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# Regulation of RNA Synthesis in Fibroblasts During Transition from Resting to Growing State

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**ABSTRACT** **Addition of serum, containing fibroblast growth factors, to a culture of resting 3T6 cells stimulates a transition to the growing state. Studies of ghost monolayers prepared with the aid of detergent at intervals after stimulation showed an increase in the rate of ribosomal RNA synthesis within 10 min. The rate continued to increase for many hours and reached a level 2.5- to 3.5-fold higher by the time DNA synthesis began. The increasing rate of ribosomal RNA synthesis appeared independent of an increase in the number of ribosomal genes, since it was not affected by prevention of DNA synthesis with cytosine arabinoside.**

**In contrast to ribosomal RNA, the overall rate of transcription of heterogeneous nuclear RNA was not directly affected by serum growth factors and does not appear to be regulated during the transition from resting to growing state. It seems, instead, to be fixed in relation to the amount of template, for it increases proportionally to DNA content.**

The rate of RNA synthesis in mouse-fibroblast line 3T6 can be measured as the incorporation of labeled ribonucleoside triphosphates by nonviable "ghost monolayers" prepared with the aid of the detergent, NP-40 (1). Estimates of the rate of RNA synthesis under these conditions are not affected by the cell-membrane permeability barrier and the slowly equilibrating cell-nucleotide pools, which complicate interpretation of incorporation rates in whole cells (2–6).

After stimulation of resting cultures of 3T6 with serum containing fibroblast growth factors, an increase in total RNA synthesis could be detected in ghost monolayers prepared less than 30 min later (1). The rate rose 2-fold by the time DNA synthesis began, and continued to rise for at least 20 hr. No attempt was made to distinguish effects on transcription of different classes of RNA.

We describe here experiments on the rates of synthesis of ribosomal RNA (rRNA) and of heterogeneous nuclear RNA in ghost monolayers prepared during the serum-induced transition from resting to growing state. These two classes of RNA respond quite differently, indicating that their synthesis is controlled independently. An increased rate of ribosome synthesis is probably an essential part of the preparation for DNA synthesis, but the overall synthesis of heterogeneous nuclear RNA, the precursor of cytoplasmic mRNA (7), is not correspondingly increased. It is likely that another level exists for the regulation of cytoplasmic mRNA content.

## MATERIALS AND METHODS

*Preparation of Cultures for Study of the Transition.* The established mouse fibroblast line 3T6 (8) was grown in the Dulbecco–Vogt modification of Eagle's medium, supplemented with 10% calf serum. For preparation of resting cultures of 3T6, cells were inoculated into 30-mm petri dishes at a density of $6 \times 10^4$ cells per $cm^2$ in medium supplemented with 0.5% calf serum. The medium was changed on the second and fourth day and the experiments were begun on the seventh day, when, as determined by radioautography, fewer than 0.1% of the cells were engaged in DNA synthesis. To initiate the transition to the growing state, calf serum was added to a concentration of 10%.

*Assay of RNA Synthesis in Ghost Monolayers.* All operations were done at 37°. The medium was removed and the monolayer was washed with 2 ml of serum-free medium. The monolayer was then treated with 1 ml of 0.5% NP-40 in assay buffer containing 0.05 M Tris·HCl (pH 7.8), 5 mM $MgCl_2$, 6 mM KCl, 75 mM $(NH_4)_2SO_4$, 0.20 M sucrose, and 1 mM freshly prepared dithiothreitol. After exactly 3 min, the NP-40 solution was removed and the ghost monolayer was washed with assay buffer. 0.80 ml of a solution of [$^3$H]UTP (10 $\mu$Ci, 35–50 Ci/mmol), 5 mM ATP, 0.2 mM CTP, and 0.2 mM GTP in assay buffer was gently pipetted onto the ghost monolayer. The reaction proceeded for 10 min and was terminated by addition of 50 $\mu$l of 0.10 M EDTA. The ghost monolayer was then washed twice with 2 ml of phosphate-buffered saline (pH 7.4), and dissolved by addition of 1 ml of 0.5% sodium dodecyl sulfate in the same buffer. 0.20 mg of *Escherichia coli* tRNA was added as carrier; the RNA was precipitated by addition of 2 ml of 20% cold trichloroacetic acid solution containing 40 mM sodium pyrophosphate. The precipitate was deposited on 0.45-$\mu$m Millipore filters, washed with 5% trichloroacetic acid, and counted by liquid scintillation.

