri dishes with 1.7×10^6 cells in medium containing 0.5% serum. The medium was changed 2 and 4 days later, and the cells were used on day 7, at which time fewer than 0.5% of the cells were synthesizing DNA.

Studies on the transition from the resting to the growing state were carried out by the addition of fresh medium containing 10% calf serum to cultures of resting 3T6. DNA synthesis began after 12 hours, and the cell divisions after 25 hours after stimulation.

Determination of Ratio of Labeling of hnRNA to mRNA

Cultures of cells were preincubated with actinomycin D and ethidium bromide for 30 min. The cells were then labeled with 5–6 ^3H -uridine (40 Ci/mmol), usually 10 $\mu\text{Ci}/\text{ml}$ for growing cells and 50 $\mu\text{Ci}/\text{ml}$ for resting cells; cells were harvested at various times. All incubations were conducted in a 37°C room to avoid temperature fluctuations.

Cultures were harvested by rinsing the monolayer twice with ice-cold PBS. 2 ml of reticulocyte standard buffer (RSB, 0.01 M NaCl, 0.003 M MgCl_2 and 0.01 M Tris pH 8.4) were added together with 20 μl diethylpyrocarbonate to inhibit nucleases. NP-40 (Shell Chemical Co., New York) was then added to a final concentration of 0.5% and the cells were scraped from the dish with a rubber policeman. The suspension was placed in a conical centrifuge tube, vortexed for 30 s and centrifuged at 800 g at 0°C for 2 min to remove the nuclei.

The supernatant was brought to 0.1 M NaCl, 0.01 M EDTA, and 0.5% sodium dodecyl sulfate (SDS) and extracted at room temperature by the phenol-chloroform method of Penman (9). Poly A(+) mRNA was then removed from cytoplasmic RNA by the oligo dT-cellulose technique as described previously (2, 5) and counted for radioactivity in Aquasol (New England Nuclear, Boston, Mass.) without sucrose gradient fractionation.

The nuclear pellet was analyzed for radioactive RNA by disrupting it in 1 ml of 0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M EDTA, and 0.5% SDS. RNA was precipitated by adding an equal volume of cold 10% TCA.

After 1 h at 0°C, the precipitates were collected on Millipore filters (Millipore Corp. Bedford, Mass.) and counted in Aquasol. The counting efficiencies of RNA in solution or on Millipore filters were found to be nearly identical.

RESULTS

Initial Ratio of Labeling of hnRNA to mRNA

These experiments are designed to measure directly the fraction of newly labeled nuclear RNA which eventually appears as cytoplasmic poly A(+) mRNA. This is accomplished by comparing the initial rate of labeling of hnRNA to initial labeling of cytoplasmic message. The results are not affected by differences in radioactive precursor pool equilibration or by the altered transport of these precursors in resting and growing cells. Ribosomal RNA synthesis is first suppressed using a low level of actinomycin (11, 12). The remaining incorporation in the nucleus occurs only in hnRNA. The contribution of mitochondrial RNA synthesis to the labeling of poly A(+) RNA in cytoplasm is suppressed using a brief pretreatment with ethidium bromide (14). This is necessary because the contribution of mitochondrial RNA, while negligible in growing cells, is significant in resting cells. After incorporation of radioactive uridine by the intact cells, nuclei and cytoplasm are separated; the radioactivity in hnRNA is measured and compared to that in poly A(+) mRNA in the cytoplasm. The ratio of the initial slope of cytoplasmic mRNA labeling to the initial slope of hnRNA labeling is the weight fraction of nuclear RNA which appears as mRNA.

The results of a typical experiment are shown in Fig. 1. When slopes of the initial rate of labeling of hnRNA are normalized, it is apparent that the ratio of labeling of mRNA to that of hnRNA is quite different for resting and growing cells. In this experiment, the ratio was 1.3% for resting 3T6 and 3.5% for growing 3T6. The results of several other experiments are shown in Table I. The ratio of relative rates of labeling was also measured following a serum-induced transition of resting cells to the growing state. 5 h after exposure to 10% serum, the ratio of mRNA to hnRNA labeling had risen to about double that of resting cells.

The rate of incorporation of label into hnRNA decreases significantly after 40 min. This is apparently due mostly to the turnover of hnRNA and occurs when the rate of labeling and the rate of decay become similar. The decreased incorporation does not appear to be due to the exhaustion of

label, since we shall see in the next section that transfer RNA, a relatively stable species, continues to be labeled linearly for at least 2 h. The later

