PROTEINS IN NUCLEOCYTOPLASMIC INTERACTIONS

V. Intranuclear Localization of Proteins in *Amoeba proteus*

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The cellular roles of the two major classes of *Amoeba proteus* nuclear proteins, the rapidly migrating proteins (RMP) and slow turnover proteins (STP) (7), are almost totally obscure. An understanding of their function would be promoted by knowledge of the structural associations of the proteins. However, microscopic examination of appropriate radioautographs of squashed and sectioned cells provides little information regarding the structures in which these proteins

might be localized (1). Studies of similar cells that were centrifuged prior to fixation, on the other hand, show promise of providing some information of intranuclear localization (5). We report here a more detailed study of the localization of these proteins based on studies of centrifuged cells.

It should be recalled that RMP is defined as that class of proteins that is detectable radioautographically in the host cell nucleus after the implantation of a radioactive protein-labeled nucleus into a nonradioactive, nucleated ameba. When the distribution of radioactivity among the cell compartments reaches equilibrium following the transfer of the radioactive nucleus, RMP is distributed approximately equally in each of the two nuclei and cytoplasm, but, since a nucleus is only about 2% of the cell volume, the concentration in each nucleus is about 25–50 times that of the cytoplasm. At equilibrium, the grafted nucleus has approximately five times as much *total* radioactive protein as does the host nucleus (7). This excess in the grafted nucleus is due to the presence of a nonmigrating class of proteins, designated STP. The characteristics just described are the criteria used for identifying the two classes of protein throughout this paper.

MATERIALS AND METHODS

Amoeba proteus, the organism used in this study, was cultured according to the method of Prescott and Carrier (12).

For most experiments, *Tetrahymena pyriformis* were grown in 2% proteose-peptone containing 50 μCi per ml of lysine-^{3}H (L-lysine-4-^{3}H, 7.0 Ci/mmole, Schwarz BioResearch Inc., Orangeburg, N. Y.) or leucine-^{3}H (L-leucine-4,5-^{3}H, 55.5 Ci/mmole, New England Nuclear Corp., Boston, Mass.). (In a few cases, the amebas were labeled by growth in defined medium (3) in which tritiated alanine, lysine, leucine, serine, tryptophan, tyrosine, and valine substituted for their unlabeled counterparts.) After 3–4 days of incubation, radioactive *Tetrahymena* were harvested in ameba medium by centrifugation. The amebas were fed these *Tetrahymena* for 4–5 days, after which the amebas averaged 1200–1500 cpm/cell in a windowless, low-background, gas-flow Geiger Counter (7). Labeled amebas usually were starved for 24 hr before they were used in experiments.

Nuclei were transplanted from one cell to another by the method of Jeon and Lorch (9). After transplantation, the host cells were incubated at 16°C for 4 or 24 hr. For centrifugation, the cells were carefully layered over 40% Ficoll (Sigma Chemical Co., St. Louis, Mo., mol wt ca. 400,000) in a 0.2 ml centrifuge tube and spun at 12,000 *g* for 20 min in a water-cooled microcentrifuge (Microchemical Specialities Co., Berkeley, Calif.). Within approximately 1 min after the centrifuge stopped, the cells were placed in Karnovsky's glutaraldehyde-formaldehyde fixative (10) for 1 hr, washed overnight in distilled water, postfixed for 30 min in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2, mounted in a block of 2% agar according to the method of Flickinger (4), dehydrated in a graded series of ethanol concentrations, passed through propylene oxide, and embedded in Araldite. Serial sections were cut at 1 μ thickness.

For radioautography, the sectioned material on slides was coated with Kodak NTB-3 or Ilford L-4 liquid emulsion (11). After suitable periods of exposure, slides were developed, fixed, rinsed in water, and air dried. Following radioautographic development, slides were stained with buffered toluidine blue (0.5% w/v) or with buffered Giemsa solution, rinsed, air dried, and mounted with Euparol (Arthur H. Thomas Co., Philadelphia, Pa.).

Radioautographic grain counts were made at a magnification of 1000, with the aid of a squared grid in the ocular. Necessary corrections for background were made for every count. Areas of approximately 300 μ^2 were assayed for each determination.