*Inhibitors of RNA and DNA Synthesis.* Actinomycin D was added to the culture medium at 0.04 $\mu$g/ml, 1 hr before preparation of the ghost monolayer. No additional actinomycin was added to the assay mixture. $\alpha$-Amanitin was used at 1 $\mu$g/ml in the NP-40 solution, the assay buffer, and the reaction mixture. When DNA synthesis was to be prevented, cytosine arabinoside was used at 5 $\mu$g/ml in the culture medium.

*Analyses.* DNA was determined by the method of Burton (9), with calf-thymus DNA as the standard. RNA was determined by absorption at 260 nm of the material that precipitated in cold 0.50 N perchloric acid and dissolved after alkaline hydrolysis (1 N NaOH, 37°, 18 hr), with 25 as the absorbance of a 1 mg/ml-solution of a mixture of 18% AMP, 19% UMP, 33% GMP, and 30% CMP (their proportion in rRNA) in 0.5 N perchloric acid.

Abbreviation: rRNA, ribosomal RNA.

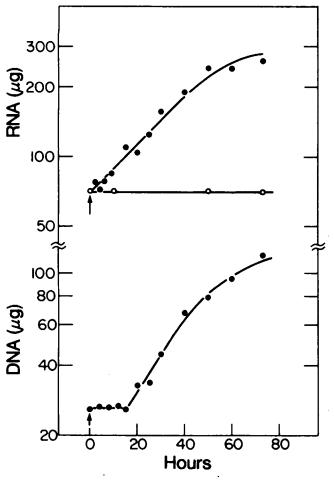


FIG. 1. Accumulation of RNA and DNA in cultures of 3T6 after serum stimulation. (*Open circles*) Resting cultures in 0.5% serum; (*closed circles*) cultures to which medium containing 10% serum was added at zero time (*arrow*) and about every 12 hr thereafter. Each point gives nucleic acid content of two cultures containing a total of about $2.4 \times 10^6$ cells at time zero.

Nucleoli were separated from nucleoplasm by the method of Penman (10). For sedimentation analysis of RNA synthesized in ghost monolayers, the RNA was extracted by the phenol–chloroform method (10) and centrifuged through a 15–30% sucrose gradient containing 10 mM Tris·HCl (pH 7.2)–0.10 M NaCl–1 mM EDTA–0.5% Na dodecyl sulfate, at 20,000 rpm for 15 hr in an SW27 rotor at 23°.

## RESULTS

*Changes in Nucleic Acid Content of Intact Cells During the Transition.* A growth cycle was initiated in confluent 3T6 monolayers resting in 0.5% serum. After addition of medium containing 10% serum there was no change in DNA content for about 15 hr; then, as replication proceeded, the DNA content began to increase (Fig. 1). Though the medium was changed twice daily, the rate of accumulation of DNA began to decline after about two cell generations, as the cells approached a new saturation density, about 4-fold higher than the initial one.

The RNA content of the cells began to rise much earlier than the DNA content (Fig. 1). When the ordinate was plotted on an exponential scale, there was no lag, but the scatter of the points is such that the increase is not significant until about 6 hr after addition of serum. By the time DNA accumulation began, the RNA content had increased over 40%. Later, DNA accumulated more rapidly than RNA, so that at the end of the experiment, when the cells were again approaching a resting state, the RNA/DNA ratio was not much different from that at the beginning. The protein content of the cultures increased proportionally to RNA content.

*Determination of Rate of Synthesis of Large-Molecular-Weight RNA in Ghost Monolayers.* The major classes of large-molecular-weight RNA are rRNA, synthesized in the nucleolus, and heterogeneous nuclear RNA (the mRNA precursor), synthesized in the nucleoplasm. rRNA (actually preribosomal RNA) is synthesized by polymerase I (11, 12), which is strongly inhibited by low concentrations of actinomycin D (13, 14). Heterogeneous nuclear RNA is synthesized by polymerase II, which is strongly inhibited by α-amanitin

