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Regulation of Protein Synthesis at the Translational Level in Neuroblastoma Cells

(cell growth/neurite outgrowth/polysomes/initiation/elongation)

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ABSTRACT **Protein synthesis in neuroblastoma cells has been studied in a cell-free system. The activity of lysates from cells grown in suspension and monolayer has been compared. A higher level of activity has been found in monolayer cells. The activity of some components of the lysate that are involved in protein synthesis has been analyzed. The data suggest that the controlling step of protein synthesis in this system might be the initiation process. The correlation between activation of protein synthesis and neurite outgrowth in monolayer cultures is discussed.**

Neuroblastoma cells can be grown in two different culture conditions, with morphological aspects resembling different stages of neuronal maturation (1, 2). If neuroblastoma cells are grown in suspension culture, they appear as spherical, immature neuroblasts; in monolayer cultures, when the cells attach to the dish, they become able to extend neurites and, therefore, look like mature neurons. Several biochemical correlates of this "maturation" *in vitro* have been described, such as increases in ribosomal RNA (3) and cell surface, as well as changes in surface glycoproteins (4, 5) and antigens (6). In addition, changes in functional properties of the cell surface, such as acetylcholine sensitivity, have been reported (7).

On the basis of these findings, it seemed of interest to investigate the protein-synthesizing machinery and its control mechanisms during the transition of neuroblastoma cells from suspension to monolayer cultures. We began this investigation by assaying the protein synthesis activity of the two types of neuroblastoma cells under conditions *in vivo*. Unfortunately no definite results could be obtained because the rate of amino-acid uptake was highly variable in different experiments. For this reason, we decided to approach the problem by using a cell-free protein-synthesizing system. In the present paper we report on studies of protein synthesis *in vitro* in neuroblastoma cells placed in the two different conditions of growth.

MATERIALS

[^{3}H]Leucine (30–40 Ci/mmol) was purchased from New England Nuclear; [^{3}H]methionine (7.4 Ci/mmol) and [^{35}S]-methionine (250 Ci/mmol) were obtained from Amersham.

METHODS

Culture Conditions. Neuroblastoma clone 41A$_3$ was grown either in monolayer or suspension culture in F10 medium supplemented with 15% (v/v) horse serum and 2.5% (v/v) fetal calf serum (8).

Cell Fractionation. Neuroblastoma cells in monolayer cultures were removed by pipetting the medium up and down. Monolayer and suspension cells were then collected by centrifugation and washed twice with 0.9% (w/v) NaCl. About 20 × 10^6 cells were suspended in 400 μl of Medium A [50 mM Tris·HCl buffer (pH 7.4), 25 mM KCl, 4 mM $MgCl_2$, and 1 mM dithiothreitol], homogenized in a Dounce homogenizer, and centrifuged at 15,000 × g for 10 min at 4°. The supernatant ("lysate") was dialyzed in a cold room (4°) against 500 volumes of Medium A for 3 hr, with the medium changed every hour. Total native ribosomes and soluble fraction (cell sap) were obtained by centrifuging the lysates at 150,000 × g for 90 min.

Standard Cell-Free Protein Synthesis Assay. The standard assay was similar to that described by Metafora *et al.* (9). The incubation mixture contained, among other things, 0.1–1 A_{260} unit of lysate or, alternatively, 0.3–1 A_{260} unit of ribosomes and 50–80 μg of cell sap proteins, as indicated.

Assay of Total Soluble Activity. For measurement of the activity of the soluble factors involved in protein synthesis, the incubation mixture contained 30 μg of heterologous, deoxycholate-treated ribosomes from rat brain cortex (10) and increasing amounts of neuroblastoma cell sap. Incubation and further processing were as described for the standard cell-free protein synthesis procedure. The proteins were determined by the method of Lowry *et al.* (11).

Assay of Total Transfer Activity. The activity of the elongation factors I and II was measured as described (12). The incubation mixture contained 70 μg of heterologous ribosomes from sea urchin embryos (12) and increasing amounts of neuroblastoma cell sap proteins.