In some cases, conclusions were based on examination of squashed whole cells as well as on sections of cells.

RESULTS

Examination of radioautographs of cells centrifuged 4 hr after the implantation of a protein-^{3}H nucleus showed a striking difference between host nucleus and grafted nucleus in the distribution of radioactivity (Fig. 1). The bulk of the radioactivity is sedimented to the centrifugal end of the donor nucleus, whereas there is no apparent stratification of radioactivity in the host cell nucleus. This is a consistent pattern observed in approximately 240 cells, whether the cells were fed or fasted. The results agree with earlier observations (5). Also in agreement with earlier work is the lack of any consistent stratification of radioactivity in the cytoplasm; there is a slight indication of such stratification in a few cells.

That the nucleus showing stratification of radioactivity is the grafted one is established by the fact that it is the more radioactive one—a clear distinction evident from simple examination of the radioautographs. It follows, then, that only STP is stratified—although all STP is not necessarily sedimentable.

Although simple light microscopic examination of centrifuged cells suggests that RMP is not

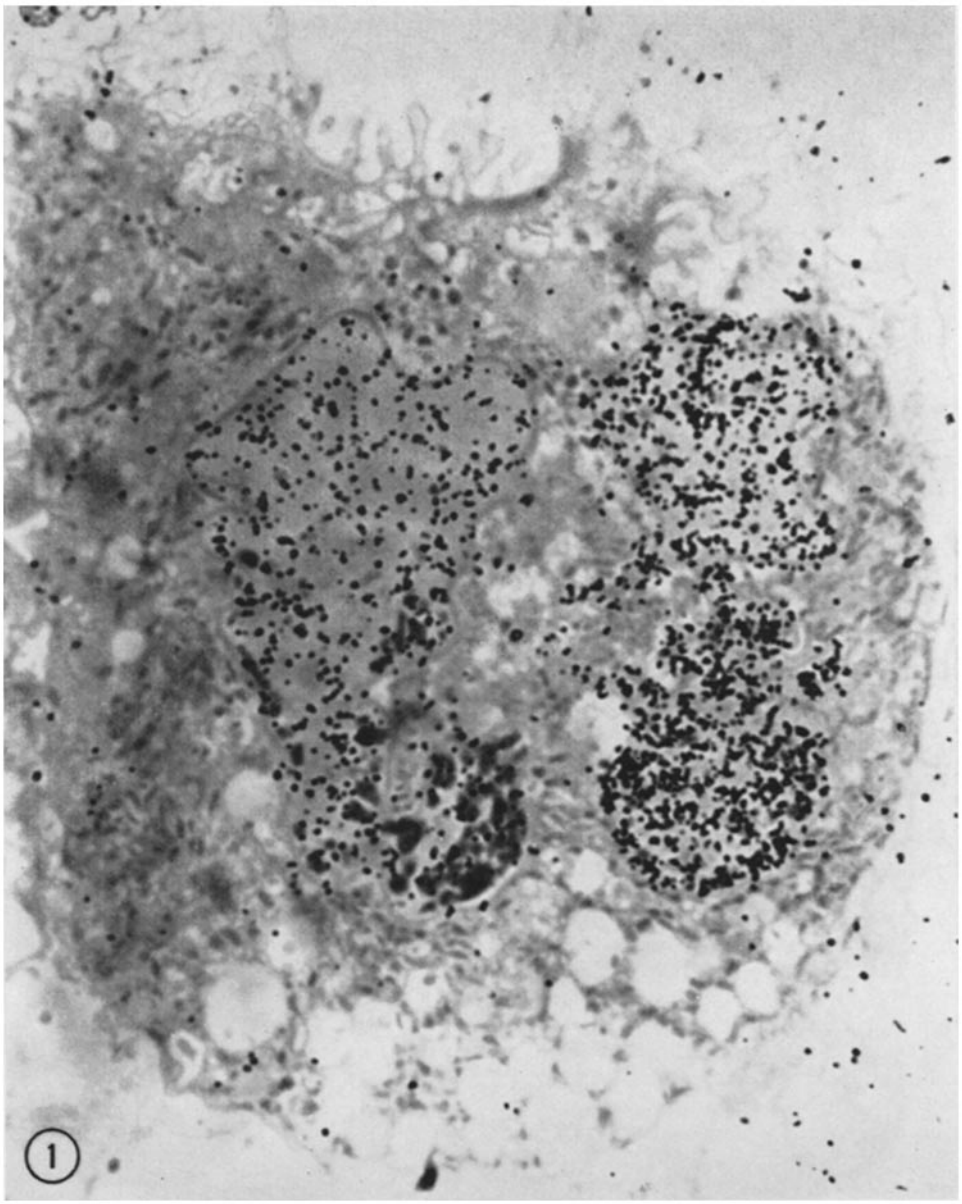

FIGURE 1 Radioautograph of cell that had received a protein-^{3}H nucleus approximately 4 hr prior to centrifugation and fixation. Cell was sectioned at 1 μ and stained with Giemsa's after development of radioautograph. The centrifugal end of the cell is at the bottom. The grafted nucleus is on the right (and is relatively more radioactive than usual at the centripetal end). Approximately $\times$ 1000.

sedimentable, quantitative assessment of the distribution seemed in order. Thus, radioautographic grain count determinations were made on the centripetal and centrifugal parts of host and donor nuclei of sectioned experimental cells. The results shown in Table I confirm that STP is sedimentable and that RMP is not, under the conditions we employed. (Since, to minimize ambiguity, the assays shown in Table I were made only on the centripetal and centrifugal thirds of the nuclei, in another experiment we investigated the possibility that there was some stratification in the middle

TABLE I

The Distribution of Radioactivity in Different Parts of Grafted and Host Nuclei

	Centrifugal	Centripetal
Grafted nucleus	50.1 ± 2.8	11.9 ± 0.6
Host nucleus	7.9 ± 0.7	7.0 ± 1.0

Number of cells = 7