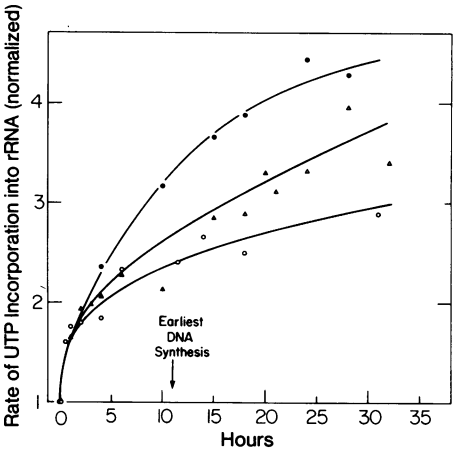


FIG. 2. Ribosomal RNA synthesis in ghost monolayers prepared at different times during the transition. Exps. *1* (●) and *2* (○) by actinomycin D sensitivity; Exp. *3*(△): by α-amanitin resistance. *Arrow* indicates earliest DNA synthesis in intact monolayers by radioautography of thymidine-labeled nuclei.

(12, 15–17) but not by a low concentration of actinomycin D (14, 18). Under the same conditions, synthesis of smaller molecular weight RNA (4, S, 5 S, etc) is not inhibited by either drug and may be performed by a third polymerase (11, 19).

In ghost monolayers we determined the relative rates of synthesis of the two types of large-molecular-weight RNA in two independent ways.

(*a*) Synthesis of heterogeneous nuclear RNA was determined by the rate of UTP incorporation after treatment with a low concentration of actinomycin D (0.04 μg/ml). Synthesis of rRNA was obtained as the difference between this rate and that in the absence of the drug (actinomycin D method).

(*b*) Synthesis of rRNA was determined by the rate of UTP incorporation in the presence of α-amanitin. Synthesis of heterogeneous nuclear RNA was obtained as the difference between this rate and that in the absence of the drug (α-amanitin method).

Only incorporation into the ghost monolayer was measured, since no large-molecular-weight RNA was released into the assay buffer during the 10-min incubation period. When, after drug treatment and incorporation of UTP, the nucleoli were separated from the nucleoplasm, we confirmed that actinomycin D eliminated nearly all nucleolar incorporation and α-amanitin eliminated nearly all nucleoplasmic incorporation. A combination of actinomycin D and α-amanitin abolished 95% of total incorporation into the ghost monolayer. Synthesis of tRNA, which accounts for about 20% of total RNA of intact cells is resistant to both drugs, but in contrast to the larger molecular weight RNA, most tRNA synthesized in isolated nuclei passes out into the medium (19). Probably for the same reason virtually all incorporation into the ghost monolayers was either preribosomal or heterogeneous nuclear RNA.

Of the total incorporation by ghost monolayers prepared from resting cells, heterogeneous nuclear RNA accounted for about two-thirds and rRNA for about one-third. This was true whether the estimate was made by the methods using drugs, or by separation of nucleoplasm from nucleoli and counting of each fraction. Since the relative activity of the two polymerases depends on the salt concentration and on the divalent cation (20, 21), the values obtained from ghost mono-

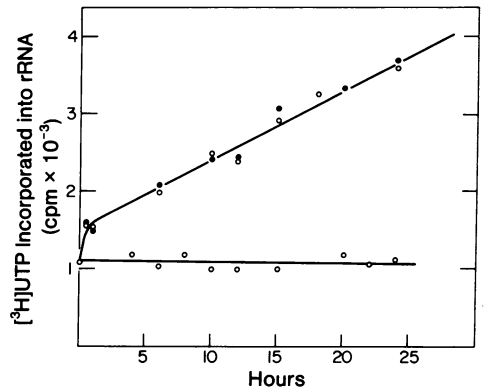


FIG. 3. Stimulation of ribosomal RNA synthesis in the presence of cytosine arabinoside. The *upper curve* shows the increase in rRNA synthesis induced by serum, as measured in ghost monolayers by the α-amanitin method. To one group of cultures cytosine arabinoside (5 μg/ml) was added with the serum and again 12 hr later. This addition completely prevented DNA synthesis, for the cells neither incorporated labeled thymidine nor increased their DNA content. The increase in rate of rRNA synthesis under these conditions (*closed circles*) was similar to that produced in the absence of cytosine arabinoside (*open circles*). *Lower curve*, unstimulated control.

layers in what might be considered intermediate salt concentrations and in the presence of $Mg^{++}$ might not necessarily reflect the ratio of the two kinds of transcription *in vivo*. For this reason we determined, in intact resting 3T6 cells, the incorporation, over a 10-min period, of tritiated uridine into nucleoplasmic and nucleolar RNA. The ratio between the two was very close to that observed in the ghost monolayers.

The size of the RNA synthesized and retained in the ghost monolayers was examined by centrifugation in a sucrose gradient and compared with that synthesized by isolated nuclei (12). About one-third of the RNA had a sedimentation coefficient greater than 28 S and two-thirds greater than 18 S. Virtually no labeled 4 S RNA was present.

*Synthesis of rRNA During Transition from Resting to Growing State.* The change in rate of rRNA synthesis after stimulation with serum (Fig. 2) was quite similar for the actinomycin D method (Exps. *1* and *2*) and the α-amanitin method (Exp. *3*). Zero-time values, to which all data are normalized, are the means of determinations on about 10 cultures resting in 0.5% serum. The rate of rRNA synthesis began to rise very quickly after exposure of the cells to 10% serum; it was increased by 50% in 30 min and by 100% in 3 hr. After that, it continued to increase but to different degrees in the three experiments. By the time DNA synthesis began, rRNA synthesis in the ghost monolayers was elevated by a factor of 2.5–3.5 in all experiments, and was still increasing at 30 hr.

By 11–13 hr after stimulation by serum, DNA synthesis began and ultimately all the cells participated, but replication of DNA had no effect on the rate of rRNA synthesis. If all DNA synthesis was prevented by addition of cytosine arabinoside to a concentration of 5 μg/ml, there was no effect on the rate of rRNA synthesis for at least 24 hr (Fig. 3). Perhaps this result was to be expected, in view of earlier findings of continued RNA accumulation in thymidineless conditions (22).

The rate of increase of rRNA synthesis was greatest at the earliest times after addition of the serum. In separate experiments, measurement by the α-amanitin method on six resting and six stimulated cultures showed an increase of 13 ± 2% within 10 min.

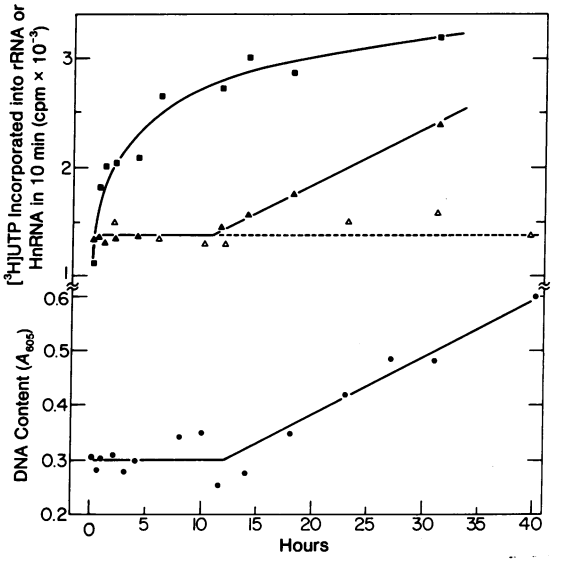


FIG. 4. Comparison of ribosomal RNA synthesis and heterogeneous nuclear RNA synthesis in ghost monolayers prepared during the transition. *Filled squares*, rRNA synthesis; *filled triangles*, heterogeneous nuclear RNA synthesis; *open triangles*, heterogeneous nuclear RNA synthesis after continuous exposure of the cultures to cytosine arabinoside. The earliest cell divisions occurred at about 25 hr. *HnRNA*, heterogeneous nuclear RNA.

*Synthesis of Heterogeneous Nuclear RNA During the Transition.* The rate of synthesis of heterogeneous nuclear RNA in the ghost monolayers was obtained from the same experiments. Fig. 4 shows the results obtained in Exp. *2* by the actinomycin D method. The top curve gives the rate of rRNA synthesis (from which one of the plots in Fig. 2 was derived). In contrast to the rapidly increasing rate of rRNA synthesis after addition of 10% serum, heterogeneous nuclear RNA synthesis underwent no change for over 10 hr. It then rose steadily for the remaining 20 hr (*middle curve*, Fig. 4). When the rise is compared with that of total DNA content of the cultures, it is clear that the two are parallel and that the rate of transcription of heterogeneous nuclear RNA is proportional to the DNA content of the culture at the time the ghost monolayer was prepared. To confirm this relation, we completely prevented DNA synthesis in some of the cultures by addition of cytosine arabinoside. In these cultures there was no increase in the rate of transcription of heterogeneous nuclear RNA.

