Electron Microscopic Autoradiography of Rabbit Reticulocytes Active and Inactive in Protein Synthesis

Abstract. *Electron microscopic autoradiography indicates that only a fraction of ribosome-containing reticulocytes (from phenylhydrazine-treated rabbits) is capable of active protein synthesis in vitro. Ribosomes, and, indeed, polyribosomes, do not appear to limit the rate of protein synthesis.*

Reticulocytes obtained from rabbits made severely anemic with phenylhydrazine have been used extensively in the study of the control and mechanism of protein synthesis in mammals. Since these cells are enucleate, have lost DNA (*1*), and appear incapable of RNA synthesis (*2*) interactions of the nucleocytoplasmic type are absent. Over 90 percent of the protein made in such reticulocytes is hemoglobin (*3*). The synthesis of complete hemoglobin molecules has also been demonstrated in cell-free extracts from such cells (*4*). The role of ribosomes and aggregates of ribosomes or polyribosomes has been emphasized in regard to the components necessary for hemoglobin synthesis in reticulocytes.

In a typical preparation of rabbit reticulocytes, almost all protein synthesis was confined to approximately one-third of the reticulocytes as demonstrated by autoradiographic techniques (*5*). Moreover, these active reticulocytes were relatively uniform in amino acid–incorporating activity, as judged by counts of silver grains over cells that had been allowed to incorporate tritiated amino acids into protein and that were then placed on glass slides as smear preparations, fixed, and coated with photographic emulsion. For example, in an experiment in which less than five grains were found over 60 percent of the reticulocytes, six to ten grains were found over only 5 percent of the cells. Of the active reticulocytes, about two-thirds gave rise to 30 or more grains, but no more than 70 grains were found over a single cell.

When autoradiography was combined with acridine-orange staining for particulate RNA, it was found that only about 50 percent of the acridine-orange positive cells were capable of protein synthesis. Thus the presence of ribosomes was insufficient as an indication of protein-synthesizing cells. However, these results could be interpreted as consistent with the idea that polyribosomes were necessary for protein synthesis.

To examine directly the possible suggested correlation between the presence of polyribosomes and protein-synthesizing ability of reticulocytes, we turned to electron microscopic autoradiography.

A reticulocyte preparation was allowed to incorporate tritiated leucine for 30 to 45 minutes, essentially according to Borsook *et al.* (*6*). After removal of unincorporated leucine and "chasing" with cold leucine, the cells were fixed with 3 percent glutaraldehyde in 0.1*M* cacodylate buffer (*7*) at 4°C. The fixed cells were then washed, dehydrated by passage through increasing concentrations of acetone, embedded in Vestopal, and, after sectioning, were stained with uranyl acetate and lead citrate. Sections were then coated with a thin layer of Ilford L-4 emulsion by a modification of the loop technique of Caro and van Tubergen (*8*) and developed after a suitable exposure period—about 1 week under the conditions used.

A representative active reticulocyte is shown in Fig. 1. The salient features of this cell are: (i) ribosomes, present as polyribosomes; (ii) mitochondria; (iii) many tubular elements; (iv) small vesicles, some of which are open to the