Numbers are means of the number of radioautographic grains per 100 μ^2 and the standard errors of the means. The cells were centrifuged and fixed 4 hr after the implantation of a protein-^{3}H nucleus. The centripetal-centrifugal axis through each nucleus was divided into three, and the centripetal counts were made from the upper "third" and the centrifugal counts from the lower "third" of each nucleus.

third of the host nucleus and found essentially equal labeling throughout the nucleus.) We conclude that quantitative assays confirm the earlier qualitative impressions. Before continuing, the reader should note that the data given in Table I indicate a difference in activity for the centripetal ends of the two nuclei. This will be elaborated upon later.

Since the nucleoli are sedimented by the centrifugation procedures we used, labeled STP was thought to be associated with those structures. However, examination of preparations like that shown in Fig. 1 could not establish whether such was indeed the case.

In order to obtain a clearer view of labeled STP localization, cells were depleted of radioactive RMP prior to centrifugation. The depletion was effected by grafting a protein-^{3}H nucleus into an unlabeled, enucleate host, and, 6 hr later, transferring the nucleus to a second unlabeled, enucleate host and, another 6 hr later, grafting the nucleus into an unlabeled nucleate host—the final host before centrifugation. Since labeled RMP distributes itself approximately equally between the nucleus and cytoplasm after about 3 hr (8), the grafted nucleus in the final host should have about ¼ of the original amount of labeled RMP and almost all of the radioactive STP. Counts of radioautographic grains over 40 nucleoli in each of donor and host nuclei of six such final hosts showed the donor nucleoli to have approximately five times the activity of the host cell nucleoli, establishing that some STP is associated with nucleoli.

Estimations of radioautographic grain density over nucleolar and nonnucleolar regions of centrifuged nuclei are, however, unreliable because the small size of the nucleoli makes radioautographic resolution difficult and the number of grains over each nucleolus is necessarily small. Despite these limitations, the analyses do suggest that some of the sedimentable radioactivity is *not* associated with nucleoli. Better optical resolution is necessary to permit decisive conclusions; preliminary electron microscopic observations (Chatterjee and Kloetzel, unpublished) indicate that some sedimentable label is not nucleolar. Daniels and Breyer (2) have shown that other fine structures also are stratified by centrifugation; and, thus, there is opportunity to study other structural associations of these labeled proteins.

