Characterization of RNAs That Do and Do Not Migrate between Cytoplasm and Nucleus*

Lester Goldstein and Olive H. Trescott

DEPARTMENT OF MOLECULAR, CELLULAR, AND DEVELOPMENTAL BIOLOGY,
UNIVERSITY OF COLORADO, BOULDER, COLO. 80302

Communicated by K. R. Porter, July 13, 1970

Abstract. We have investigated the nature of the RNA that moves from cytoplasm to nucleus against a concentration gradient in *Amoeba proteus*. We find that: In the presence of actinomycin D an unlabeled nucleus grafted into a [3H]RNA cytoplasm acquires RNAs with sedimentation constants 30 S, 19 S, and 4–6 S that are not related to the general population of cytoplasmic ribosomal and transfer RNAs. RNAs of sedimentation constants 39 S and 16 S may also enter the nucleus from the cytoplasm, but not in the presence of actinomycin D. Nuclei were transplanted from [3H]RNA cells through several unlabeled cytoplasms to dilute out migrating [3H]RNA. This resulted in the 4–6 S [3H]RNA being retained as the predominant labeled material of the nucleus and establishes that a substantial portion of 4–6 S nuclear RNA does not leave the interphase nucleus. We conclude that nuclear RNAs may be classified in a new way: (1) RNA that is to become cytoplasmic RNA and presumably moves only from nucleus to cytoplasm; (2) RNA that migrates back and forth between nucleus and cytoplasm; and (3) RNA that does not leave the nucleus during interphase.

Goldstein and Plaut1 reported in 1955 that RNA moved from the nucleus where it is made, to the cytoplasm but seemed not to migrate from cytoplasm to nucleus. The conclusion that RNA does not migrate into the nucleus apparently has never been challenged. