

# Distribution of Proteins between Nucleus and Cytoplasm of *Amoeba proteus*

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**ABSTRACT** By transplanting nuclei between labeled and unlabeled cells, we determined the localization of the major proteins of amebas and described certain features of their intracellular distribution. We identified ~130 cellular proteins by fluorography of one-dimensional polyacrylamide electrophoretic gels and found that slightly less than half of them (designated NP, for nuclear proteins) are almost exclusively nuclear. About 95% of the other proteins (designated CP, for cytoplasmic proteins) are roughly equally concentrated in nucleus and cytoplasm, but—because the cytoplasm is 50 times larger than the nucleus—about 98% of each of the latter is in the cytoplasm. Of the CP, roughly 5% are not detectable in the nucleus. Assuming that these are restricted to the cytoplasm only because, for example, they are in structures too large to enter the nucleus and labeled CP readily exit a nucleus introduced into unlabeled cytoplasm, we conclude that the nuclear envelope does not limit the movement of any nonstructural cellular protein in either direction between the two compartments. Some NP are not found in the cytoplasm (although ostensibly synthesized there) presumably because of preferential binding within the nucleus. Almost one half of the protein mass in nuclei *in vivo* is CP and apparently only proteins of that group are lost from nuclei when cells are lysed. Thus, while an extracellular environment allows CP to exit isolated nuclei, the nuclear binding affinities for NP are retained.

Further examination of NP distribution shows that many NP species are, in fact, detectable in the cytoplasm (although at only about 1/300 the nuclear concentration), apparently because the nuclear affinity is relatively low. These proteins are electrophoretically distinguishable from the high-affinity NP not found in the cytoplasm.

New experiments show that an earlier suggestion that the nuclear transplantation operation causes an artifactual release of NP to the cytoplasm is largely incorrect. Moreover, we show that cytoplasmic “contamination” of nuclear preparations is not a factor in classifying proteins by these nuclear transplantation experiments.

We speculate that no mechanism has evolved to confine most CP to the cytoplasm (where they presumably function exclusively) because the cytoplasm's large volume ensures that CP will be abundant there. Extending Bonner's idea of “quasi-functional nuclear binding sites” for NP, we suggest that a subset of NP usually have a low affinity for available intranuclear sites because their main function(s) occurs at other intranuclear sites to which they bind tightly only when particular metabolic conditions demand. The other NP (those completely absent from cytoplasm) presumably always are bound with high affinity at their primary functional sites.

**Fundamental to a full appreciation of interactions between nucleus and cytoplasm in the control of gene expression and other activities of the nucleus is identification of those protein**

**molecules that may be uniquely present in each cell compartment and hence putative candidates for agents responsible for functions restricted to nucleus or cytoplasm. The movement,**

or lack thereof, of these molecules between the two compartments may also provide clues to possible roles in gene expression or replication. Major, but not exclusive, attention in these matters has focused on nonhistone proteins of the nucleus because of the current consensus that these proteins are the molecules primarily responsible for the specificity of differential expression of genes in different cells or under varying conditions (see, for example, reference 1).

An interesting assortment of methods have been employed to study the distribution of proteins between nucleus and cytoplasm (see Discussion), but until recently only in a few studies have specific intracellular proteins been identified and their distribution characterized. In previous studies, molecules not normally present in cells have been examined; proteins have been characterized by homogenizing and fractionation of cells; molecules have been extracted, purified, and reinjected into cells; only one or a few kinds of proteins could be studied; etc. (see reference 2 for review). Most studies centered on the movement (or nonmovement) of molecules from cytoplasm to nucleus; rarely was the movement of molecules from nucleus to cytoplasm or their true intracellular distribution determined with confidence. As a result, these studies provided little advance in understanding of how these molecules function.

The particular advantage of our method of study is that we are, in a sense, looking at the full spectrum of proteins normally present in the nucleus and cytoplasm and examining the behavior and localization of these proteins without ever isolating any cell part or molecule before the final assay. Moreover, in all crucial experiments, that final assay does not require cell lysis; the proteins to be characterized are extracted directly from lyophilized cells. Our fundamental approach is to exchange, by micromanipulation, nuclei between [<sup>35</sup>S]methionine-labeled and unlabeled cells, so that one cell has a labeled protein nucleus in unlabeled cytoplasm and another has the opposite arrangement. We can determine (by further nuclear transplantation if necessary) which proteins are retained by each compartment and which move from one compartment to the other.

We have shown, without necessarily identifying particular proteins, that the pattern of protein distribution may reveal how some proteins may come to serve regulatory roles. A major conclusion of this work is that almost all cellular polypeptides can enter the nucleus, but only a fraction of them is retained within the nucleus at a higher concentration than in the cytoplasm. Whereas many of the latter polypeptides are present in the cytoplasm at very low concentration, some appear to be entirely absent from the cytoplasm—and only a relatively small proportion of all the protein species in the cytoplasm are undetectable in the nucleus. In the Discussion we consider how the data from several other laboratories on different experimental systems are consistent with these conclusions.

The mechanisms by which nuclear, but not cytoplasmic, proteins are retained within the nucleus is not known, although it may be significant that even isolated nuclei show the same kind of discriminations. We propose that whatever is responsible for that selectivity is related to mechanisms responsible for differential gene expression and general changes in nuclear activity.

## MATERIALS AND METHODS

### *Cells and Cell Culturing*

Used throughout this work was the free-living amoeba, *Amoeba proteus*, cultured as described earlier (3).

### *Radioactive Labeling of Cells*

Amebas were labeled by feeding them [<sup>35</sup>S]methionine-labeled tetrahymenas (4).

The cells were labeled with <sup>3</sup>H-amino acids as follows. Tetrahymenas from a log-phase culture growing on 2% proteose peptone were inoculated into a defined medium (5) in which 150 μCi/ml of [<sup>3</sup>H]leucine, 50 μCi/ml of [<sup>3</sup>H]lysine, and 150 μCi/ml of [<sup>3</sup>H]alanine were substituted for their unlabeled analogues and incubated for about 24 h at 29°C. Amebas were fed for 2 d with the labeled tetrahymenas washed free of the defined medium, and then fed unlabeled tetrahymenas for another 2 d. At the end of that period, the amebas averaged 7.5–10 × 10<sup>4</sup> dpm/cell.

### *Assay of Radioactivity*

Radioactivity was assayed as described previously (6).