Speculation that RMP might be concerned with the regulation of gene transcription led to experiments (still unpublished) that suggested that the concentration of RMP in the nucleus (where it is approximately 25–50 times more concentrated than in the cytoplasm) is due to binding of RMP to chromatin. The results of the following experiment argue against this possibility.

Since we found that RMP is not stratified by the centrifugal conditions we employed, we investigated whether nuclear DNA is. Because we know of no dye that can adequately stain DNA in the *A. proteus* nucleus, we labeled the DNA with thymidine-^{3}H, centrifuged the cells as described, and then localized the radioactivity (hence the DNA) by radioautography (Chatterjee, unpublished). The thymidine-^{3}H activity was found to be largely stratified as a band just above the layer of nucleoli. RMP which does not stratify, thus cannot be associated with the bulk of the chromatin.

Although the radioautographs show that much STP is stratified, assays of radioautographic grain densities suggest that some STP, like RMP, is not stratified by centrifugation. Table II shows the radioautographic grain densities over sections of the centripetal thirds of paired host and donor nuclei of 19 cells. Evidently, the centripetal ends of the donor nuclei contain approximately 1.67 times the radioactivity of the centripetal ends of the host nuclei, indicating that some STP does not stratify. (Although based on a smaller sample, the data of Table I show much the same.) This conclusion is further supported by the results of experiments in which the grafted radioactive nucleus is depleted of labeled RMP by passage through two unlabeled cytoplasms prior to implantation into

the final unlabeled, nucleate host (as described above). The data for 20 such cells, given in Table III, show, as would be predicted, that the centripetal ends of the grafted nuclei have proportionately more radioactivity than in the experiment shown in Table II. The more radioactive nuclei have only two times as much radioactivity as the host nuclei in the centripetal ends, however, whereas we expected a greater difference following depletion of 75% of the RMP. That the difference is not greater suggests that some STP also is being lost from nuclei during the serial transfers, as was indicated in earlier experiments (8).

Earlier experiments showed that an unlabeled nucleus could acquire labeled STP as well as labeled RMP from enucleate protein-^{3}H cytoplasm (6), a finding suggesting the existence of a cytoplasmic pool of STP. So that we could learn something of the nature of this pool, unlabeled nuclei were grafted into protein-^{3}H cytoplasm and, 16 hr later, grafted into unlabeled, nucleate cells. 4 hr later, such cells were either centrifuged or each of the two nuclei of each cell was transferred to an unlabeled host and fixed immediately. The latter were used for Geiger counter determinations of the distribution of radioactivity. The data from three experiments, involving 35 cells for this latter procedure, show that the activity of the nucleus that had been in protein-^{3}H cytoplasm averaged just about twice as much activity as the final host cell nucleus, indicating that the grafted nucleus had acquired, by our definition, some STP from the labeled cytoplasm. Radioautographs of the centrifuged cells comprising the other part of the experiments showed little, if any, stratification of radioactivity. Thus, it appears that the labeled STP acquired by nuclei from the cytoplasm is largely of the nonsedimentable type.

TABLE II

A Comparison of the Radioactivity in the Centripetal "Third" of Host and Donor Nuclei

Donor	Host	Mean ratio
37.0 ± 2.2	22.3 ± 1.0	1.67 ± 0.07 to 1
Number of cells = 19		

The cells were centrifuged and fixed 24 hr after implantation of protein-^{3}H nuclei. Mean Ratio refers to mean of individual ratios of centripetal "third" of donor nucleus to centripetal "third" of host nucleus of each cell. Other details as for Table I.

TABLE III

Same as Table III, Except that Donor Nucleus was Depleted of Labeled RMP by Passage of Protein-^{3}H Nucleus through Two Unlabeled Cytoplasm Prior to Implantation into Fixed Host Cell

Donor	Host	Mean ratio
23.7 ± 2.6	12.2 ± 1.4	2.0 ± 0.1 to 1
Number of cells = 20		

Cells centrifuged and fixed 24 hr after protein-^{3}H nuclei implanted into final hosts. Other details as for Tables I and II.

