THE CYTONUCLEOPROTEINS OF AMEBAE

II. Some Aspects of Cytonucleoprotein Behavior and Synthesis

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ABSTRACT

Radioactivity, apparently in cytonucleoproteins, from an amino acid-labeled nucleus implanted into a non-radioactive cell appeared in the host nucleus within 10 minutes, and the typical equilibrium ratio 70:30 donor nucleus radioactivity:host nucleus radioactivity was reached in 4 to 5 hours at 25°C. If such binucleates grew and divided, no localization of radioactivity was observable in cells fixed during mitosis, but the protein label remained concentrated in the daughter interphase nuclei for at least 4 generations. Continued migration of cytonucleoproteins was observed if these daughter nuclei were transplanted to other unlabeled cells. The Q10 (19°C to 29°C) of the migration rate of radioactive cytonucleoproteins was ca. 1.3, suggesting that passage through the cytoplasm occurred by diffusion. Both non-migratory nuclear proteins and cytonucleoproteins appear to be synthesized in the cytoplasm.

INTRODUCTION

We have described, in the preceding paper (1), a number of "static" properties of cytonucleoproteins, a class of substances that appear to be in constant non-random movement between nucleus and cytoplasm of amebae. In the course of the investigations on cytonucleoproteins we also encountered another class of materials, apparently restricted to the nucleus, and we have called these non-migratory nuclear proteins. In this paper we report on some studies of the more dynamic properties of these proteins, in an attempt to gain some insight into their cellular function. We have examined the kinetics of the cytonucleoprotein migration from nucleus to cytoplasm and back to nucleus, the effects of temperature on these kinetics, the behavior of the proteins during and after cell division, and the site of synthesis of these proteins. Some clues have come from these investigations, but by and large the role of the two classes of proteins in the physiology of the cell remains obscure.

MATERIAL AND METHODS

The material and most of the methods used in the following experiments have been described in the preceding paper (1).

ENUCLEATE AMEBAE: To obtain large numbers of enucleate cell fragments, amebae were cut approximately in half with the tip of a No. 26 gauge hypodermic needle wielded manually. A bend was made in the shank of the hypodermic needle a few millimeters back from the beveled tip at an angle such that the side of the tip could be used as a knife...
in a stroking motion, which cut the cells in two. This operation was performed in a petri dish at a magnification of ca. 30 to 50. Nucleate and enucleate halves of the amebae were separated by means of the differences in their response to light (2).

**Radioactivity Assay:** The radioactivity of C\(^{14}\) and S\(^{35}\)-labeled animals was assayed with a windowless, low background, gas flow Geiger counter (Nuclear Chicago Corporation, Des Plaines, Illinois, Model 186). Before they were dried for assay of radioactivity, labeled animals were washed in chaser media until the activity of the wash medium was no greater than that of background. Individual samples of 50 to 200 living, washed amebae were dried on stainless steel planchets and assayed for radioactivity both before and after extraction with 5 per cent trichloroacetic acid.

**Results**

A. **Kinetics of Cytonucleoprotein Migration**

1. **Rate at Which Cytonucleoprotein Label Reaches Equilibrium Distribution Between Host and Donor Nuclei**

We noted in the preceding paper that we could distinguish between the donor and host nuclei (after the implantation of a labeled nucleus into an unlabeled cell) by the higher radioactivity of the donor nucleus. We were confident of this because of the results reported below on the distribution of radioactivity between donor and host nuclei as a function of time following the transplantation operation. These experiments were primarily directed at estimating the rate of migration of cytonucleoproteins and at determining whether the distribution of labeled proteins between the two nuclei would reach a stable equilibrium.

Donor amebae were incubated in 67 \(\mu\)C leucine-H\(^{3}\)/ml for 51/\(2\) hours and then placed in 100 X chaser for a minimum of 10\(1/2\) hours. Nuclei from these leucine-H\(^{3}\)-labeled cells were grafted into unlabeled amebae that had been preincubated in chaser, and the binucleate cells were then placed in 100 X chaser and incubated at room temperature (25\(^\circ\) ± 1\(^\circ\)C). The experimental cells were fixed at predetermined intervals and processed for autoradiography.