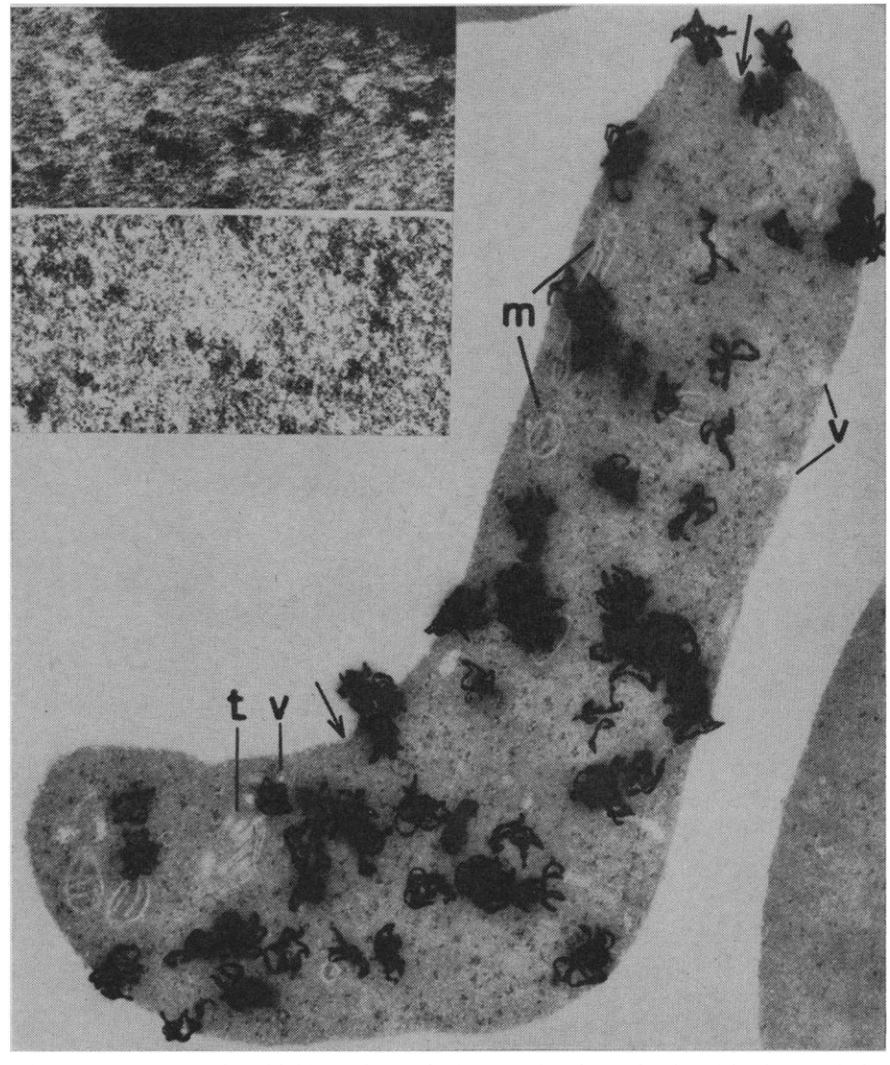

Fig. 1. A representative, highly active reticulocyte, showing mitochondria (*m*), tubular elements (*t*), small vesicles (*v*), and many ribosomes in clusters of varying sizes. At the arrows can be seen modifications of the plasma membrane which resemble the vesicular membranes. To the right, a section of a totally inactive reticulocyte, which contains ribosomes (× 27,500). Insets: Two larger associations of ribosomes taken from an active reticulocyte. Both clusters of ribosomes in linear arrays (upper inset) and long rows of single ribosomes (lower inset) are seen regularly in active cells. Part of a silver grain is seen in the upper inset (× 165,000).

extracellular space and which appear to have a modified plasma membrane; and (v) a relatively low density in the cytoplasm.

The ribosomes in active cells are not only clustered but appear generally in aggregates larger than five or six, the size predominating in cell-free preparations. Furthermore, the polyribosomes are often of the "open" configuration (*9*). Rifkind *et al.* (*10*) have also noted such large ribosomal aggregates. In inactive reticulocytes or those occasional cells with low activity (that is, one-fifth to one-tenth the activity of most active cells), ribosomes are also found and frequently at relatively high concentration. The ribosomes in inactive cells are usually not clustered; but we regularly found such cells, in which most ribosomes were present as polyribosomes (*11*). However, these polyribosomes are usually dimers or trimers.

Mitochondria are invariably found in active cells. These mitochondria have a rather dense matrix and are relatively small—0.1 to 0.2 μ in length—although occasionally longer ones may be found. In sections from cells that have been extracted with acidic acetone after fixation and dehydration, dense particles are often seen in mitochondrial matrices. Mitochondria are generally absent in inactive cells but are occasionally seen in those inactive cells containing a high concentration of ribosomes.

Sections of active cells often show deep invaginations of the plasma membrane and large vacuoles. Serial sections indicate that these invaginations are finger-like and that some of the large vacuoles are, indeed, vacuoles and not cross-sectioned invaginations. Our results are consistent with the observations of Bessis and Bricka (*12*) who described young reticulocytes as motile cells which exhibited amoeboid-like movements.

Small infoldings showing structural modifications of the plasma membrane are found almost exclusively in active cells. Tubular and vesicular elements are always found in active cells, but also appear frequently in inactive cells with many ribosomes. By serial sectioning it was found that at least some vesicular elements were continuous with the cell membrane.

Attempts have been made, in studies on the maturation of reticulocytes, to correlate loss of protein-synthesizing activity with loss of ribosomes (*13*). However, our results clearly demonstrate that many totally inactive cells contain large numbers of ribosomes, and, in some cases, polyribosomes. Ribosomes, and, indeed, polyribosomes, do not appear to be rate-limiting. Biochemical experiments which complement these autoradiographic studies strongly suggest that primary control of hemoglobin synthesis may very well reside in mitochondria or in elements of the plasma membrane or in both (*14*).

