ELECTRON MICROSCOPE LOCALIZATION OF NUCLEAR RNA'S THAT SHUTTLE BETWEEN CYTOPLASM AND NUCLEUS AND NUCLEAR RNA'S THAT DO NOT

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ABSTRACT

Nuclear transplantations and electron microscope autoradiography (EMRA) were utilized in order to localize and characterize small nuclear RNA's (snRNA) in ameba nuclei. A class of nonmigrating low molecular weight nuclear RNA's is associated with the structureless region of the nucleoplasm but not with the nucleoli, nuclear helices, or chromatin. Thus, the role of these RNA's in genetic regulation is questionable. A class of migrating RNA's (presumed, but not directly shown, to be low molecular weight) that shuttles between nucleus and cytoplasm is also not associated with nucleoli or helices but some radioactivity is associated with the chromatin. It may be, therefore, that the shuttling RNA's are in some way involved in genetic transcription or replication.

INTRODUCTION

Reports of the existence of a variety of small (ca. 4-10S) nuclear RNA's (snRNA) apparently unrelated to ribosomal, transfer (tRNA), and messenger RNA's have come from a number of laboratories (18, 19, 8, 23, 7). Essentially nothing is known of the function of most snRNA's. Although a small proportion may be precursors of tRNA's and 5S ribosomal RNA's (18), most undoubtedly have other cellular roles. Except perhaps for the obvious tRNA precursors, none of the snRNA's have amino acid acceptor activity (8) and none have methylation patterns characteristic of other RNA's (23). In HeLa cells at least nine different snRNA species, other than the tRNA and ribosomal precursors, are recognized (19), and there is no reason to believe that any of these are artifacts of preparation. While some snRNA's seem to be rather stably associated with the nucleus over several cell generations, most of the available experimental data are insufficient for determining whether this stability is due to the existence of molecules that shuttle between nucleus and cytoplasm but which are present in relatively high concentrations in the nucleus or molecules that do not migrate from the nucleus.

Studies performed in this laboratory indicate that snRNA's may consist of both types: some that shuttle between cytoplasm and nucleus and some that appear not to leave the nucleus (7) except during mitosis (15). Using these behavioral characteristics to identify the particular classes of molecules, we have determined the localization of snRNA's in the expectation that we would thus obtain additional clues regarding the functions of these molecules.

Although the behavior and localization of snRNA's suggests that they may be involved in the

1 Goldstein, L., G. E. Wise, and M. Beeson. 1972. Submitted for publication
control of gene replication or expression, our results reveal that most snRNA's are not associated with nucleoli, chromatin, or nuclear helices—the best characterized nuclear structures. These happen to be the only nuclear structures so far implicated in transcription and/or replication. There is an indication that a small proportion of the shuttling RNA's may be associated with chromatin at any one moment during interphase and this suggests some involvement in the regulation of polynucleotide synthesis.

**MATERIALS AND METHODS**

**Cultures**

Stock cultures of *Amoeba proteus* were grown according to the method of Prescott and Carrier (14).

**Cell Labeling**

The food source for the amebas, *Tetrahymena pyriformis*, was inoculated into a synthetic medium deficient in pyrimidines (4) except for 250 μCi/ml of [3H]uridine (29.3 Ci/mM, New England Nuclear Corp., Boston, Mass.). The ameba culture was fed radioactive tetrahymena for 44 h and then divided into three groups, each of which was then treated differently. Some amebas, the unchased group, were removed from food for 4 h (although some [3H]-tetrahymenas were still present in food vacuoles) and then centrifuged, fixed, and processed for electron microscope autoradiography (EMRA). A second group was fed unlabeled tetrahymenas for 24 h, centrifuged, fixed, and processed for EMRA. The third group was fed unlabeled tetrahymenas for 24 h and then served as donors of [3H]RNA containing nuclei that were used in the transplantation experiments to be described.