Assays of autoradiographic grain concentrations demonstrated that for 4 to 5 hours after the implantation of a protein-labeled nucleus there is a continuous increase in the proportion of radioactivity acquired by the host cell nucleus (Fig. 1). Important for many interpretations, moreover, was the fact that the host cell nucleus never acquired as much activity as the donor nucleus; we were thus confident that we could distinguish a labeled donor (more active) nucleus from a host (less active) nucleus.

The data plotted in Fig. 1 show that the distribution of radioactivity between the two nuclei did, indeed, reach an equilibrium, at which point the host cell nucleus contained approximately 30
per cent of the total activity of both nuclei. Although the equilibrium was reached only 4 to 5 hours after the introduction of the labeled nucleus, the migration is probably a quite rapid process. This rapidity is suggested by the appearance of some radioactivity in the host nucleus within a few minutes after the operation. Our autoradiographic grain counts suggest that the combined activity of the two nuclei remained nearly constant throughout the course of the experiment: 8 cells fixed from 0 to 10 minutes after the nuclear transfers showed a combined nuclear grain concentration of 21.0 ± 3.6 grains per 81 μ2, whereas the average for 65

TABLE I

<table>
<thead>
<tr>
<th>Temperature Coefficients of Rate of Acquisition of Radioactivity by Host Cell Nucleus Following Implantation of Radioactive Protein Labeled Donor Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation temperature</td>
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<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Implantation of labeled nucleus</td>
</tr>
<tr>
<td>Hrs.</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>Δ1-2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time after nucleoplasm of labeled nucleus</th>
<th>19°C</th>
<th>29°C</th>
<th>Q10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hrs.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14.4 (25)</td>
<td>18.9 (18)</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>20.6 (22)</td>
<td>27.25 (22)</td>
<td>1.3</td>
</tr>
<tr>
<td>Δ1-2</td>
<td>6.2</td>
<td>8.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

When several steps are involved in a single process, the measured Q10 may be that of the slowest step in the series. We therefore cautiously suggest that the migration of cytonucleoproteins through the cytoplasm may be by means of simple diffusion, which usually has a Q10 in the region of 1.3 to 1.4. There are, of course, other important aspects of the over-all migration process, such as, for example, the penetration of the nuclear membrane and the possibility of some sort of transient binding of the cytonucleoproteins within the nucleus. A higher concentration of cytonucleoproteins in the nucleus than in the cytoplasm could not be maintained by simple diffusion processes alone.

B. The Fate of Cytonucleoproteins and Non-Migratory Nuclear Proteins Over the Course of Several Generations

Does the presence of cytonucleoproteins in relatively high concentration in the nucleus indicate that they are part of the genome of the cell? An answer might come from studies conducted over several cell generations. Thus, nuclei from directly labeled cells were transplanted to unlabeled cells and these now binucleate amebae were allowed to feed, grow, and multiply in a non-radioactive environment. About 20 transfers each were made with nuclei labeled with tritiated tryptophan, arginine, and leucine. Samples of the progeny of about 50 cells was examined after each of the 4 cell divisions.

In all cases, even after 4 cell divisions, the radioactivity was still very largely localized in the host nuclei, although in a few instances the proportion of label in the cytoplasm may have been higher than in cells that had not divided. Nevertheless, these results gave the impression that the cytonucleoproteins are quite stable. To determine whether these proteins are completely stable, as DNA is, for example, would require quantitative assays of a precision beyond the capacity of our current methods.