### *Nuclear Transplantation*

Nuclei were transplanted according to the method of Jeon and Lorch (7), with the following modifications. To minimize the adsorption by one cell of any labeled material that might have been released from another cell during nuclear transplantation, the operations were performed in a cell extract made by homogenizing 1 part by volume of amebas in 3.3 parts by volume amoeba medium and removing all particulate matter by centrifugation. This "operation medium" was roughly equivalent to a 1% protein solution. In some instances, to facilitate a closer examination of the nucleus being transplanted and to exclude any nuclei having any adhering cytoplasm, the nuclei being transplanted were allowed to remain in the external medium for ~10 s rather than being pushed directly from one cell into the other. We determined that for ~80% of the nuclei this extracellular exposure had no effect on the subsequent survival and reproduction of the recipient cell. For some experiments it was necessary during the transplantation operations to distinguish between two different nuclei in one cell. This was accomplished by arranging for the two nuclei to differ in size (6).

### *Isolation of Nuclei*

Nuclei were isolated by lysing cells in a medium of 4 mg spermidine phosphate and 0.5 ml Triton X-100 per 100 ml water (8).

### *Cell Fixation, Embedment, and Radioautography*

Amebas were fixed in a mixture of glutaraldehyde and paraformaldehyde in a sodium cacodylate buffer (9), followed by a rinse and soaking for at least 2 d in the cacodylate buffer. To facilitate handling of groups of cells, they were embedded in 2% agar by an adaptation of Flickinger's method (10). After trimming, the agar block containing the cells was "postfixed" in 1% buffered OsO<sub>4</sub> for 1 h and dehydrated over a period of 2 d in a graded series of ethanol concentrations followed by propylene oxide. The extended soaking and dehydration were carried out to minimize chemical fogging of radioautographic film. Finally, the block was embedded in Araldite.

For light microscope radioautography (AR), the trimmed Araldite block was sectioned at 0.5 μm, and the sections were placed on subbed microscope slides. The slides were coated with either Ilford L-4 or Kodak NTB-3 nuclear emulsion diluted 1:1 with water and, after suitable exposure in the dark at 4°C, developed in Kodak K-19 and fixed in Kodak Fixer. For microscope examination, the slides were stained with 1% methylene blue in a 1% sodium borate buffer, and destained in water and then in ethanol.

### *Extraction and Polyacrylamide Gel Electrophoresis (PAGE) of Proteins*

These procedures were carried out essentially as described by others (11, 12). For estimation of molecular weights, the following purified proteins were run in lanes adjacent to the protein mixtures to be analyzed: cytochrome *c* (~12,300 mol wt), carbonic anhydrase (~30,000 mol wt), ovalbumin (~46,000 mol wt), bovine serum albumin (~69,000 mol wt), phosphorylase *b* (~92,500 mol wt), and myosin (~200,000 mol wt), all of which were obtained from New England Nuclear Corp., Boston, Mass.

### *Fluorography*

Destained PAGE gels were processed for fluorography by an adaptation of the Bonner and Laskey method (described in reference 4).

## RESULTS

### Initial Characterization of Proteins of Nucleus and Cytoplasm

The initial characterization was achieved by exchanging nuclei between  $^{35}\text{S}$ -labeled and unlabeled amoebas. After ~24 h of incubation, the nuclei were transplanted to unlabeled cells, which were shortly thereafter lyophilized for extraction purposes, as were the enucleate cytoplasms from which the nuclei had been removed (Fig. 1). This resulted in four different groups of cell parts (~200 in each group): (1) the original  $^{35}\text{S}$ -labeled cytoplasms; (2) the original  $^{35}\text{S}$ -labeled nuclei; (3) the original unlabeled cytoplasms, which acquired any  $^{35}\text{S}$ -labeled proteins that exited from 2; and (4) the original unlabeled nuclei, which acquired any  $^{35}\text{S}$ -proteins that entered from 1. The results of PAGE of the four fractions are shown in Fig. 2. What is immediately apparent is that the patterns of labeled proteins in fractions 1, 3, and 4 are remarkably similar, whereas the pattern for 2 differs markedly from the others. The difference between the proteins of the cytoplasm (seen in fraction 1 for example, and hereinafter called CP [for cytoplasmic proteins]) and the proteins that do not exit from the nucleus under

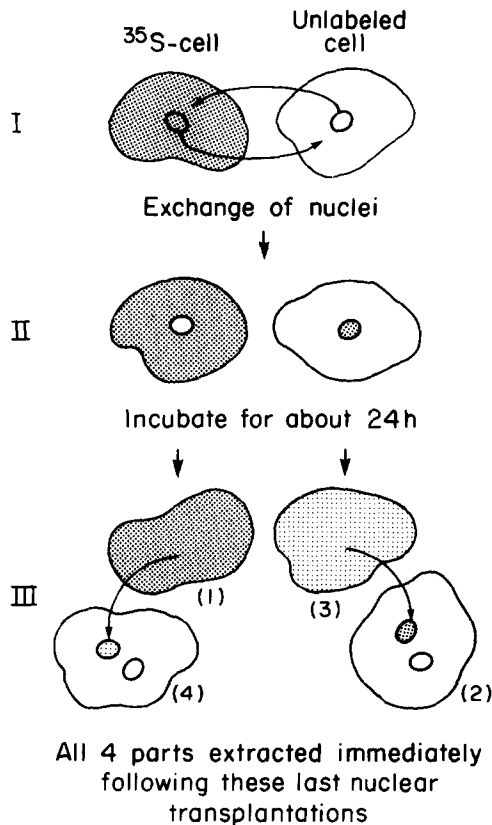


FIGURE 1 Diagram of initial experiment to characterize proteins of nucleus and cytoplasm. At stage I, nuclei were exchanged between [ $^{35}\text{S}$ ]methionine-labeled cells (stippled to represent radioactivity) and unlabeled cells (not stippled). At stage II, cells were incubated in nonradioactive medium for ~24 h. At stage III, the nuclei of the two kinds of cells were transplanted to unlabeled cells, which were shortly thereafter lyophilized for extraction purposes—as were the enucleate donor cytoplasms. The four pooled components (with numbers corresponding to those used in the figure and the text) are: (1) the original  $^{35}\text{S}$ -cytoplasms; (2) the original  $^{35}\text{S}$ -nuclei; (3) the original unlabeled cytoplasms, which had acquired  $^{35}\text{S}$ -proteins from 2; and (4) the original unlabeled nuclei, which had acquired  $^{35}\text{S}$ -proteins from 1.

these conditions (seen in fraction 2 and hereinafter called NP [for nucleic proteins]) is even more striking when seen in higher resolution gels. The simplest interpretation of this basic experiment is that most CP move freely between nucleus and cytoplasm, whereas NP are largely (if not exclusively) confined to the nucleus.