**Nuclear Transplantation**

To enrich the nuclei for putative nonmigrating [3H]RNA, [3H]RNA-labeled nuclei were transplanted into enucleate, unlabeled amebas by the method of Jeon and Lorch (10). 24 h later these nuclei were again transplanted into fresh enucleate, unlabeled hosts. 4 h after this second transfer, the amebas were centrifuged, fixed, and processed for EMRA. After this sequence of nuclear transfers, because the migrating [3H]RNA's would have been diluted by exchange with unlabeled material from the cytoplasm, about 80% of the [3H]RNA in the nuclei was in the form of the nonmigratory 4–6S RNA (7). In steady-state labeled nuclei about 15% of the radioactivity is in low molecular weight RNA.

To localize the migrating nuclear RNA's, well-chased [3H]RNA-labeled nuclei were transplanted into unlabeled amebas, thus forming binucleate cells. 4–5 h after the transfer these amebas were centrifuged and prepared for EMRA in order to determine the localization of the [3H]RNA that had migrated into the recipient cell nucleus.

**Centrifugation**

To centrifuge amebas, about 0.05 ml of a 40% Ficoll solution (made in ameba medium) was placed in the bottom of a 0.2 ml centrifuge tube and the amebas were layered atop this solution. The tubes were spun for 20–30 min in a Misco (Berkeley, Calif.) microcentrifuge at about 13,000 g. Within 1–2 min after the power to the centrifuge was stopped, the cells were removed from the tube and fixed for electron microscopy. If the cells are not fix ed after centrifugation, they will destratify and appear normal in all visible respects.

**Preparation for Electron Microscopy**

Centrifuged amebas were fixed for about 45 min in Karnovsky's mixture of glutaraldehyde and paraformaldehyde in 0.1 M cacodylate buffer (11) at pH 7.3. After fixation the amebas were left overnight in distilled H2O and embedded in agar (5) the following day. The agar blocks of amebas were postfixed in 1% OsO4 in 0.1 M cacodylate buffer for 1 h, followed by dehydration in a graded series of ethanol and propylene oxide. The blocks then were embedded in Araldite and 600–800 Å sections were cut on a Porter-Blum MT2 ultramicrotome.

**Electron Microscope Autoradiography (EMRA)**

This technique has been described earlier (16, 20). The grids were left under Ilford L4 nuclear emulsion for 6–30 days in the dark before being developed. After the sections were processed for EMRA, they were stained with uranyl acetate followed by lead citrate and viewed with a Philips 300 electron microscope.

**RESULTS**

The "Steady-State" Pattern

The radioactivity of a directly labeled nucleus, in which all of the classes of RNA (designated 39, 32, 19, 16, 4-6S) are labeled with [3H]uridine for approximately the length of one cell cycle, is more heavily concentrated over the nucleolus than over the nucleoplasm (Fig. 1). A higher magnification electron microscope autoradiograph shows this more clearly (Fig. 2).

130 THE JOURNAL OF CELL BIOLOGY • VOLUME 56, 1973
Figure 1  Electron microscope autoradiograph of an $[^3H]RNA$-labeled nucleus in which all the classes of RNA are labeled. Nucleus has been centrifuged and sectioned parallel to the axis of centrifugation. The majority of silver grains are over the nucleoli (N) at the centrifugal end of the nucleus, although there are some grains over the nucleoplasm (NU) in the centripetal regions. Exposure time, 6 days. Bar, 1 $\mu$m. $\times$ 6100.
The Localization of Nonmigrating RNA

After the sequential transfer of a [3H]RNA-labeled nucleus through two unlabeled cytoplasms, the nonmigrating 4-6S RNA is almost the only labeled class of molecules remaining in the nucleus as shown by sucrose gradients of RNA extracted from such nuclei (7). In such nuclei, the labeling pattern observed by electron microscope autoradiographs is markedly different from that in nuclei in which all classes of RNA are made radioactive. We see in Fig 3 that most of the nonmigrating 4-6S nuclear RNA is in the nucleoplasm with little indication of any association with nucleoli. At higher magnification, the autoradiographic grains are seen to be over a rather structureless region of the nucleoplasm (Fig. 4) rather than over the network of chromatin or the nuclear helices. That the interconnected network of fibrils (labeled C in Fig. 5) is chromatin was established previously by examination of [3H]thymidine-labeled nuclei (20).