Although the radioactivity continues to be largely localized in the nuclei after cell divisions,
this is not evidence that the cytonucleoproteins migrate as before. To obtain such evidence, nuclei from uniformly leucine-H\(^3\)-labeled amebae were grafted into unlabeled amebae and these organisms were fed unlabeled food until they divided. After division, the nucleus from one of the mononucleate daughter cells (two of the daughter cells were usually mononucleate, the third usually binucleate) was grafted into an unlabeled ameba, which, after at least another 6 hours incubation, was fixed and processed for autoradiography. Ten cells of this experimental series were examined and all revealed that the labeled material continued to migrate from one nucleus to another. We found, however, that the autoradiographic grain concentration of the host nuclei averaged only 20 per cent of the total activity of host and donor nuclei. If the cytonucleoproteins and the non-migrating proteins are distributed to all four 1st division daughter nuclei equally, we would have expected the 2nd host nucleus to acquire the usual 30 per cent of the total nuclear activity, and the data in the next section do suggest that the non-migrating proteins are not retained by any one nucleus after mitosis. There are two obvious possibilities to account for the acquisition of 20 per cent rather than 30 per cent of the total nuclear activity by the 2nd host nucleus. Either the donor nucleus of the 2nd transplantation has converted more cytonucleoproteins into non-migrating proteins after the division than before, or more of the cytonucleoproteins are lost to the cytoplasm as a result of mitosis. We have had the impression that the latter explanation is most likely because of apparently increased cytoplasmic activity in some cells, but these were only preliminary, subjective observations. There are, of course, other possibilities to account for the deviation from the expected distribution, but they are not worth considering at present.

A few nuclear transfers also have been made following the 2nd division after the transplantation of a labeled nucleus into an unlabeled cell, and preliminary results show that labeled materials continue to migrate from one nucleus to another at least in some cells.

C. Localization of Cytonucleoproteins and Non-Migrating Proteins During Mitosis

If either or both of the classes of proteins are associated in an intimate and somewhat permanent fashion with the chromosomes, it might be apparent from a study of the localization of the label during mitosis. When an ameba that had an implanted protein-labeled nucleus was found by chance to be in mitosis, no matter what the experimental series, it was immediately fixed on a slide. We acquired 7 such amebae, all fixed approximately in metaphase. Two of the cells were in the first metaphase after receiving a labeled nucleus, 1 was in the second metaphase, 3 were in the third metaphase, and 1 was in the fourth metaphase. After fixation the cells were processed for autoradiography.

In all 7 cases, no localization of radioactivity was observed; the label appeared to be uniformly distributed through the metaphase cells. Thus, it is probable that in the absence of an intact nuclear membrane the labeled proteins are not restricted to the region of the cell occupied by the chromosomes. This does not necessarily mean, of course, that the nuclear membrane is responsible for the interphase localization of the cytonucleoproteins and the nonmigrating nuclear proteins. Prescott (7), using a different experimental approach, has observed a similar (and probably identical) phenomenon in amebae.

D. Site of the Synthesis of Cytonucleoproteins and Non-Migrating Proteins

An extensive series of experiments was conducted to determine whether the two classes of proteins under investigation are synthesized in the nucleus or in the cytoplasm. Some of the results are reported here. The basic experimental design was as follows. Enucleate amebae, created by pushing nuclei out of cells with a probe, were allowed to incorporate amino acids into protein (6) and then were implanted with unlabeled nuclei. After suitable incubation, the nuclei, which might have acquired labeled proteins from the cytoplasm, were retransplanted to unlabeled whole cells. Autoradiography was performed to detect radioactivity, if any. Distribution of radioactivity, as in earlier experiments, would suggest that both classes of proteins are synthesized in the cytoplasm; equal distribution of label between host and donor nuclei would suggest that only cytonucleoproteins are synthesized in the cytoplasm; lack of activity in the nuclei would suggest that: (a) the nucleus is the site of synthesis of the two classes of proteins, or (b) synthesis of these proteins in the cytoplasm can not proceed in the absence of the nucleus.
The procedure for the initial experiments in this series is diagrammed in Fig. 2. After enucleation the cells were incubated in 50 μc leucine-H³/ml for 6 to 16 hours, followed by washing in unlabeled media, and then placed in 100 X chaser for at least as long as they had been in leucine-H³. (Experiments described below indicate that the pool of unincorporated precursors reaches a minimum after approximately 2 hours in chaser medium.) After incubation in chaser, each enucleate cell was implanted with an unlabeled nucleus from an ameba that had been incubating in a medium identical with the above chaser. Twenty-four hours later the nucleus was transferred (Transfer 2) to the final host cell, which was fixed after another 24 hours' incubation and processed for autoradiography.