As can be deduced from the patterns of 3 and 4 in Fig. 2, most (probably 90–95%) CP are present in nuclei as well as cytoplasms. In addition, as will be seen below, many NP species are present in the cytoplasm at low concentration (roughly 1/300 of that in the nucleus). This may make the chosen CP and NP nomenclature seem inappropriate, but we base the nomenclature on where the greatest amount of a particular protein is found and almost always the difference between the two compartments in the amount of any one polypeptide species is an order of magnitude or more.

A number of additional points need to be noted. Of the roughly 70 different proteins seen in each compartment, few seem to have the same electrophoretic mobility in nucleus and cytoplasm, a matter to be clarified by two-dimensional PAGE now in progress. That the present one-dimensional PAGE gels display only the quantitatively major proteins of each compartment (which actually must contain hundreds to thousands of different polypeptides) must be recognized; thus, these gel patterns provide only a crude quantitative comparison of the proteins of nucleus and cytoplasm. Moreover, the reader is cautioned to avoid quantitative comparisons between adjacent lanes on any gels, since the lanes may well have been loaded

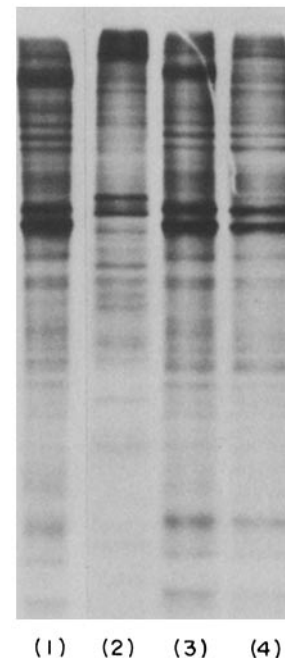


FIGURE 2 Fluorograms of protein patterns obtained from experiments described in text and Fig. 1. Using same numerology as before: lane 1 contains material of the original  $^{35}\text{S}$ -cytoplasms (40 parts); lane 2 contains material of the original  $^{35}\text{S}$ -nuclei (38 parts); lane 3 contains the material of the original unlabeled cytoplasms (28 parts); and lane 4 contains the material of the original unlabeled nuclei (40 parts). For lane 1, although 40 cytoplasms were extracted, only 1/40 of the extract was loaded on the gel. In the actual slab gel, several lanes separated lane 1 from the other three lanes shown and thus this figure is a montage; that is the reason the alignment of the bands of lane 1 with those of lanes 3 and 4 does not seem as good as might be expected.

with material from different numbers of cell parts. In general, the material run in each lane was extracted from between 40 and 200 nuclei or the equivalent of just a few cytoplasms. The patterns of Coomassie Blue-stained gels of cytoplasmic material are sufficiently similar to the patterns of the fluorograms of CP to ensure that we probably are observing "steady-state" labeling in the latter.

In sum, these observations are best explained as follows: NP are almost exclusively confined to the nucleus, whereas CP move freely in both directions across the nuclear envelope (NE).

### Simple Quantitative Relationships between the Proteins of Cytoplasm and Nucleus

The amoeba nucleus occupies ~2% of the volume of *Amoeba proteus* (13) and we have repeatedly found (our unpublished results) that the nucleus contains ~4% (range: 3–5%) of cellular protein. (This means, of course, that the concentration of protein is about twice as high in nucleus as in cytoplasm.) Given those values, it is interesting that in the experiments described in the previous section, the amount of protein acquired by an unlabeled nucleus in labeled cytoplasm is roughly 2% of the cytoplasmic total and the amount of labeled proteins (essentially CP) exiting from the labeled nucleus is repeatedly found to be ~40% nuclear total, or almost 2% of the labeled protein of the original <sup>35</sup>S-labeled cell. Thus, the nuclear concentration of CP is approximately equal to the cytoplasmic concentration. This is similar to an earlier finding with respect to the major amoeba protein actin (14).

### Are Any CP Excluded from the Nucleus?

Because all, or virtually all, proteins (including NP) are synthesized in the cytoplasm of most cells (15), it would appear from the data of our first experiment that the NE is no barrier to the movement of any protein, whether NP or CP, from cytoplasm to nucleus. This result seems surprising, especially because the proteins of cytoplasmic organelles, for example, might be expected not to move freely through the cell. It may be, however, that proteins of cytoplasm structures are quantitatively too rare to be detected by the PAGE analyses we have done. (We believe that our protein extraction procedure—probably better called solubilization procedure—is not selective, because at least 98% of the radioactive proteins of all preparations enters most PAGE gels.) Careful examination of PAGE fluorograms reveals, however, that some CP do not, in fact, pass from cytoplasm to nucleus. This is shown reasonably well in Fig. 3, where the proteins marked 20K, 30K, and 90K are evidently present in the cytoplasm but not in the originally unlabeled nuclei that were grafted into those cytoplasms. If this reflects the general proportion of total CP excluded from nuclei, roughly 5–10% of all CP are in that group.

An additional point of interest in Fig. 3 is a polypeptide (38K) seen in the nucleus but not in the cytoplasm, although obviously all the labeled nuclear material must have come from the cytoplasm. In other experiments one or two proteins of different molecular weights have been found to behave in a similar manner. The two simplest interpretations of such an effect are: (a) a particular CP is modified upon entering the nucleus, and (b) a protein present in low concentration in the cytoplasm is accumulated to relatively high concentration in the nucleus.

### The Possibility of Artifact

Could some of the results described thus far reflect cytoplasmic "contamination" of nuclear preparations? One can imagine that the label thought of as exiting from a grafted <sup>35</sup>S-labeled nucleus into unlabeled cytoplasm is actually only cytoplasmic material that adhered to the nucleus as it was being transplanted and, conversely, labeled protein ostensibly acquired by an unlabeled nucleus in labeled cytoplasm might simply reflect the acquisition of adhering radioactive cytoplasm. Much evidence makes those possibilities seem unlikely.

That transplanted nuclei are unlikely to be contaminated with cytoplasm is implicit in the finding that an unlabeled nucleus in <sup>35</sup>S-labeled cytoplasm does not acquire the full spectrum of CP (Fig. 3). Moreover, we know from direct microscope observation during the nuclear transplantation operation—especially if, for increased clarity, the nucleus is left in the external medium for a few seconds before implantation into the recipient cell—that little, if any, cytoplasm is detectable on the nucleus. Whatever cytoplasm may accompany the nucleus during transplantation, it must be far less than the volume of the nucleus; yet the quantitative relations noted above would require that the amount of "contaminating" cytoplasm ought to equal the volume of the nucleus—if cytoplasmic "contamination" is responsible for patterns 3 and 4 in Fig. 2. In earlier nuclear transplantation experiments, Craig and Goldstein (16) could detect no ribosomal protein as part of transplanted nuclei, a result consistent with the absence of cytoplasmic contamination on such nuclei.