In addition to the nuclear structures mentioned above, ameba nuclei contain helical structures (13). The helices do not contain DNA (20, 22) but do contain RNA (21) and protein (17). However, as seen in Fig 6, the absence of radioactivity indicates that the helices apparently contain no low molecular weight RNA.

The Localization of Shuttling RNA’s

The shuttling RNA’s that migrate into a nucleus from the cytoplasm into which a [3H]RNA-containing nucleus had been implanted also are not associated with the nucleoli. After the transplantation of a [3H]RNA nucleus, the host nucleoli are virtually unlabeled in marked contrast to the heavy labeling of the nucleoli of the transplanted nucleus (Fig 7).

Although the amount of migrating RNA label over the chromatin seen in Fig 7 is relatively small, it appears to be significantly greater than the amount of nonmigrating RNA associated with
FIGURE 3  Section cut parallel to the axis of centrifugation of an [3H]RNA nucleus that has been transferred through two unlabeled cytoplasms to enrich it in 4-6S RNA. This electron microscope autoradiograph shows that the 4-6S nonmigratory RNA is localized in the nucleoplasm (NU) with few, if any, silver grains present over the nucleoli (N). Compare absence of grains over nucleoli in this nucleus with the heavy labeling of nucleoli in Fig. 1. Exposure time, 14 days. Bar, 1 μm. × 6400.
High magnification autoradiograph of centripetal region of nonmigratory RNA (4-6S)-enriched nucleus showing that this RNA is in a rather structureless region of the nucleus. Exposure time, 14 days. Bar, 1 μm. × 24,700.

Electron microscope autoradiograph of nonmigratory RNA (4-6S)-enriched nucleus showing the paucity of grains over the chromatin (C) as compared to the surrounding nucleoplasm. Chromatin is a network of interconnected fibrils. Exposure time, 14 days. Bar, 1 μm. × 19,700.
chromatin (see Fig. 5). This suggests that the shuttling RNA's may play a relatively direct role in polynucleotide synthesis.

In contrast to the direct evidence (7) that almost all of the nonmigrating nuclear RNA's are less than approximately 10S, the suggestion that the shuttling RNA's also are small is less certain. There are two reasons for believing that the shuttling RNA's are relatively small molecules. One is that intranuclear distribution of labeled RNA in the host nucleus of Fig 7 is similar to that of the sequentially transferred nucleus of Fig 3 and the labeled RNA of the latter nucleus is very largely of low molecular weight; almost certainly, at least some of the labeled material of the sequentially transferred nucleus is shuttling RNA, in addition to RNA that does not migrate. The other reason is that unpublished preliminary work from this laboratory indicates that the [32P]labeled RNA that goes from a grafted nucleus to an unlabeled host cell nucleus is largely in the form of low molecular weight material as shown by autoradiograms of electrophoretic gels.

**DISCUSSION**

Our observations indicate that the so-called nonmigrating low molecular weight nuclear RNA of *A proteus* is essentially nonnucleolar and non-chromosomal. The almost exclusive nucleoplasmic localization of this class of RNA's makes previous speculation about their function seem unsupportable. Thus, because of the lack of association with chromatin, the nonmigrating RNA's appear not to be involved in the regulation of genetic transcription (8), although the appearance in our autoradiographs of a few labeled molecules in the vicinity of chromatin does not exclude that involvement absolutely.