Polysome Sucrose Density Gradient Analysis. Cells were lysed in hypotonic Tris buffer [20 mM Tris·HCl (pH 8.5), 50 mM KCl, 5 mM Mg acetate] containing 0.5% Nonidet. The postmitochondrial supernatant was centrifuged through a 15–40% sucrose density gradient in hypotonic Tris buffer, during 75 min at 39,000 rpm in an SW 41 Spinco rotor. The polysome profiles were recorded at 254 nm with an Isco continuous flow cell UV analyzer.

Acrylamide Gel Electrophoresis. For analysis of the synthesized polypeptide chains by acrylamide gel electrophoresis, a scaled-up incubation mixture (500 μl) containing 840 μg of ribosomes either from suspension or monolayer cells and 900 μg of the homologous cell sap proteins, was incubated for 30 min at 37°. The "monolayer system" was labeled with 300 μCi of [^{3}H]methionine and the "suspension system" with 75 μCi of [^{35}S]methionine. The released, newly synthesized proteins were chromatographed on 15% sodium dodecyl sulfate/

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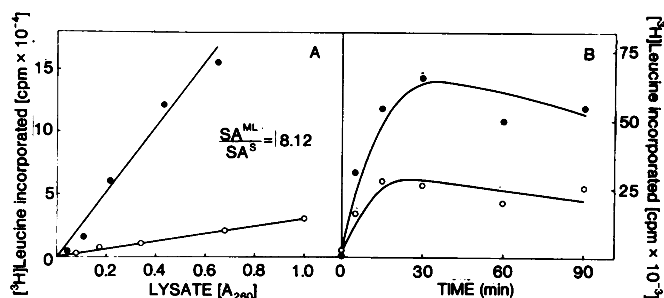

FIG. 1. [^{3}H]Leucine incorporation by neuroblastoma cell lysates. (A) Concentration dependence. The activity was assayed in standard reaction mixture as described under *Methods*. ●, Monolayer; ○, suspension. SA^{ML} and SA^{S}, specific activity of monolayer and suspension, respectively. (B) Time course. A scaled-up (200 μl) standard reaction mixture containing 1.6 A_{260} units of lysate was incubated at 37°. At the indicated time, 25 μl were taken and processed as described under *Methods*. ●, Monolayer; ○ suspension.

acrylamide gel columns (6.5 cm long) at 2 mA per tube. The gel slices were counted after NCS treatment in a Liquifluor/toluene scintillation mixture.

RESULTS

Lysate activity

Changes During Growth. In suspension cultures, the lysate specific activity showed little changes as the cells proceeded

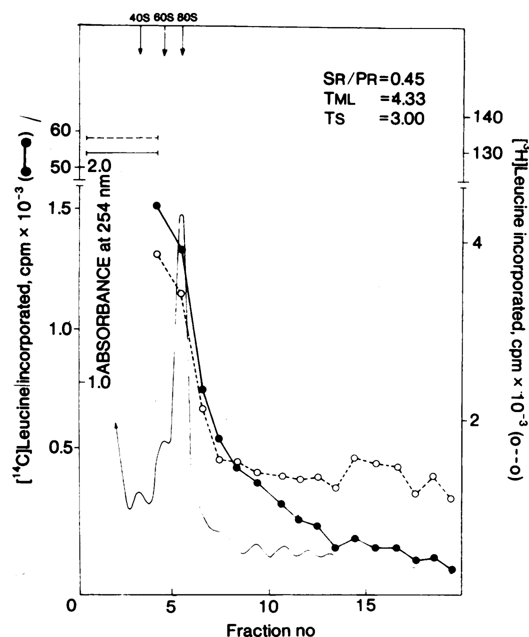

FIG. 2. Estimation of relative rates of elongation. 10^6 monolayer and suspension cells were respectively labeled with [^{3}H]-leucine (5 μCi/ml; specific activity, 5 Ci/mmol) and [^{14}C]leucine (1 μCi/ml; specific activity, 306 mCi/mmol) for 5 min. The cells were then mixed and lysed with 0.5% Nonidet in 20 mM Tris·HCl (pH 8.5), 50 mM KCl, 5 mM Mg acetate. The postmitochondrial supernatant, after addition of 1% Triton X-100 (final concentration), was placed on a 15–40% sucrose gradient as described under *Methods*. Further processing and calculations of the average rates of elongation (S_R/P_R) in the two cell types and of the average transit time (T) were according to Palmiter (19). ○, Monolayer; ●, suspension.

TABLE 1. *Lysate activity as a function of cell growth*

Growth conditions \ Phase of cell growth	Specific activity (pmol of [^{3}H]leucine incorporated/A_{260})		
	Lag	Logarithmic	Stationary
Monolayer	5.8	9.7	21.0
Suspension	5.6	5.2	6.8

Cells were subcultured in monolayer and suspension and samples collected as described under *Methods:* (*i*) prior to the start of cell division (lag); (*ii*) during exponential cell growth (logarithmic); and (*iii*) after density-inhibition of cellular proliferation (stationary). Lysates were prepared and assayed as described in the *text*. The specific activity was calculated on the linear part of a lysate concentration curve ranging from 0.1 to 1 A_{260} unit/assay.

from the lag phase to the stationary phase of growth. On the contrary, in monolayer cultures, the specific activity of the lysate increased, as the cells started growing after attachment to the dish (logarithmic phase) to reach the highest value in stationary phase, when neurite outgrowth was *at its maximum* (Table 1). Since the highest difference in protein synthesis was observed in the stationary phase, all subsequent experiments were carried out on cells at this stage of growth.

Time Course. In lysates from neuroblastoma cells, the incorporation of [^{3}H]leucine into proteins was linear during 20 min and reached a plateau after 30 min of incubation (Fig. 1B). The leveling off of the incorporation may be related to the inactivation of some factor(s) and/or production of inhibitors during incubation (13, 14); in addition, the ATPase activity of the microsomal membranes (15–17) might deplete the ATP from the incubation mixture. A slight decrease in incorporation was found after longer times of incubation, suggesting the presence of some proteolytic activity in the system. Fig. 1 clearly shows that both the rate and extent of protein synthesis are markedly higher in the lysate from monolayer cells.

Concentration Curve. In order to measure the specific activity of the lysates of the two cell types, we followed [^{3}H]-leucine incorporation as a function of lysate concentration (Fig. 1A). The incorporation remained linear over the range of concentrations used. In the experiment reported in Fig. 1A, the specific activity of the monolayer was 8-fold higher than that of the suspension. However, a certain variability in the specific activity of the lysates was observed, the average specific activity ratio, monolayer/suspension, being 2.5.

These results prompted us to investigate which of the components of the protein-synthesizing machinery present in the lysate was responsible for the higher activity of monolayer cells as compared to suspension. Therefore, the two main components, ribosomes and cell sap (containing soluble factors) were fractionated and their specific activities measured.

Cell sap activity

The total activity of the cell saps was measured over a range of concentrations, with a constant amount of heterologous ribosomes. Table 2 shows that the specific activity of monolayer cell sap is about twice as high as that of suspension. The decay of [^{14}C]phenylalanine-tRNA and [^{3}H]poly(U)

TABLE 2. *Activity of cell sap factors*

Culture conditions	Total soluble specific activity (pmol of leucine incorporated/mg of protein)	Transfer factors specific activity (pmol of Phe incorporated/mg of protein)	Aminoacyl-tRNA synthetase specific activity (cpm of [^{14}C] aminoacids charged/A_{280})
Monolayer	172	41.2	490,000
Suspension	92	36.6	476,000

Monolayer and suspension cultures in stationary phase were used. Cell saps were prepared as described under *Methods*. Total soluble activity was measured as described under *Methods*, with increasing amounts of cell sap (3–80 μg of proteins) and a fixed amount (30 μg) of deoxycholate-treated ribosomes from rat brain cortex. Transfer factor activity was measured as described under *Methods*. Aminoacyl-tRNA synthetase activity was measured as previously (9) in the presence of an excess of rabbit reticulocyte tRNA. Phe, phenylalanine.

was measured (18) and found to be the same, thus ruling out the possibility that the difference in the total activity of cell sap could be ascribed to different ribonuclease activities.

Several factors involved in protein synthesis are present in the cell sap, namely, initiation factors, elongation factors, termination factors, transfer RNAs, and aminoacyl-tRNA synthetases. The activities of some of these factors were measured in an attempt to identify which of them might be responsible for the higher activity of monolayer cell sap.