The final host cell autoradiographs (over 100 amebae in 9 different experiments) revealed that the label invariably had migrated from donor to host nucleus and was localized primarily in the two nuclei, as was the case in earlier experiments. The data thus suggest that cytonucleoproteins are synthesized in the cytoplasm.

Since the non-migrating proteins do not appear outside the nucleus (by definition) in our experiments, we had assumed that these, at least, would not be synthesized in the cytoplasm. We were thus surprised, after determination of the autoradiographic grain concentrations, to find that the host nucleus acquired an average of ca. 28 per cent of the total nuclear activity in the above experiments. This proportion is close to the 30 per cent found when a nucleus from a directly labeled cell is grafted into an unlabeled cell and
therefore suggests that non-migratory nuclear proteins also are synthesized in the cytoplasm.

As clear as the above data may seem, the interpretation would be completely erroneous had the labeled enucleate cell not been free of labeled precursors at the time of the implantation of the unlabeled nucleus. If labeled precursors were still present at that time, synthesis of radioactive cytonucleoproteins and non-migrating nuclear proteins might very well have occurred in the implanted nucleus. Despite the fact that a labeled enucleate cell was incubated in chaser for lengthy periods before and after the implantation of a nucleus, we could not be certain that the labeled precursor pool was significantly obliterated at the time of the nuclear transfer operation. A first attempt to resolve this issue involved experiments similar to those depicted in Fig. 2, with two modifications of procedure. One modification was to vary the chaser incubation period (in the range of 1/2 to 29 1/2 hours) for enucleate cells prior to the implantation of an unlabeled nucleus; the other modification was to fix the final host cell immediately after transfer 2. The second modification permitted easier assay of the radioactivity acquired by the transplanted nucleus. The first modification was the crucial one and was based on the following reasoning. If the nuclei were synthesizing the two proteins from unincorporated labeled precursors, the amount of radioactivity detectable in the final host cell nucleus probably would be inversely proportional to the length of the period the enucleate cell was in chaser after incubation in labeled medium and before the nuclear transfer operation. If the nuclei were accumulating preformed labeled proteins from the enucleate cytoplasm, however, we might expect that the radioactivity of the final host cell would occur by the end of the 29 1/2 hours (or whatever period we might have chosen) in chaser.

In the light of the aforementioned possibility, an examination of the radioactive precursor pool was performed. We asked whether the chaser effectively diluted out the labeled amino acids. For this, large numbers of enucleate half amebae were incubated in 5 μc phenylalanine-C14/ml for 19 hours, followed by washing and incubation in 100 X chaser. At intervals over a 24 hour period, samples containing 75 enucleate cells each were dried on individual planchets and radioactivity determined by Geiger counting before and after extraction with 5 per cent trichloroacetic acid (TCA) for 6 minutes at 5°C. The total activity (before TCA extraction) and the TCA-insoluble activity are plotted in Fig. 4 as a function of the length of time enucleate cells were in chaser. The difference between the two curves represents the TCA-soluble material and is presumed to contain the precursor pool but may contain non-precursor material as well. The data in Fig. 4 show that:

(a) there is a rapid initial loss of TCA-soluble

Figure 3 The degree of nuclear labeling as a function of the period of incubation in chaser medium of leucine-H3-labeled enucleate cells prior to the implantation of an unlabeled nucleus. Enucleate cells were placed in chaser medium immediately upon removal from leucine-H3 medium.