That the nonmigrating nuclear RNA's are also not concerned with nucleolar function seems even more probable, since essentially none of these RNA's are localized in nucleoli. This does not necessarily mean, however, that they are uninvolved in ribosomal RNA synthesis, a primary role for ameba nucleoli in the latter function is questionable in the light of evidence that *A proteus* nucleoli are apparently devoid of DNA (20).
Electron microscope autoradiograph of a binucleate cell created by transplanting a $[^3H]$RNA nucleus into an unlabeled cell. The nucleoli (N) of the grafted nucleus (G) are heavily labeled, whereas the nucleoli of the host nucleus (H) are not labeled. Numerous silver grains are present in the nucleoplasm (NU) of the host nucleus and some grains appear to be associated with the chromatin (C). This micrograph conclusively shows that intact $[^3H]$RNA molecules can migrate into an unlabeled nucleus. Exposure time, 14 days. Bar, 1 μm. × 8100.
lack of association between the nonmigrating RNA's and the nuclear helices suggests that these RNA's are not involved in RNA transport. The one function attributed to the helices that seems most reasonable at the moment is that of the transport of gone messages to the cytoplasm (21).

This leaves few recognizable options for the function of the nonmigrating RNA's. The distribution of the nonmigrating RNA's resembles the intranuclear distribution of the so-called Rapidly Migrating Proteins (3) but electron microscopy of the latter will be required before any identity in localization can be reasonably established. However, even if the two classes of molecules can be shown to be associated within the nucleus, our understanding of the function of the nonmigrating RNA's will be little advanced; our understanding of the cellular role of the Rapidly Migrating Proteins is very meager also. The possibility that the nonmigrating RNA's have a role in the formation of any nuclear structure seems remote on the basis of observations made in this study.

Speculation about the function of the RNA's that shuttle between cytoplasm and nucleus is somewhat more gratifying because we know a few more interesting facts about these molecules and there are even some hypotheses that can accommodate a few of the facts. Thus, the models of Britten and Davidson (1) and of Bonner's group (12) invoke particular classes of RNA to regulate specific gene transcriptions. The shuttling RNA's described in this paper can be incorporated into these models. Thus, we can imagine that the RNA's shuttle between cytoplasm and nucleus for the purpose of being able to detect changes in the cytoplasmic environment in a manner analogous to the detection of environmental changes by repressor proteins in bacteria (9). Perhaps more important than the shuttling behavior in implicating these RNA's in the regulation of transcription is the finding that they may be associated with chromatin, even though only transiently. Although the amount of shuttling RNA associated with chromatin at any one moment seems small, the fact that it is proportionally more concentrated in chromosomal material than is nonmigrating nuclear RNA appears to be significant. It would be useful to know whether shuttling RNA's are also present in multicellular organisms, for if that is so, the possible roles for these molecules would be considerably broadened. The work of Brown and Coffey (2) showing that the entry of polyribo-
nucleotides into rat liver nuclei can affect the template properties of the intranuclear DNA lends encouragement to further exploration along the aforementioned lines.

The possibility that the passage of radioactivity from one nucleus to another simply represents the incorporation into host nuclear RNA of products of the turnover of [3H]RNA from the grafted nucleus was discounted by earlier work (6). It can be noted here, however, that contrary to what is observed when the usual radioactive precursors are made available to a cell (such as the labeling of nucleoh seen in the lower nucleus of Fig 7), the label that appears in a host nucleus in these kinds of experiments is almost totally absent from nucleoh (as seen in the upper nucleus of Fig 7) — although the amount of nucleoplasmic label (unfortunately not shown in the grafted nucleus) is approximately the same in both nuclei. Thus, the passage of radioactivity from one nucleus to another seems unlikely to be in the form of the usual precursors available to the cell — otherwise the nucleoh of the host nucleus should have been much more heavily labeled than the nucleoplasm.

Implicit in all the experiments reported here is the fact that all of the 3H is always in the form of RNA. This fact was established in earlier work (7).

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