Because the presence of cytoplasmic material would vitiate our interpretation of most of the results with nuclei, we sought to establish with greater certainty whether such contamination occurs. Accordingly, we explored the possibility of contamination in yet another way, which we consider decisive. We

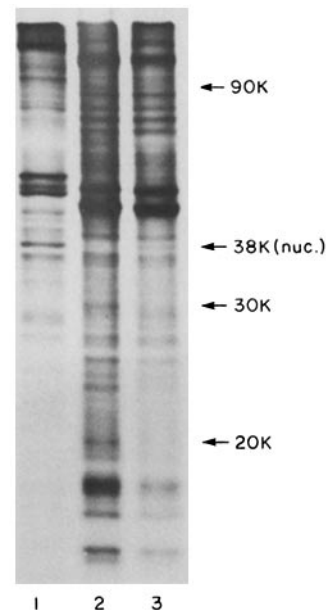


FIGURE 3 Fluorogram illustrating that some CP do not enter the nucleus. Lane 1, the contents of the original <sup>35</sup>S-labeled nuclei after CP were allowed to exit. Lane 2, the contents of the original <sup>35</sup>S-cytoplasms. Lane 3: the <sup>35</sup>S-labeled contents acquired by unlabeled nuclei placed in the cytoplasms used in lane 2. The arrows marked 20K, 30K, and 90K point to the position of bands detectable in lane 2 but not in lane 3. The arrow marked 38K points to the position of a band detectable in lane 3 but not in lane 2.

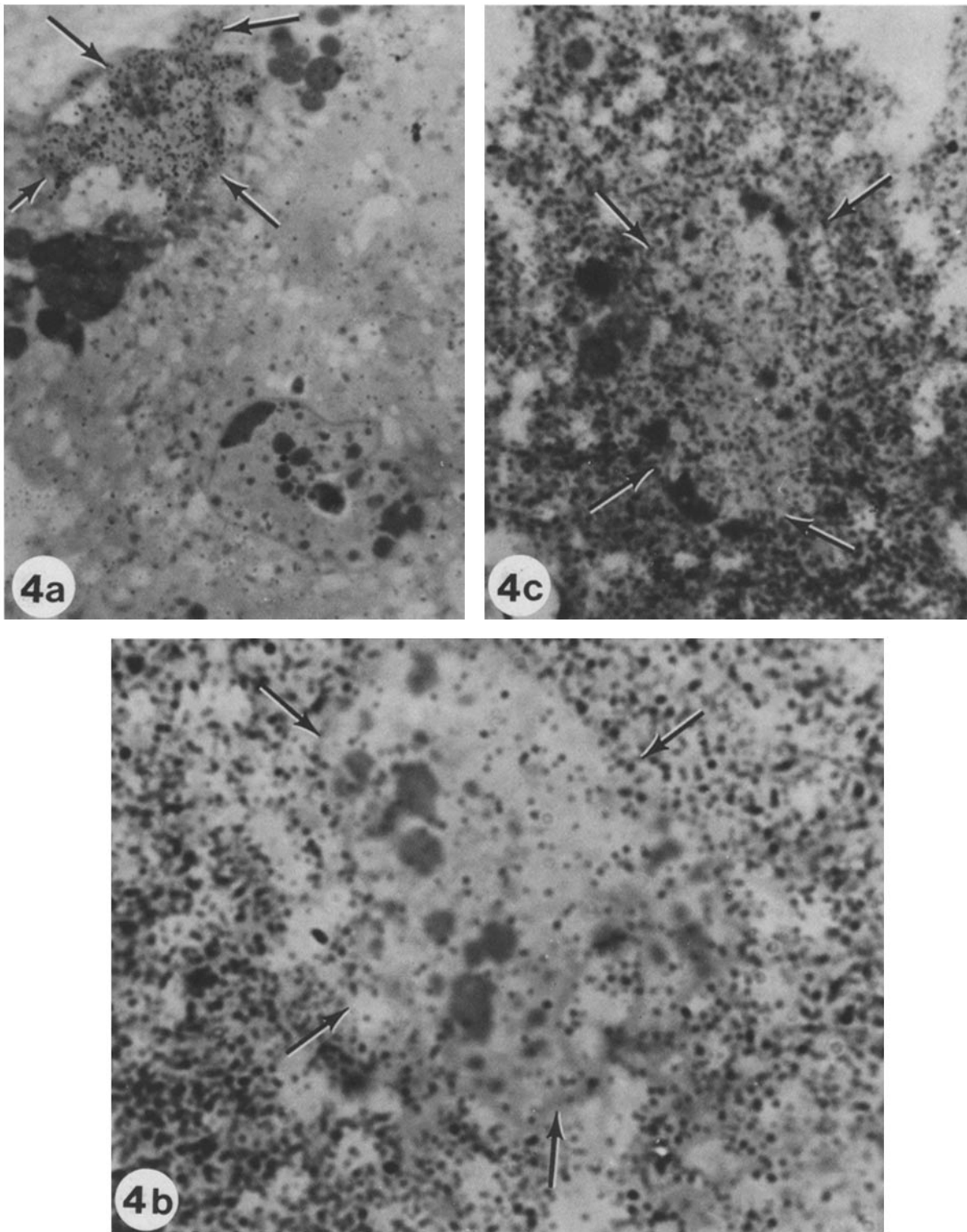


FIGURE 4 Photomicrographs demonstrating that cytoplasmic "contamination" cannot be a significant factor in the results reported in this paper. Fig. 4 a shows a radioautogram of a 0.5- $\mu$ m section through a cell into which a [ $^3$ H]methionine-labeled nucleus was implanted 5–10 s before the recipient cell was fixed.  $\times \sim 600$ . The grafted and host nuclei are seen; note that little or no cytoplasmic radioactivity is adhering to the former nucleus. (The labeled nucleus is momentarily misshapen as a result of the nuclear transplantation operation.) Fig. 4 b shows a radioautogram of a 0.5- $\mu$ m section through an enucleate [ $^3$ H]methionine-labeled cell into which an unlabeled nucleus was implanted  $\sim 3$  min before fixation.  $\times \sim 1,600$ . Note that by that time some radioactivity already is present within the grafted nucleus. Fig. 4 c shows a radioautogram of a 0.5- $\mu$ m section through a similar cell as for Fig. 4 b, except that the recipient cell was fixed  $\sim 6$  h after implantation of the nucleus.  $\times \sim 1,000$ . The level of radioactivity within the nucleus is almost equal to that in the cytoplasm. The arrows in the figures point to various locations on the NE of the nuclei to provide some idea of the nuclear boundaries.