The activities of elongation factors I and II and of aminoacyl-tRNA synthetases (Table 2) were the same in the cell saps of monolayer and suspension cells. However, the elongation rate does not depend only on the activity of the elongation factors; other cellular components, such as GTP and aminoacyl-tRNAs, are involved in the elongation of peptide chains. For this reason the ability of the two cell types to elongate peptide chains was measured *in vivo* (Fig. 2) by the double-labeling "ratio method" described by Palmiter (19). The relative ratio of elongation, expressed by the S_R/P_R ratio (Fig. 2), showed that suspension cells were about twice more active in elongating polypeptide chains than monolayer cells. This higher activity of suspension cells is also evident from the average transit time (T) values calculated according to Palmiter for each cell type (Fig. 2).

TABLE 3. *Specific activity of neuroblastoma ribosomes*

Culture conditions	Specific activity (pmol of [^{3}H]leucine incorporated/mg of ribosomes)	
	Native ribosomes	Deoxycholate ribosomes
Monolayer	100	160
Suspension	56	97

Native ribosomes were prepared as described from cultures in stationary phase. Deoxycholate ribosomes were obtained by adding deoxycholate to 1% final concentration to the lysate and pelleting them at 180,000 × *g* for 6 hr through a cushion of 37% sucrose in Medium A. Increasing amounts of ribosomes (0.3–1 A_{260} unit) were assayed for protein-synthesizing activity with a constant amount (80 μg) of rat brain cortex cell sap (10). The specific activity was calculated from the linear part of the concentration curve.

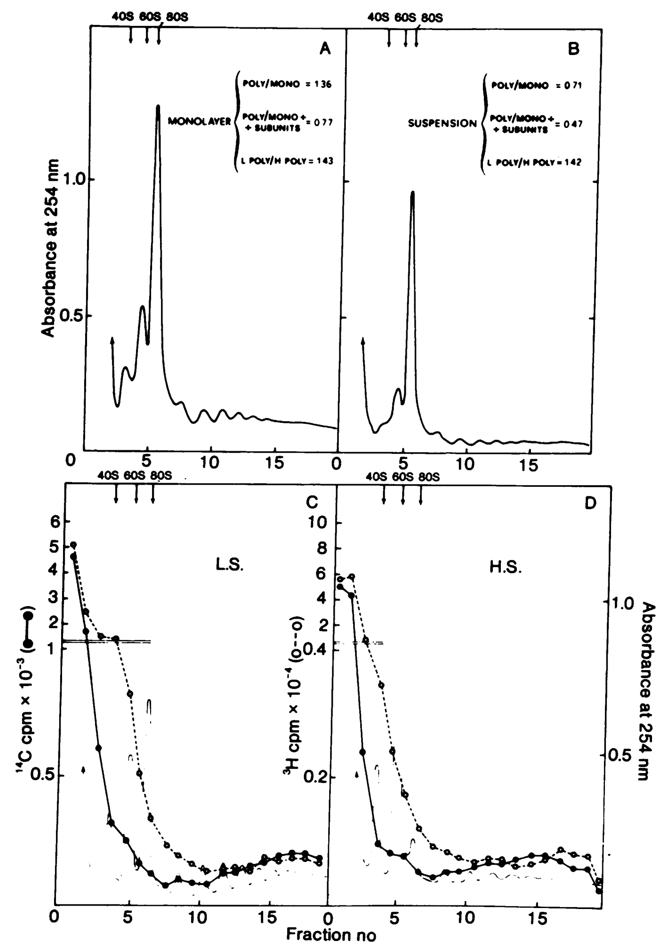

FIG. 3. Polysomal profiles from monolayer and suspension cells. (A and B) Postmitochondrial supernatants were prepared from 10^6 monolayer or suspension cells and placed on 15–40% sucrose density gradients. The gradients were scanned as described under *Methods*. Inserts: The areas under the 254 nm absorbance peaks were gravimetrically measured and their ratios calculated. L. Poly, light polysomes; H. Poly, heavy polysomes. (C and D) Effects of high salt treatment on ribosome profiles. Cells were labeled and processed as described in the legend of Fig. 2. The postmitochondrial supernatant was divided into two aliquots. One of them was placed on a 15–40% sucrose gradient, in hypotonic Tris buffer. To the other was added KCl to 300 mM final concentration, and it was placed on a sucrose gradient in 20 mM Tris·HCl buffer (pH 8.5) containing 300 mM KCl and 3 mM Mg acetate. After centrifugation, the absorbances were recorded, and 0.6-ml fractions were collected and processed as described in the legend of Fig. 2. Solid curve, absorbance at 254 nm; ●, suspension; O, monolayer. L.S., low salt; H.S., high salt.

