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 the 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-3H (L-lysine-4-3H, 7.0 Ci/m mole, Schwarz BioResearch Inc., Orangeburg, N. Y.) or leucine-3H (L-leucine-4,5-3H, 55.5 Ci/m mole, 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 µ² 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-3H 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
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

**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 $\mu$ 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.
TABLE 1
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 µ² and the standard errors of the means. The cells were centrifuged and fixed 4 hr after the implantation of a protein-3H 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.

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-3H, centrifuged the cells as described, and then localized the radioactivity (hence the DNA) by radioautography (Chatterjee, unpublished). The thymidine-3H 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

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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 centri-
petal ends of the grafted nuclei have proportion-
ately 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 follow-
ing depletion of 75% of the RMP. That the differ-
ence 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-3H cyto-
plasm (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-3H 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 determina-
tions 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-3H 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.

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 dis-
tribution of radioactivity in thin sections of the
experimental nuclei), the nucleoli (RMP radio-
activity is not stratified as are nucleoli), or the
chromatin (thymidine-3H 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 sedi-
mentable 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 cyto-
plasmic 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 micro-
scopic investigations and/or isolation and purifica-
tion of the proteins by more traditional biochem-
ical means.

**Table II**

| 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-3H 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
Depicted of Labeled RMP by Passage of Pro-
tein-3H 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-3H
nuclei implanted into final hosts. Other details as
for Tables I and II.
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