transplanted nuclei from  $^3\text{H}$ -protein-labeled cells into unlabeled cells and fixed the recipients as rapidly as possible and looked for the presence of accompanying  $^3\text{H}$ -cytoplasm by AR; we also transplanted unlabeled nuclei into  $^3\text{H}$ -protein-labeled cells and fixed them at different times thereafter to determine by AR the rate and degree of entry into nuclei of  $^3\text{H}$ -proteins from the cytoplasm. (We used  $^3\text{H}$  rather than  $^{35}\text{S}$  to obtain better AR resolution.) Fig. 4 shows that typically little or no labeled cytoplasmic material accompanies a nucleus transplanted from a  $^3\text{H}$ -protein-labeled cell and that labeled protein is detectable inside an unlabeled nucleus within a few minutes after its implantation into  $^3\text{H}$ -labeled cytoplasm. The concentration of  $^3\text{H}$ -protein within such nuclei by 6 h after implantation is almost as great as in the surrounding cytoplasm, but it should be recalled that we do not normally assay nuclear contents until ~24 h after transplantation. Thus, the material normally assayed must be considered to be largely inside the nuclei and not from adhering cytoplasm.

These, and earlier, observations give us confidence that cytoplasmic "contamination" is an insignificant factor in the results reported here.

### The *In Vitro* Affinity of Proteins for the Nucleus

Because almost all (if not all) NP are synthesized in the cytoplasm and then cross the NE into the nucleus and because we show here that probably >90% of CP cross the NE in both directions, why NP appear to move only unidirectionally is an intriguing question—but is not answered in this paper. Because molecules up to 200,000 dalton (and probably larger) traverse the NE in both directions, NE pore size seems not to be a factor in retaining NP within the nucleus (but may influence the rate of movement [2]). In fact, no convincing evidence exists that the NE regulates the passage of materials between nucleus and cytoplasm. A few observations may, however provide clues concerning the nuclear affinity for NP.

One clue to the nuclear retention of NP, but not CP, came from examination of nuclei isolated directly from fully labeled cells. Fig. 5 shows that essentially all CP present in nonisolated nuclei are lost from nuclei that are isolated from cells lysed in Triton X-100 and spermidine solutions; probably no NP are. The arrows in Fig. 5 point to the two most abundant CP (the lower one of which is actin of 43,000 mol wt) that are present in nonisolated nuclei but absent from isolated nuclei. The next most abundant CP (~50,000 mol wt) probably is lost as well, but that is difficult to resolve because of the presence of two NP of roughly the same molecular weight. Almost certainly the great majority (if not all) of CP are lost from nuclei isolated in Triton-spermidine solutions and the amount lost is roughly 40% of the total protein present in nonisolated nuclei.

Particularly striking about this finding is the apparent selective retention of NP under nonphysiological conditions, even after the loss of the outer nuclear membrane,<sup>1</sup> and it emphasizes the likelihood that NP affinity for the nucleus is not dependent on the NE. This association with the nucleus persists *in vitro* even for the many NP species whose *in vivo* affinity constant for the nucleus is relatively low, as will be seen in the next section.

<sup>1</sup> K. G. Murti (St. Jude's Childrens Research Hospital, Memphis, Tennessee), personal communication.

### Not All NP are Irreversibly Bound within Interphase Nuclei *In Vivo*

Some years ago we showed by nuclear transplantation experiments (17) the existence of two general classes of NP: one that seems never to leave interphase nuclei and one that seems to shuttle nonrandomly between interphase nucleus and cytoplasm, although present in much higher concentration in nucleus than cytoplasm. (Most NP are almost entirely in the cytoplasm during mitosis [cf., for example, reference 18].) Because the apparent shuttling behavior of some NP was considered a possible artifact resulting from the nuclear transplantation operation itself (19), a reinvestigation of that possibility, using the present biochemical methodology, seemed in order.

Legname and Goldstein (19) suggested that when a labeled nucleus was grafted into an unlabeled nucleated cell, the nucleus of the latter acquired some labeled protein released from the grafted nucleus because of "surgical trauma." To avoid that possibility, we changed the previous experimental sequence. Unlabeled nuclei were implanted into [ $^{35}\text{S}$ ]methionine-labeled whole cells, thereby avoiding the artifactual release of labeled proteins from grafted nuclei. After ~24 h incubation, by which time the originally unlabeled nuclei ought

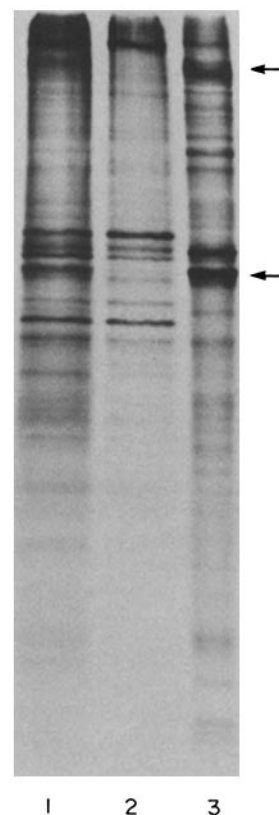


FIGURE 5 Fluorographic comparison of the proteins in isolated and nonisolated nuclei. Lane 1: all the labeled proteins in unlabeled cells into which nuclei from [ $^{35}\text{S}$ ]methionine-labeled cells were implanted shortly before being frozen for extraction. Lane 2: all the labeled proteins of nuclei isolated by the Triton X-100 and spermidine method from cells of the same population as used for lane 1. Lane 3: the labeled proteins in the cytoplasm of the cells from which the nuclei of lane 1 came. The arrows point to the position of two major CP bands detectable in lane 1 but not in lane 2.

to have acquired a full complement of labeled CP and “shuttling” NP, the latter nuclei (which were distinguishable from the original host cell nuclei by a difference in size [6]) were transplanted to unlabeled enucleate cytoplasms to allow the  $^{35}\text{S}$ -CP to exit into the new hosts. After an additional overnight incubation, the nuclei were transplanted to unlabeled hosts for assay purposes and all the pooled cell parts of the experiment were extracted and subjected to PAGE analysis. The results of such an experiment (Fig. 6) reveal that the labeled proteins retained by the original unlabeled nuclei are similar to some of the proteins of the original  $^{35}\text{S}$ -labeled nuclei—as expected from earlier work. (That the former nuclei appear to contain small amounts of certain of the “major” proteins of the latter in the region noted below probably is attributable to a small error in distinguishing between a host and grafted nucleus in the same cell.) The region of greatest difference in proteins of the two kinds of nuclei is bracketed in Fig. 6. The most prominent bands, roughly 30,000–48,000 mol wt, in this region of lane 3 must represent “nonshuttling” NP. Thus, these results confirm that a specific subset of NP do in fact leave interphase nuclei, but also continually return to the nuclei (obviously making no distinction between host and grafted nuclei) in which the affinity for them is higher than in cytoplasm.