Ribosome activity

Activity of the ribosomes from the two cell types was measured in a cell-free system containing heterologous soluble factors. The specific activity of native ribosomes (free and membrane-bound) was about twice higher in monolayer than

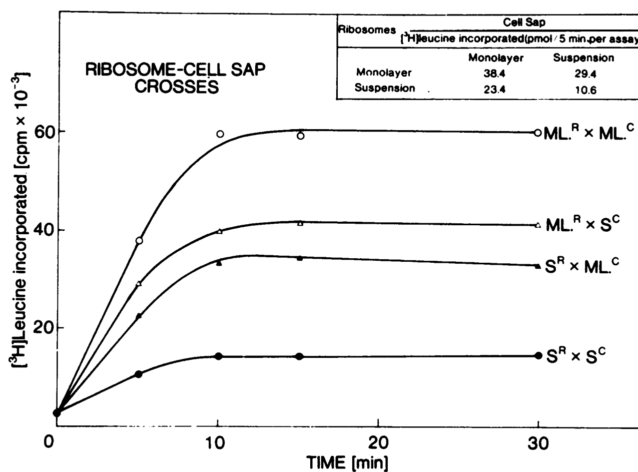

FIG. 4. Cross experiments with cell sap and ribosomes from monolayer and suspension cells. A scaled-up (500 μl) standard reaction mixture, containing 350 μg of ribosomes and 500 μg of cell sap proteins from both monolayer and suspension cells, was incubated at 37°. At the indicated time, 100-μl aliquots were taken and processed for determination of radioactivity. O, Ribosomes from monolayer (ML^R) + cell sap from monolayer (ML^C); ●, ribosomes from suspension (S^R) + cell sap from suspension (S^C); Δ, ribosomes from monolayer (ML^R) + cell sap from suspension (S^C); ▲, ribosomes from suspension (S^R) + cell sap from monolayer (ML^C). *Insert*, activity after 5 min of incubation.

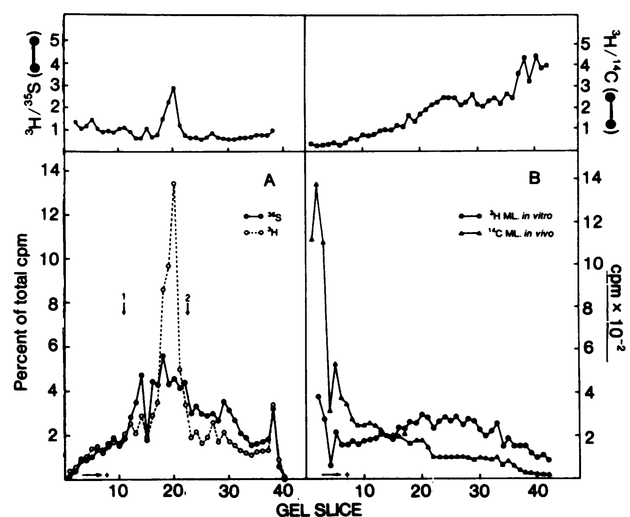

FIG. 5. (A) Acrylamide gel electrophoresis of proteins synthesized in reconstituted cell-free systems. Polypeptide chains were synthesized in a reconstituted cell-free system, and released chains were processed as described under *Methods*. 30,000 cpm of [^{3}H]- and [^{35}S]methionine-labeled proteins were loaded on the gel. The electrophoresis and further processing of the gel was performed as described. The monolayer reconstituted system was labeled with [^{3}H]methionine and the suspension one with [^{35}S]methionine. (B) Comparison by acrylamide gel electrophoresis of proteins synthesized in the intact cell and in the lysate. Monolayer cells were incubated with 5 μCi/ml of [^{14}C]-leucine in the F10 medium without leucine for 30 min at 37°. Postribosomal supernatant was prepared as described and mixed with similar amounts of radioactive polypeptide chains released from ribosomes of a monolayer lysate labeled *in vitro* with [^{3}H]-leucine.