DISCUSSION

The experiments reported here show that, in *Amoeba proteus* centrifuged at 12,000 *g* for 20 min, only the slow turnover proteins (STP) are caused to stratify; the rapidly migrating proteins (RMP) are apparently not moved by these forces. We can also conclude the following:

RMP

The bulk of RMP apparently is *not* associated with the nuclear envelope (there is uniform distribution of radioactivity in thin sections of the experimental nuclei), the nucleoli (RMP radioactivity is not stratified as are nucleoli), or the chromatin (thymidine-^{3}H label in the nucleus is stratified but RMP is not). An understanding of the structural basis for concentrating nuclear RMP is thus still remote. Experiments employing much higher centrifugal forces, like that used by Daniels and Breyer (2), for example, may be useful in the future.

STP

STP apparently can be divided into three classes: (*a*) Nucleolar-associated STP (that is sedimentable with the nucleoli); (*b*) Sedimentable but not nucleolar-associated STP; (*c*) Nonsedimentable STP. Probably, STP is even more heterogeneous than these experiments show. Of the pool of cytoplasmic STP available for transport to the nucleus during interphase, most seems to be in the form of nonsedimentable STP. Further understanding of STP will depend largely on electron microscopic investigations and/or isolation and purification of the proteins by more traditional biochemical means.

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REFERENCES

1. BYERS, T. J., D. B. PLATT, and L. GOLDSTEIN. 1963. The cytonucleoproteins of amebae. I. Some chemical properties and intracellular distribution. *J. Cell Biol.* **19**:453.
2. DANIELS, E. W., and E. P. BREYER. 1966. Stratification within centrifuged amoeba nuclei. *Z. Zellforsch. Mikrosk. Anat.* **70**:449.
3. ELLIOTT, A. M., L. E. BROWNELL, and J. A. GROSS. 1954. The use of *Tetrahymena* to evaluate the effects of gamma radiation on essential nutrilites. *J. Protozool.* **1**:193.
4. FLICKINGER, C. J. 1966. Methods for handling small number of cells for electron microscopy. *In* Methods in Cell Physiology. D. M. Prescott, editor. Academic Press Inc., New York. **2**:311.
5. GOLDSTEIN, L. 1965. Interchange of protein between nucleus and cytoplasm. *In* The Use of Radioautography in Investigating Protein Synthesis. C. P. Leblond and K. B. Warren, editors. Academic Press Inc., New York. 79.
6. GOLDSTEIN, L., and D. M. PRESCOTT. 1967. Protein interactions between nucleus and cytoplasm. *In* The Control of Nuclear Activity. L. Goldstein, editor. Prentice-Hall Inc., Englewood Cliffs, New Jersey. 273.
7. GOLDSTEIN, L., and D. M. PRESCOTT. 1967. Proteins in nucleocytoplasmic interactions. I. The fundamental characteristics of the rapidly migrating proteins and the slow turnover proteins of the *Amoeba proteus* nucleus. *J. Cell Biol.* **33**:637.
8. GOLDSTEIN, L., and D. M. PRESCOTT. 1968. Proteins in nucleocytoplasmic interactions. II. Turnover and changes in nuclear protein distribution with time and growth. *J. Cell Biol.* **36**:53.
9. JEON, K. W., and I. J. LORCH. 1968. New simple method of micrurgy of living cells. *Nature (London).* **217**:463.
10. KARNOVSKY, M. J. 1965. Formaldehyde-glutaraldehyde fixative of higher osmolality for use in electron microscopy. *J. Cell Biol.* **27**:137A. (Abstr.)
11. PRESCOTT, D. M. 1964. Autoradiography with liquid emulsion. *In* Methods in Cell Physiology. D. M. Prescott, editor. Academic Press Inc., New York. **1**:365.
12. PRESCOTT, D. M., and R. F. CARRIER. 1964. Experimental procedures and cultural methods for *Euplotes eurystomus* and *Amoeba proteus*. *In* Methods in Cell Physiology. D. M. Prescott, editor. Academic Press Inc., New York. **1**:85.