Figure 4 The degree of nuclear labeling as a function of the period of incubation in chaser medium of leucine-H3-labeled enucleate cells prior to the implantation of an unlabeled nucleus. Enucleate cells were placed in chaser medium immediately upon removal from leucine-H3 medium.
radioactivity that seems to be complete within 2 to 3 hours after enucleate cells are placed in chaser; (b) there is a substantial amount of labeled TCA-soluble material that remains undiminished after the initial loss; and (c) there is little, if any, increase in TCA-insoluble radioactivity over the 24 hour period studied. In a parallel series, enucleate fragments from a similar group preincubated for 19 hours in phenylalanine-C\textsuperscript{14} were left in the radioactive amino acid medium for an additional 24 hours rather than being transferred to chaser. The amount of TCA-insoluble radioactivity in these enucleate cells more than doubled over the final 24 hours. Additional experiments with whole cells, whole enucleate cells (nucleus pushed out of the cell with a probe), and enucleate half cells (as above) with a number of different radioactive amino acids confirm the above data. The important conclusions are: (a) a sizeable TCA-soluble radioactive pool persists much beyond 24 hours (data not shown); and (b) most importantly, this pool probably does not contain precursors for the synthesis of TCA-insoluble material since there is little, if any, increase in TCA-insoluble radioactivity during this period of time, while such an increase does occur in cells kept in labeled media. These results are consistent with the assumption that chaser is effective in suppressing further incorporation of labeled precursors into protein and thus support the conclusion that cytonucleo-

proteins and non-migrating nuclear proteins are synthesized in the cytoplasm.

Having established the effectiveness of chaser, we proceeded to determine whether the amount of radioactivity acquired by an unlabeled nucleus grafted into a labeled cytoplasm would be higher in the complete absence of chaser. (It had been observed that the initial rapid loss of TCA-soluble radioactivity during a chase period (Fig. 4) was about the same whether plain ameba medium or 100 X chaser was used.) The experiment followed the outline of Fig. 2. In one series, the leucine-H\textsuperscript{3}-labeled enucleate was “chased” for a minimum of 10 hours in plain ameba medium. In a second series, the enucleate chase was in ameba medium plus 100 X chaser. The final host cells were fixed immediately after transfer 2. The average autoradiographic grain concentration over the donor nuclei of the 17 final host cells in the series exposed to ameba medium only was 30.2 ± 4.2 grains/100\mu\textsuperscript{2}, and that for the 14 final host cells of the series exposed to ameba medium plus chaser was 26.6 ± 2.3 grains/100\mu\textsuperscript{2}. These concentrations are not significantly different according to the t test, and it thus appears that 100 x chaser is no more effective in preventing nuclear labeling than plain ameba medium. We interpret these results to mean that the labeled molecules that enter the nucleus from the cytoplasm are not subject to competition by added amino acids and, therefore, that they probably are proteins rather than precursors. These experiments, then, also support the conclusion that cytonucleoproteins and non-migrating nuclear proteins are synthesized in the cytoplasm.

DISCUSSION

The kinetics of distribution of the protein label (and the unequal distribution at equilibrium) between nuclei strongly favors, as do many other data of this and the preceding paper, the view that the cytonucleoproteins are continuously shuttling back and forth between nucleus and cytoplasm. These circumstances, along with the marked differences between nucleus and cytoplasm in the concentration of cytonucleoproteins, suggest that the over-all shuttling activity is not a random process and bolsters the hypothesis that the cytonucleoproteins are involved in important nucleo-cytoplasmic interactions, perhaps in the communication of signals from one compartment to the other.
Our studies of the temperature coefficient of cytonucleoprotein migration intimate that the shuttling movement in the cytoplasm is by diffusion. This possibility is supported also by earlier evidence (1) that the cytonucleoproteins are probably not associated with any cytoplasmic structure.

The dispersal of the nuclear proteins throughout the cytoplasm at mitosis and their recollection in the daughter nuclei also has been observed in A. proteus by Prescott (7), whose techniques differed from ours. These data thus show that neither the cytonucleoproteins nor the non-migratory proteins are relatively permanent components of the chromosomes as are DNA and, perhaps, histone. We might also note that although a considerable portion of the nuclear membrane remains intact around the metaphase figures of A. proteus (3, 9), there is no concentration of radioactivity in that area. This confirms our earlier observation (1) that there is apparently no association of these proteins with the nuclear membrane.