in suspension cells (Table 3). Since it has been reported (14, 20) that, in *in vitro* systems, membrane-bound ribosomes are more active than free ribosomes during short incubation times, the difference in activity of native ribosomes could be due to a change in the relative amounts of free and membrane-bound ribosomes in the two cell populations. Therefore, neuroblastoma ribosomes were treated with 1% deoxycholate (final concentration), so that all ribosomes would be present as free particles. Again, the specific activity of monolayer ribosomes was higher than that of suspension ribosomes. The deoxycholate treatment resulted in an increase of activity in both kinds of ribosomes (Table 3). The difference in the specific activities of the two total ribosomal populations could then be accounted for by a difference in the relative content of polysomes and monosomes. Density gradient analysis showed that, indeed, the polysomes/monosomes ratio was about twice higher in monolayer than in suspension cells (Fig. 3A and B). This ratio remains about the same when calculated as polysomes/monosomes + subunits. The higher relative content of monosomes in suspension cells was not due to ribonuclease degradation of the polysomes, as shown by the following experiment. Monolayer and suspension cells were labeled for 5 min, respectively, with [^{14}C]- or [^{3}H]leucine, mixed, and lysed as described under *Methods*. The postmitochondrial supernatant was then run on sucrose density gradients in low and high salt buffer. The results (Fig. 3C and D) show that the monosome peak was almost totally dissociated into subunits by high-salt treatment, thus showing that no nascent chains were associated with the single ribosomes. This was also shown by the radioactivity profile, which did not show any peak in the monosome region of the gradient and was not modified by the high salt treatment.

The polysomal patterns, reported in Fig. 3A and B, show also that while the total ribosomal population in monolayer cells was about twice that present in suspension cells, there was no significant change in the polysome size among the two cell types.

Cross experiments with cell sap and ribosomes

Experiments where cell sap activity was tested in cross experiments with monolayer and suspension ribosomes (Fig. 4) clearly confirmed that both ribosomes and some of the soluble factors are involved in determining the higher activity of the monolayer lysate.

Acrylamide gel electrophoresis of proteins synthesized *in vitro*

Labeled polypeptide chains synthesized by reconstituted (cell sap + ribosomes) cell-free systems from monolayer and suspension cells were subjected to acrylamide gel electrophoresis in order to ascertain whether qualitative differences could be detected.

The overall patterns of the released proteins appeared to be similar in the two systems (Fig. 5A). However, quantitative differences were evident in three regions of the gel (fractions 13–15, 16–23, and 23–33). In comparison to suspension, the monolayer system synthesized a larger amount of proteins migrating around the 35,000 dalton region and a smaller amount of proteins of lower molecular weight.

It was relevant, at this point, to compare the products of the reconstituted cell-free systems with the soluble proteins synthesized in the intact cell. Fig. 5B shows that the *in vitro* system synthesized polypeptides with a lower average molecular weight.

DISCUSSION

The data reported in this paper indicate that the transition of neuroblastoma cells from suspension to monolayer conditions of growth involves a marked increase in the protein-synthesizing activity. Morphologically, the transition from suspension to monolayer is characterized by neurite outgrowth, thus suggesting a correlation with the observed increase in protein synthesis activity. Such a correlation is strengthened (*i*) by the data reported in Table 1, showing that the maxima of protein synthesis activation and neurite outgrowth coincide (stationary phase), and (*ii*) by the observation (data not shown) that in monolayer cultures in which neurite outgrowth happened to be very poor, protein synthesis was not activated. Previous findings, showing that neurite outgrowth can occur in the presence of cycloheximide, are not necessarily in contrast with the suggested correlation (21): in these experiments, cycloheximide was added to the cells 24 hr after plating, that is several hours after the cells had attached to the culture dish. On the contrary, if the drug is present at the time of cell attachment, neurite outgrowth is inhibited (22). It is likely that cell attachment brings about a rearrangement of the cell surface, which in turn might be the triggering signal for protein synthesis activation. On the other hand, neurite outgrowth is not inhibited by actinomycin D (22), indicating that this process does not depend on transcriptional events. On the basis of previous reports, suggesting that the flow of mRNA from the nucleus to the cytoplasm is the same in the two growth conditions (23), it seems likely that the activation of protein synthesis observed in the transition suspension → monolayer occurs at the translational level.