### The Cytoplasmic Pool of Shuttling NP

If certain NP shuttle between nucleus and cytoplasm, some should be present and detectable in the cytoplasm. To look for that, we transplanted unlabeled nuclei into enucleate  $^{35}\text{S}$ -cytoplasms and, after a 1-d incubation, retransplanted the nuclei to unlabeled cytoplasms to allow the acquired  $^{35}\text{S}$ -CP to exit the nuclei. The contents of those nuclei were then subjected to PAGE analysis, with the result shown in Fig. 7. We see that labeled shuttling NP are indeed present in the cytoplasm and can be concentrated by implanted nuclei, presumably by exchange with their unlabeled counterparts in the implanted nuclei.

The amount of cytoplasmic shuttling NP is, however, small. We measured the amount of shuttling  $^{35}\text{S}$ -NP in directly labeled nuclei (the original radioactive nuclei of the above recipient  $^{35}\text{S}$ -cytoplasms) and the amount acquired by the unlabeled nuclei placed in  $^{35}\text{S}$ -cytoplasm and determined that the cytoplasm contains about one fifth to one seventh the total cellular shuttling NP. Because the nucleus occupies only 2% of the cell volume, the concentration of shuttling NP is ~250–350 times greater in the nucleus than in the cytoplasm, which means the shuttling occurs in the face of an enormous concentration differential.

### NP Liberated by Micromanipulation

Earlier studies referred to above (19) suggested that some NP were induced to exit transplanted nuclei by the effects of micromanipulation and consequently were called “injury proteins.” We took precautions in the current work to minimize contributions from labeled injury proteins and believe they were not a factor in our observations. Nevertheless, we sought to determine which proteins might be responsible for the “injury” effect and what they might have contributed to the experimental results. In these efforts we found that the injury protein effect is at most a minor one.

Taken as evidence of operation-induced injury proteins was the observation that labeled proteins were released to the cytoplasm from a transplanted nucleus and did not return to

the nucleus (19), where they ostensibly belong. From the results reported here, we can be certain that most of the labeled protein exiting to the cytoplasm must have been CP and that ~98% of the labeled material would remain in the cytoplasm as a result of simple exchange with unlabeled CP of the recipient cytoplasm. Thus, the bulk (if not almost all) of the “injury proteins” are presumably not that at all.

By sequentially transplanting  $^{35}\text{S}$ -protein nuclei through unlabeled cytoplasms, followed by PAGE analysis, we determined that the labeled material in the first host cytoplasm was predominantly CP, as expected, and that the labeled material found in subsequent host cytoplasms was the expected small amounts of shuttling NP. Thus, we found no evidence of “injury proteins.”



FIGURE 6 Fluorogram of experiment demonstrating the existence of a subclass of “shuttling” NP. Nuclei from unlabeled cells that had divided a few hours earlier (and hence were small) were implanted into [ $^{35}\text{S}$ ]methionine-labeled cells that had been subjected to several cytoplasmic amputations with intervening growth on unlabeled food (and hence had large, labeled nuclei). After ~24 h of incubation, the nuclei were segregated into two groups according to size by transplantation to unlabeled hosts, and 1 d later the small nuclei (which were expected to have acquired putative radioactive “shuttling” NP from the initial  $^{35}\text{S}$ -host but to have lost the acquired labeled CP to the unlabeled host) were retransferred to unlabeled hosts, which were immediately frozen for extraction, as were the cytoplasms from which they came. Lane 1: the labeled CP acquired by (and subsequently released from) the original unlabeled nuclei while in the original  $^{35}\text{S}$ -cell. Lane 2: the labeled proteins remaining in the original (small) unlabeled nuclei after the material (CP) in lane 1 had exited. Lane 3: the labeled proteins remaining in the original  $^{35}\text{S}$ -nuclei after labeled CP were allowed to exit into unlabeled cytoplasms (see lane 4). Lane 4: the labeled material that exited from the original  $^{35}\text{S}$ -nuclei. Lane 5: the labeled proteins of the original  $^{35}\text{S}$ -cytoplasms. Attention is directed to the region delineated by the bracket (material of ~30,000–48,000 mol wt) in which the greatest difference between lanes 2 and 3 is evident. (The minor amount of material in this molecular weight range in lane 2 is the result of a small error in segregating small from large nuclei present in the original labeled cell.) We conclude that the prominent bands in the region of lane 3 include many of those that must be “nonshuttling” proteins.

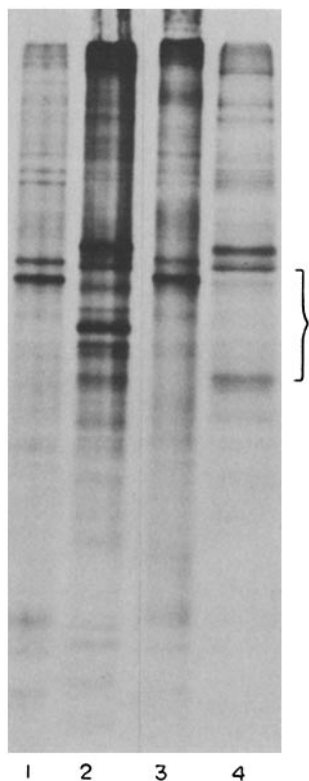


FIGURE 7 Fluorogram of experiment demonstrating the existence of a cytoplasmic pool of "shuttling" NP. Unlabeled nuclei were implanted into enucleate  $^{35}\text{S}$ -cytoplasms, and 1 d later the nuclei were retransplanted to fresh, unlabeled enucleate cytoplasms to allow the acquired radioactive CP to exit from those nuclei. 1 d later, the nuclei were transferred to unlabeled host cells and shortly thereafter frozen for extraction purposes, as were the enucleate cytoplasms from which they came. Although the foregoing was the most important part of the experiment, we also transplanted the original  $^{35}\text{S}$ -labeled nuclei through a similar series of unlabeled cytoplasms to examine the labeled material that exited those nuclei and those that remained. Lane 1: the labeled CP acquired by (and released from) the unlabeled nuclei placed in the original  $^{35}\text{S}$ -cytoplasms. Lane 2: the labeled protein remaining in the original  $^{35}\text{S}$ -nuclei after the material of lane 3 had exited. Lane 3: the labeled material (CP) of the original  $^{35}\text{S}$ -nuclei that had exited into unlabeled cytoplasms. Lane 4: the labeled material remaining in the original unlabeled nuclei after the material of lane 1 had exited. The bracket delineates the region of 30,000–48,000 mol wt, in which the greatest difference between lanes 2 and 4 can be seen.