## DISCUSSION

These experiments demonstrate that the rate of synthesis of RNA by ghost monolayers reflects the state of the cells at the time the detergent was added, and that the entrance of resting cells into a division cycle is accompanied by very different changes in synthesis of rRNA and of heterogeneous nuclear RNA.

Synthesis of rRNA is regulated during the transition, and increases in rate long before DNA synthesis begins. This increase is not the result of changes in turnover rate of the RNA synthesized; in ghost monolayers prepared from both resting and serum-stimulated cultures, the RNA synthesized was stable during a 15-min chase with unlabeled UTP.

Replication of DNA, which began at about 12 hr, had no obvious effect on the rate of synthesis of rRNA, though doubling of the number of ribosomal genes must have occurred, perhaps during a discrete portion of the ensuing S period (23). Blocking DNA synthesis with cytosine arabinoside did not affect the increasing rate of rRNA synthesis for at least 24 hr after serum stimulation. Synthesis of rRNA is therefore not template limited. On the other hand, the overall rate of transcription of heterogeneous nuclear RNA, the mRNA precursor (7), is proportional to DNA content and appears not to be regulated, as is that of rRNA. The rate of synthesis of heterogeneous nuclear RNA behaves as if it were limited by template, not polymerase, and taking place at a maximal and therefore fixed rate.

The effects of serum stimulation on rRNA synthesis in ghost monolayers are of the same magnitude as that calculated by Emerson (4) and Weber (6) for the differences between resting (dense) and growing (sparse) cultures of intact chick fibroblast, using appropriate corrections for the labeling of the nucleotide pool. Similar experiments also suggested a significant though not very large difference in the rate of transcription of heterogeneous nuclear RNA (4). We attribute this to difficulties in correcting the incorporation rates by intact cells for the changing radioactivity of the nucleotide pool.

From our own experiments, we cannot conclude that no species of heterogeneous nuclear RNA is subject to change in its rate of synthesis during preparation for growth, because we determined the combined synthetic rate of a very large number of different heterogeneous nuclear RNA molecules, and a change in the rate of synthesis of a few would escape detection. Even changes in the rate of synthesis of numerous heterogeneous nuclear RNA molecules are not excluded if increases in some are balanced by decreases in others, but this possibility seems unlikely.

During preparation for DNA synthesis, cell protein increased by 50%. Since most cell proteins are probably included in this increase most cytoplasmic mRNA species must be involved; yet there was no increase in the overall rate of synthesis of heterogeneous nuclear RNA. If the amount of cytoplasmic mRNA were determined only by the rate of transcription of heterogeneous nuclear RNA, the transition from resting to growing state would be accompanied by a drop in the mRNA/ribosome ratio. Indeed the growth cycle itself would result in cyclical changes in this ratio, for the rate of synthesis of mRNA would increase only during the S period, while that of rRNA would increase throughout the cycle. If the amount of cytoplasmic mRNA does not increase during the transition from resting to growing state, increased protein accumulation would have to take place through changes in the efficiency of utilization of existing mRNA. Stanners and Becker (24) found that growing cells do initiate protein synthesis more efficiently than resting cells but they also concluded, from rather indirect experiments, that the ratio mRNA/ribosome was the same.

An increase in cytoplasmic content of mRNA could take place without an increase in transcription of heterogeneous nuclear RNA, if the rate of breakdown of the mRNA were reduced. The newer methods of evaluating the life-time of mRNA have shown that it turns over at an appreciable rate in growing cells (25, 26), but its stability in resting cells has not been examined. Finally, if the stability of cytoplasmic mRNA does not change during transition from resting to growing state, an increase in cytoplasmic mRNA could take place as a result of processing of a larger amount of heterogeneous nuclear RNA into mRNA.

An increase in ribosome content is likely to result from every stimulus to growth or protein synthesis (27), and this increase is probably achieved mainly through an increase in the rate of ribosome synthesis. Many other changes take place during such transitions, and it is important to fix the order in which they occur. The increase in rate of rRNA synthesis produced by fibroblast growth factor of serum is so rapid that there is probably not sufficient time for the increase to be brought about through prior changes in protein synthesis. It seems more likely that, as in bacteria (28, 29), the rate of transcription of the ribosomal genes is controlled by a small molecule, and that the serum factor affects its concentration.

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