The data reported in this paper indicate that both soluble factors (Table 2, Fig. 4) and ribosomes (Table 3, Fig. 4) are involved in the activation of protein synthesis. Elongation of peptide chain does not seem to be responsible for this activation, since suspension cells show a higher relative average elongation rate (Fig. 2) than monolayer cells. The higher specific activity of the ribosomes prepared from monolayer cells is due to a higher polysomes/monosomes ratio. This in turn could be dependent either on an increase of mRNA flow from the nuclei to the cytoplasm or on an activation of the initiation step of polypeptide assembly process. The former possibility seems to be ruled out by previous findings (23); this leads to the suggestion that initiation might be the critical step responsible for protein synthesis activation. In other systems (24–27), initiation has also been shown to be an important regulating step of protein synthesis.

The lower elongation rate in lysates from monolayer cells, in spite of their higher activity in protein synthesis, might be due to a qualitative change in the mRNA population. Our analyses by acrylamide gel electrophoresis of polypeptide chains synthesized and released in the cell-free system indicate that, at least, the relative amount of proteins synthesized by lysates from the two cell types varies; this would be in favor of some qualitative change in mRNA populations.

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1. Schubert, D., Humphreys, S., Baroni, C. & Cohn, M. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 316–323.
2. Augusti-Tocco, G., Sato, G. H., Claude, P. & Potter, D. D. (1970) *Symp. Int. Soc. Cell Biol.* **9**, 109–120.
3. Casola, L., Romano, M., Di Matteo, G., Augusti-Tocco, G. & Estenoz, M. (1974) *Dev. Biol.*, **41**, 371–379.
4. Augusti-Tocco, G., Parisi, E., Zucco, F., Casola, L. & Romano, M. (1973) in *Tissue Culture of the Nervous System*, ed. Sato, G. (Plenum Press, New York), pp. 87–105.
5. Glick, M. C., Kimhi, Y. & Littauer, U. Z. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1682–1687.
6. Akeson, R. & Herschman, H. (1974) *Nature* **249**, 620–623.
7. Harris, A. J. & Dennis, M. (1970) *Science* **167**, 1253–1255.
8. Augusti-Tocco, G. & Sato, G. H. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 311–315.
9. Metafora, S., Terada, M., Dow, L. W., Marks, P. A. & Bank, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1299–1303.
10. Zomzely-Neurath, C., York, C. & Moore, B. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2326–2330.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 165–275.
12. Felicetti, L., Metafora, S., Gambino, R. & Di Matteo, G. F. (1972) *Cell Differ.* **1**, 265–277.
13. Acs, G., Neidle, A. & Schneiderman, N. (1962) *Biochim. Biophys. Acta* **56**, 373.
14. Murthy Venkatachala, M. R. (1972) *J. Biol. Chem.* **247**, 1936–1943.
15. Branton, D. & Deamer, D. W. (1972) in *Membrane Structure* (Springer-Verlag, Wien, New York), p. 38.
16. Dunn. A. J. (1970) *Biochem. J.* **116**, 135–145.
17. Korner, A. (1961) *Biochem. J.* **81**, 168.
18. Metafora, S., Felicetti, L. & Gambino, R. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 600–604.
19. Palmiter, R. D. (1972) *J. Biol. Chem.* **247**, 6770–6780.
20. Andrews, T. M. & Tata, J. R. (1971) *Biochem. J.* **121**, 683–694.
21. Seeds, N. W., Gilman, A. G., Amano, T. & Nirenberg, M. W. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 160–167.
22. Schubert, D. & Jacob, F., (1970) *Proc. Nat. Acad. Sci. USA* **67**, 247–254.
23. Augusti-Tocco, G., Casola, L. & Romano, M. (1974) *Cell Differ.* **3**, 313–320.
24. McCormick, W. & Penman, S. (1969) *J. Mol. Biol.* **39**, 315–333.
25. Fan, H. & Penman, S. (1970) *J. Mol. Biol.* **50**, 655–670.
26. Vaughan, H. M., Jr., Pawlowski, P. J. & Forchhammer, J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2057–2061.
27. Schochetman, G. & Perz, R. P. (1972) *J. Mol. Biol.* **63**, 577–590.