## DISCUSSION

We have shown that cellular proteins (at least for *Amoeba proteus*) can be classified into four major groups with respect to localization within nucleus and/or cytoplasm. Assuming that the 70 or so primarily CP observed by one-dimensional PAGE and another 60–70 primarily NP similarly observed are representative of most of the thousands of cellular protein species, they can be classified as follows: (a) 98% of the mass of the majority of CP species are in the cytoplasm (the remainder is in the nucleus) and the concentration of those species is approximately the same in both compartments; (b) roughly 5–10% of CP species are found only in the cytoplasm; (c) a large portion (probably more than half) of NP by mass are in species (nonshuttling) that are virtually absent from the cytoplasm during interphase; and (d) the remaining NP species (shuttling) are apparently less tightly bound within nuclei than the former

class and are present in the cytoplasm, but at only  $\sim 1/300$  the nuclear concentration.

The restriction of NP almost exclusively to nuclei is easy to rationalize as reflecting the need to keep those proteins concentrated near where they are to function. This could even be the case for the shuttling NP, which may appear in the cytoplasm simply because of a lesser binding affinity than the nonshuttling NP for the nucleus. This kind of shuttling behavior has been observed also in mammalian cells by use of other techniques (cf. reference 20), but no evidence is available that it reflects any physiological function. Such behavior is, however, also consonant with transport function, the regulation of gene expression, and other functions.

Unexpected, at least to us, was the finding that such a high proportion (probably  $>90\%$ ) of CP species are present in nucleus at concentrations similar to those in the cytoplasm. This suggests that those proteins are in the nucleus for the trivial reason that the NE is no barrier to their passage through the cell. However, a similar argument was once made about actin (14), the most abundant of amoebas' proteins, but subsequent evidence (see, for example, reference 21) suggests that actin may have a function in nuclei different from that in the cytoplasm. Moreover, if the presence of CP in nuclei is physiologically trivial, why are not all CP found in nuclei? We find it difficult to believe that  $>90\%$  of CP species function in the nucleus, but also difficult for us to understand is why only a small percentage is excluded from the nucleus. This matter is considered further below. Peterson and McConkey (22) found that approximately half of HeLa cell CP species (roughly 500 found on their two-dimensional gels) are not found in nuclei, but most of those are quantitatively minor. If the HeLa results and the amoeba results can be reconciled, it would seem that a much higher proportion of quantitatively minor CP species than major species are excluded from the nucleus. If that is true, the proteins displayed in our one-dimensional gels are not representative of the total protein population in amoebas. However, several uncertainties need to be resolved before this issue can be settled.

What is the biological basis for the selective accumulation and retention of NP within the nucleus? A variety of observations (cf., for example, reference 2) suggest that NP accumulation is not a function of the NE, and in this paper we show that nuclei without outer nuclear membranes do not lose the ability to retain the full complement of NP. The evidence for this latter finding also demonstrates that the process responsible for NP retention continues to function even in a nonphysiological medium and lends encouragement to hopes of being able to understand the *in vivo* process from studies with cell-free preparations. (In some isolation media, however, certain kinds of nuclei do lose NP [cf., for example, reference 23]). Especially dramatic evidence that the NE has little to do with the nuclear accumulation of proteins comes from the work of Feldherr and Pomerantz (24), who showed that the presence of large ruptures in the NE of intact *Xenopus laevis* oocytes did not effect the kinds of proteins present in their nuclei. If not the NE, intranuclear structures seem likely to be responsible for the intranuclear retention of NP.

A number of recent studies have been directed at determining the distribution of proteins between nucleus and cytoplasm in a variety of cells, cells that are sufficiently varied with respect to function, structure, relative volumes of cytoplasm and nucleus, etc., to encourage us to believe that any common features may reflect traits characteristic of most kinds of cells. Unfor-



tunately, any comparisons dependent on quantitative measurements are virtually impossible to make from the published data. Despite this limitation, we suggest that the results from the different laboratories are consistent with the following conclusions:

(a) The general pattern of proteins is markedly different in nucleus and cytoplasm.

(b) Many CP are found in the nucleus and only a small proportion are not.

(c) Some NP are virtually absent from the cytoplasm, but a large proportion of NP species are present at low levels in the cytoplasm.

(d) Only relatively few protein species seem to be neither primarily cytoplasmic nor primarily nuclear, but appear in substantial amounts in both compartments. Actin may be one such protein.

The presence of certain (most of the "major") CP in the nucleus may reflect the absence of a permeability barrier in the NE and may have no functional significance. Likewise, the relatively small amount of certain NP (which we have called shuttling proteins) in the cytoplasm may merely reflect relatively low intranuclear binding constants and also could have little or no functional significance.

A few selected cell systems serve to illustrate the kind of information that has been acquired about the cellular distribution of proteins and the basis for the generalizations we have made. Kato and Lowry (25) separated nuclei and cytoplasm of rabbit dorsal root ganglion cells by dissection of frozen-dried preparations and measured the activities of nine different enzymes in each compartment. On a dry-weight basis (in effect, a measure of specific activity) the activities in the nucleus for eight of the enzymes ranged from roughly half to roughly twice that in the cytoplasm. Given that a variety of environmental factors could differentially influence enzyme activities, the results seem to be consistent with the view that most CP are present at about the same concentration in nucleus and cytoplasm. The ninth enzyme, ATP-NMN adenyllyltransferase, once thought to be an exclusively nuclear enzyme, deviated both from the aforementioned quantitative relationship and from previous expectation. On a dry-weight basis, the cytoplasm was found to have at least seven times the nuclear activity of the enzyme and, in fact, the enzyme may be entirely absent from the nucleus; it may be one of the minority of CP that seem to be exclusively cytoplasmic.

Probably the most thorough analysis of NP and CP distribution has been carried out on homogenized and fractionated HeLa cells by Peterson and McConkey (22), who could distinguish ~500 CP and ~700 NP by two-dimensional PAGE. Although slightly less than half of the protein species found in the cytoplasm were present also in the nucleus, by mass only 8% of CP are exclusively cytoplasmic. Thus, most of the latter are relatively minor proteins and presumably of a class undetectable by the one-dimensional PAGE used in our study. These proteins may be part of organelles too large to penetrate the NE. On the other hand, of the HeLa NP roughly 40% by mass are exclusively nuclear, and that is about what is found in amoebas as well. We conclude, therefore, that the results with the separated HeLa fractions are in reasonably good agreement with what we report for *Amoeba proteus*.