We are encouraged to believe that the nuclear proteins we have been examining are somehow important for the interphase physiological activity of the genetic material, since they are clearly not associated with mitotic chromosomes (which are considered to be "physiologically" inert).

Apparently related changes in nuclear proteins at the time of mitotic activity have been observed by others. Harris (5) noted that valine-H³-labeled nucleolar proteins of rat connective tissue cells appeared in the reconstituted nucleoli of post-mitotic cells kept in non-radioactive media during and after division. Das (4) observed that a silver staining component of the nucleoli of various cell types disappeared from the nucleus concurrently with nucleolar breakdown in early mitosis. Simultaneously with these events, similarly staining material began to appear in the cytoplasm. During telophase the process was reversed; i.e., the stain disappeared from the cytoplasm and reappeared in the newly forming daughter nuclei. This behavior is, of course, quite similar to the behavior of the nuclear proteins that we have studied, except that we have not been able to show any particular affinity for the nucleoli (1). Richards (8) apparently has studied similar activities, using still another technique. He observed an increase in the mass of mouse cell nuclei in telophase at a rate that rapidly brought the combined mass of the 2 nuclei just up to the amount of the parental cell nucleus. Richards believes that this uptake was faster than would be expected from the de novo synthesis of proteins and suggests that increase was due to the accumulation of proteins that are present in the cytoplasm in mitotic cells.

It is important to note that, since the labeled cytonucleoproteins are apparently stable through cell division and are reconcentrated in daughter cell nuclei, the appearance of radioactivity within host cell nuclei in our transplantation experiments is very unlikely to be due to transferred precursor material but most probably due to the acquisition of preformed radioactive proteins.

The evidence that the non-migratory nuclear proteins and the cytonucleoproteins probably are synthesized in the cytoplasm is important, since it is possibly the best evidence that some nuclear proteins may originate outside the nucleus. These data also serve to warn against assuming that a protein that is more concentrated in a particular part of the cell is necessarily synthesized there. Zalokar has observed (10) that, following a brief exposure to leucine-H³, ovarian cells of Drosophila first display radioactivity in the cytoplasm and later in the nuclei. This suggests that protein synthesis occurs in the cytoplasm and that the protein may then migrate to the nucleus, but the author cautions that alternative mechanisms are possible.

A number of observations has led us to question whether there is any relationship between the cytonucleoproteins and the non-migrating nuclear proteins. Whether the distinctions we have made are as real as is suggested by their designated names is uncertain, since data of Prescott (7) intimate that there is no group of proteins that is permanently restricted to the nucleus during interphase. He observed that, if one repeatedly amputates cytoplasm of Tritiated protein-labeled amebae that are allowed to regenerate cytoplasm from non-radioactive food between amputations, all the nuclear radioactivity is lost after ca. 30 amputations and the removal of 99.9999998 per cent of the original cytoplasm. The process is quite slow, however, and some nuclear label (but very little cytoplasmic label) remains after 15 amputations and the loss of 99.9999998 per cent of the original radioactive cytoplasm. Although Prescott's experiments were carried out over a much longer time period than ours and thus protein breakdown may be a factor in his experiments, his results may be interpreted to mean that our so called non-migra-
Tory nuclear proteins become migratory under certain conditions. That the reverse is also possible, namely that cytonucleoproteins may become non-migratory, is suggested by the evidence that non-migratory nuclear proteins appear to comprise a larger fraction of the combined donor and host nuclear radioactive proteins in transplantation experiments performed after cell division (the distribution being perhaps 80:20 rather than 70:30 for donor to host activity). Thus, we could have two possible interactions reflecting nucleocytoplasmic relationships. One, involving the so-called cytonucleoproteins, would perhaps be concerned with relatively transient activities such as enzyme induction. The other, in which the same proteins in a non-migratory role would perhaps be involved, might be important for more stable interactions, such as those involved in embryonic differentiation. The latter circumstance is, of course, not appropriate to a unicellular organism, but perhaps there are conditions requiring relatively stable interactions even in amebae.

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