ALEXANDER MILLER
ARVID B. MAUNSBACH
Department of Zoology,
University of California, Los Angeles

References and Notes

1. N. S. Burt, R. G. E. Murray, R. J. Rossiter, *Blood* **6**, 906 (1951); B. W. Holloway and S. H. Ripley, *J. Biol. Chem.* **196**, 701 (1952).
2. P. A. Marks, C. Willson, J. Kruh, F. Gros, *Biochem. Biophys. Res. Commun.* **8**, 9 (1962).
3. J. Kruh and H. Borsook, *J. Biol. Chem.* **220**, 905 (1956).
4. R. S. Schweet, H. Lamfrom, E. H. Allen, *Proc. Natl. Acad. Sci. U.S.* **44**, 1029 (1958).
5. A. Miller, Abstracts, Annual Meeting Am. Soc. Cell Biol., 2nd, San Francisco, 5–7 November, 1962, p. 124.
6. H. Borsook, E. A. Fischer, G. Keighley, *J. Biol. Chem.* **229**, 1059 (1957).
7. D. D. Sabatini, K. Bensch, R. J. Barrnett, *J. Cell Biol.* **17**, 19 (1963).
8. L. G. Caro and R. P. van Tubergen, *ibid.* **15**, 173 (1962); A. B. Maunsbach, *J. Ultrastruct. Res.* in press.
9. A. P. Mathias, R. Williamson, H. E. Huxley, S. Page, *J. Mol. Biol.* **9**, 154 (1964).
10. R. A. Rifkind, L. Luzzatto, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.* **52**, 1227 (1964).
11. Since designation of a cell as inactive is based on negative evidence, that is, lack of silver grains, we have considered it prudent to regard as inactive only those ribosome-containing reticulocytes which are found in large sections of uniform thickness, with a regular distribution of cells, and covered with a uniform layer of emulsion (as judged by grain distribution and concentration). Most significant are observations of adjacent inactive and highly active cells which are indistinguishable on the basis of ribosomal concentration and clustering.
12. M. Bessis and M. Bricka, *Rev. Hematol.* **7**, 407 (1952).
13. P. A. Marks, R. A. Rifkind, D. Danon, *Proc. Natl. Acad. Sci. U.S.* **50**, 336 (1963); R. A. Rifkind, D. Danon, P. A. Marks, *J. Cell Biol.* **22**, 599 (1964); E. Glowacki and R. L. Millette, *J. Mol. Biol.* **11**, 116 (1965); P. Rowley, *Federation Proc.* **24**, 223 (1965); D. Danon, T. Zehavi-Willner, G. R. Berman, *Proc. Natl. Acad. Sci. U.S.* **54**, 873 (1965).
14. A. Miller, *Proc. Intern. Congr. Biochem., 6th*, New York, **I**, 135 (1964); A. Miller and A. B. Maunsbach, *J. Cell Biol.* **27**, 65A (1965); ———, *J. Ultrastruct. Res.*, in preparation.
15. Supported in part by NSF (GB-2633) and in part by Cancer Research Funds of the University of California.

12 November 1965

Polysome Morphology: Evidence for Endocrine Control during Chick Embryogenesis

Abstract. *Surgical removal of the pituitary gland had little apparent effect on the distribution or morphology of skin and feather polysomes in chicken embryos incubated for 12 days. However, polysome patterns obtained from 15-day-old hypophysectomized embryos differed markedly from those of their controls. In addition, a tetrad-shaped polysome, characteristic of the 158S peak of the 12-day-old embryo but not of the 15-day-old control, is still retained in the operated embryo at 15 days. Therefore, it appears that after day 12 of embryogenesis the structure of the four-ribosome polysome from skin and feathers is contingent on a functional hypophysis.*

During early development (day 0 to day 12), growth and general differentiation of the chick embryo are not markedly influenced by either extirpation of the pituitary or blocking of the thyroid gland by goitrogenic agents (*1*). However, sometime during the 2nd week of incubation, normal growth becomes dependent on the function of these glands. In the course of this time period, changes have been noted in the sucrose density gradient patterns of skin and feather polysomes obtained from the intact or normal embryo. Before day 13 of incubation the gradient profile is dominated by a four-ribosome peak which has been observed to be short lived, inactive in protein synthesis, and resistant to the action of ribonuclease. It has been suggested that the inactivity and ribonuclease resistance of the four-unit polysomes are the result of their being mainly composed of tight symmetrical squares. After day 13, the polysomes comprising this peak are longer lived, capable of carrying on protein synthesis, and no longer ribonuclease resistant (*2*, *3*). These changes appear to be related to their configuration, which after day 13 is elongated in the manner characteristic of functional units.

In considering the above information it appeared reasonable that the changes noted in the structure and function of skin and feather polysomes during differentiation might have been effected through endocrine mediation. The following experiments were designed to determine whether altered endocrine operation influences polysome structure and function during embryogenesis of the chick.

Fertilized chicken eggs (White Leg-