A somewhat different approach has been used to characterize the NP and CP of the dipteran *Chironomus tentans* salivary gland cells (26). Cytoplasm and nuclei were separated by microdissection from salivary glands fixed in ethanol/acetic

acid and the proteins of each group were analyzed by one-dimensional PAGE. (The authors show that fixation causes no significant translocation of proteins nor any change in the electrophoretic properties of the proteins.) Although the overall patterns of proteins in the two compartments clearly differ, the published data are unclear as to whether some proteins may be present in both nucleus and cytoplasm; they do appear, however, to be consistent with an overlap in distribution. If the situation in *Chironomus* is similar to that in amoebas, we expect such a result. When we examined amoeba nuclei that retained their normal in vivo complement of proteins, we could distinguish clearly (as noted above) only two or three of the whole range of CP we know to be present; the others are obscured because the NP are quantitatively dominant in the gels.

The experimental work that probably most resembles ours is that of Bonner on *Xenopus* oocytes (27). Unlabeled oocytes were injected with [<sup>35</sup>S]methionine-labeled materials micropipetted out of either nuclei or cytoplasm and, after suitable incubations, the proteins of the recipient nuclei and cytoplasm (separated by dissection) were analyzed by one-dimensional PAGE fluorography. (This work differs from the others in that the proteins examined in the *Xenopus* oocytes are essentially "newly synthesized" ones, whereas in other studies [including ours] the proteins analyzed presumably were labeled to a "steady state.") The results, as we interpret them from the published data (Bonner interprets them somewhat differently), can be summarized as follows: The NP clearly concentrate in the nucleus, even if injected into the cytoplasm. The cytoplasm nevertheless has a "pool" of NP that can enter and concentrate in the nucleus, as is the case with the amoeba's shuttling proteins. The overall pattern of proteins in the cytoplasm is distinctly different from the nuclear pattern. Clearly, some major CP are found in the nucleus at about the same concentration as in the cytoplasm. However, whether a majority of CP are found in the nucleus is difficult to say. Although Bonner implies they are not, one can not be certain because the quantitative relations with respect to relatively minor recognizable CP species are not analyzable from the available data. However, we think it significant that with regard to total protein, on the order of half the total radioactive protein taken from nuclei and injected into unlabeled oocytes remains in the cytoplasm. This agrees with our findings as to how much nuclear label is actually CP. Moreover, when labeled CP are injected into unlabeled oocytes, the concentration of radioactivity in the recipient nucleus is approximately equal (after appropriate corrections) to that in the cytoplasm—as is the case with amoebas. At present, unfortunately, the equality of concentration is seen in *Xenopus* oocytes for only a few proteins, one of which presumably is actin, which also is equally concentrated in nucleus and cytoplasm of amoebas (14). Whether *Xenopus* oocytes and amoebas differ significantly in any of the foregoing respects requires more refined experiments. One observation in the frog work that seems to be at striking variance with ours is that in *Xenopus* oocytes virtually all NP species are detectable in the cytoplasm; i.e., presumably no nonshuttling NP exist in the oocyte. That difference may be attributable to the presence in oocytes of a large cytoplasmic store, as for so much else, of nonshuttling NP awaiting the rapid cell multiplication that follows fertilization.

Our findings and that of Bonner that nuclei accumulate high concentrations of NP acquired from the cytoplasm confirms observations going back almost two decades. This propensity of NP for the nucleus is probably most readily demonstrated

by the fact that during mitosis almost all NP are liberated to the cytoplasm but return to the nuclei beginning at telophase (see reference 28 for references to earlier work). That NP have a particular affinity for the nucleus also is implicit in the fact that virtually all protein synthesis (of CP and NP) occurs in the cytoplasm of most cells (15). This kind of "homing" behavior has been confirmed by recent microinjection studies (29, 30). Microinjection experiments have shown such homing behavior also for the chromosomal protein HMG 1 (31), but related experiments (32) suggest that much HMG 1 is located in the cytoplasm.

Although our work falls far short of providing an understanding of the functions of the vast array of NP, we can note two advances:

(a) Whereas the great majority of CP species has no special affinity for the cytoplasm, almost all NP do have such an affinity for the nucleus. Probably the simplest interpretation of this is that, although most of the CP are in the cytoplasm because of that compartment's relatively large volume, the limited numbers of NP molecules are kept at high nuclear concentration by affinity mechanisms—presumably because the nucleus is where the NP must function.

(b) Our observations allow us to place the NP into two major groups: (i) those with high affinity for the nucleus and undetectable in the cytoplasm (except perhaps during mitosis) and (ii) those for which, as Bonner (2) puts it, the nucleus has a high capacity but a low affinity. The latter group, into which perhaps the majority of NP species fall, is what we call shuttling NP and is detectable in the cytoplasm. One group that clearly fits into the "high capacity, low affinity" class is the histones. Injection experiments (2) show that the nucleus can accumulate far more histone than can be accommodated on nuclear DNA, their "true functional" sites in contrast to their "quasifunctional" sites (see below).

Arguing that under most circumstances the low-affinity, high-capacity NP are not bound intranuclearly to sites at which those proteins function, Bonner (2) proposed a model of "quasifunctional [intranuclear] binding sites" that ostensibly have relatively low affinities for shuttling NP. Presumably this arrangement would poise those NP to function at nearby intranuclear (for example, chromosomal) sites when called upon by changed conditions. These latter sites should then be considered to be the primary binding sites for shuttling NP. Presumably, the tight nuclear affinity of the nonshuttling NP reflects the fact that these proteins are always bound to their primary functional sites during interphase.

Why do a few CP species (probably no more than 5–10% of those seen on a one-dimensional PAGE gel) not enter the nucleus? Probably it is because they are stable subunits of cytoplasmic structures that are unable to penetrate the NE. If that is not the case, we believe it will be difficult to determine what the actual nuclear exclusion mechanism is.

Some years ago Legname and Goldstein (19) suggested that the nuclear transplantation operation itself caused the release from the nucleus of "injury proteins" that might not otherwise be in the cytoplasm. Our new work shows that this interpretation is largely without basis. The main basis for the earlier suggestion was that a substantial portion of the labeled protein of a transplanted nucleus was released to the recipient cell cytoplasm and did not return to the nucleus. From the present work, we know that >90% of the released protein is probably CP that would exchange with the unlabeled CP of the recipient

cell. We also expect that a small proportion of the total that is released would be some labeled shuttling NP. We showed both of these expectations to be correct by additional experiments designed to test those expectations and, as far as we can tell, the "injury" effect has no bearing on the observations reported here.

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