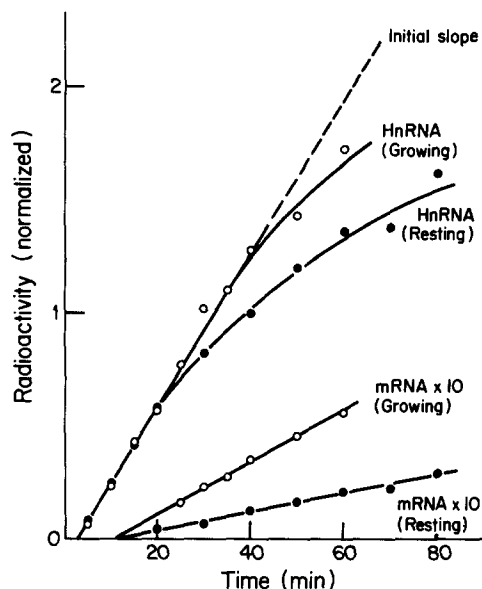


FIGURE 1 Initial rates of labeling of hnRNA and poly(A)⁺ mRNA in resting and growing 3T6. Identical cultures of resting or growing 3T6 cells were preincubated for 30 min with 0.088 $\mu\text{g/ml}$ actinomycin and 1 $\mu\text{g/ml}$ ethidium bromide. At time = 0, the medium was replaced with medium containing the drugs and [³H]uridine. At various times thereafter, cultures were harvested and analyzed for labeled nuclear RNA and poly(A)⁺ mRNA as described in Materials and Methods. The initial slopes for incorporation into nuclear RNA of resting (●—●) or growing (○—○) cells were normalized to emphasize the differences in the efficiency of conversion into mRNA.

TABLE I
Efficiency of Conversion of hnRNA into Poly(A)⁺ mRNA

Inhibitors	Experiment	Resting	Growing	In transition
Actinomycin, 0.088 $\mu\text{g/ml}$ and ethidium bromide, 1 $\mu\text{g/ml}$	1	0.71	3.73	
	2	1.14		
	3	1.05	3.54	1.98
	4	1.30		
Actinomycin, 0.044 $\mu\text{g/ml}$ and ethidium bromide, 0.35 $\mu\text{g/ml}$	5	2.87	4.57	
	6	1.28		

The initial rate of synthesis of mRNA is expressed as a percentage of the rate of synthesis of hnRNA (conversion efficiency). Labeling of pre-rRNA and mitochondrial RNA was prevented by inhibitors. The conversion efficiency was determined during the transition from resting to growing states, 5 h after the addition of 10% serum to resting cultures.

kinetics of hnRNA in growing cells are perturbed by the incorporation of a small amount of radioactive nucleoside into DNA, but this is appreciable only long after the time at which the initial rates are measured.

A possible source of error might arise from the drugs used to inhibit ribosomal and mitochondrial RNA formation. The effects of these drugs would be expected to be similar in resting and growing cells. However, to test the possibility of differential inhibition of mRNA formation in resting compared to growing cells, the experiments were repeated at drug concentrations which were significantly lower but still adequate to effect the inhibition of the undesired RNA species. The results are also shown in Table I. Though there may be a small increase in the overall conversion efficiency at the lower drug concentration, the difference between the conversion efficiency of resting and growing cells remains of similar magnitude. We therefore do not believe that the difference can be the result of differential action of the drugs on cells in the two states, although this possibility cannot be completely excluded.

Kinetics of Processing of Nucleoplasmic RNA

The kinetics of emergence of mRNA into the cytoplasm seen in Fig. 1 already suggest that there is no difference in the time required for nuclear processing of mRNA in resting and growing cells.

The kinetics of emergence are examined more completely in the experiment shown in Fig. 2. In these experiments, no inhibitors are used. For at least 3 h, messenger accumulation proceeds linearly in both resting and growing cells.

The labeling of tRNA is probably the best indicator of cellular precursor-pool specific activity. The metabolism of tRNA is apparently quite simple, and its processing time in the nucleus is quite short. The linear accumulation of labeled cytoplasmic tRNA indicates that in both this experiment and the previous one shown in Fig. 1, the radioactive precursor pools remained essentially constant in specific activity during the experiment. The relatively constant specific activity results from the rapid uptake of the trace amount of labeling used and this is complete within 15 min (G. Zieve, unpublished data). Thereafter, the total soluble specific activity cannot rise but rather declines slowly, in this case remaining nearly constant for the duration of the experiment. This implies that differences between resting and growing cells are not due to radically different radioactive pool behavior.

The regulation of processing of rRNA appears more complex than that of mRNA and tRNA. It is not possible to assign a single dwell-time to ribosomal RNA (precursor) molecules in the nucleolus since the dispersion in processing time of both 18 and 28S RNA appears quite large (Fig. 2). However, it is quite clear that the processing of both molecules is considerably slower in the rest-

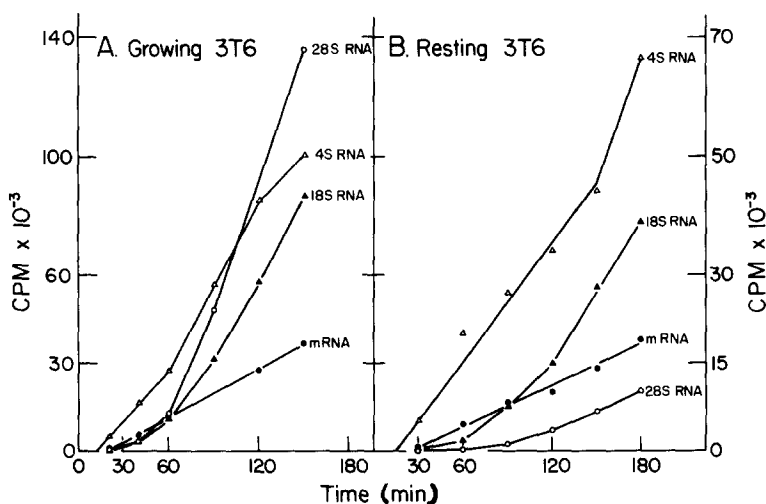


FIGURE 2 Appearance of newly synthesized RNA in the cytoplasm of resting and growing cells. Identical cultures of resting or growing 3T6 were labeled with [³H]uridine at time = 0 and harvested at various times thereafter. Samples were analyzed for the various cytoplasmic RNA species as described previously (5).

ing cell. This is particularly true of 28S RNA, which is severely retarded relative to 18S RNA. Thus, while the 28S:18S ratio is approaching the steady-state value by 3 h in the growing cells, the ratio in resting cells is still very low and only approaches the steady-state value much later.

DISCUSSION

In a previous communication (6) we reported that the principal means by which the content of poly A(+) mRNA is controlled in resting and growing 3T6 cells is the regulation of the efficiency of conversion of nuclear poly A(+) RNA into cytoplasmic poly A(+) mRNA. We determined that in growing or serum-stimulated 3T6 cells the efficiency of export of poly A from the nucleus to the cytoplasm was 2- to 3-fold higher than in resting cells.

In this report we examined the ratio of labeling of hnRNA to that of poly A(+) mRNA in resting and growing cells by comparing initial rates of labeling of the two species. We find that about 3-4% of labeled nuclear RNA (excluding ribosomal) emerges into the cytoplasm of growing cells and this is 2 to 3 times greater than the value found for resting cells. The only assumptions necessary for this conclusion to be valid are that the hnRNA turnover time is not short compared to the period over which the initial slope is measured, and that the dispersion in nuclear dwell-times for cytoplasmic mRNA is not great compared to the observation time. Both these assumptions seem reasonable. The most rapidly decaying component of hnRNA in mammalian cells has a half-life of at least 30 min, a period sufficiently long to permit determination of the initial slope of labeling. This estimate is borne out by the approach to equilibrium seen in Fig. 1.

Resting and growing 3T6 cells require about the same amount of time for nuclear processing of mRNA. Labeled mRNA begins to emerge from the nucleus about 15-20 min after the addition of labeled uridine to the culture medium. This is consistent with our previous observation that the lag between the addition of labeled adenosine and the appearance of labeled poly A in the cytoplasm was the same (about 7 min) for resting or growing cells (6).

These experiments cannot give information bearing on the possibility that cytoplasmic mRNA is the product only of a small subclass of hnRNA whose transcription rate is regulated while that of the majority of hnRNA is not. In the absence of

any means of defining such a subclass, our main conclusion from these experiments is that whereas the overall rate of synthesis of hnRNA is not linked to the growing or resting state of the cells, the proportion of total hnRNA which reaches the cytoplasm is. Only further work will clarify the exact mechanisms which are operative. The data certainly suggest that, especially in the resting cells, not every hnRNA molecule gives rise to a cytoplasmic poly A(+) message.

It was recently determined that about 94% of the mRNA sequences found in 3T6 cells are common to both resting and growing states (13). Therefore, it appears that the major difference in production of mRNA in the two states is in the quantity exported to the cytoplasm rather than in the variety of sequences produced. However, control of transcription is likely for a small number of gene products that may be synthesized exclusively in one state (e.g., histones in growing cells). Of course, the control of transcription would be more marked in cells undergoing an alteration in phenotype such as occurs during development.

We also examined the kinetics of nuclear processing of pre-rRNA and found that the rate of processing of 18S and 28S RNA, especially the latter, was significantly reduced in resting 3T6 cells. This is evidenced by an increased time required for the emergence of labeled rRNA from the nucleus following the addition of labeled uridine to the culture medium and a severely reduced ratio of labeled 28S rRNA to 18S rRNA in resting compared to growing cells after 3 h.

The greatly reduced rate of processing of 28S RNA in resting cells must tend to increase nucleolar content of 28S and 32S RNA relative to the rate of ribosomal precursor formation. However, 45S pre-rRNA synthesis is greatly reduced in these cells, probably by a factor of 3 to 4. Thus, the greatly increased processing time of the 28S RNA may very well result in an intra-nucleolar content of this species not very different from that found in growing cells.

Although we have not determined the efficiency of nuclear processing of pre-rRNA into cytoplasmic rRNA in 3T6 cells, it is clear from studies with human lymphocytes (4) that this efficiency is significantly higher in cells stimulated to enter the growing state following exposure to phytohemagglutinin than it is in nonstimulated cells. We have suggested (1) that 18S rRNA must be "wasted" in the nucleus of resting 3T6 cells, since this species of RNA was more stable than 28S rRNA in the

cytoplasm and since there is no excess accumulation of 18S RNA in resting cells. Thus, in contrast to the situation for mRNA, it appears that rRNA content is regulated by changes in the rate of transcription (7), stability (1), and nuclear processing of the rRNA precursor molecules into the mature cytoplasmic species.

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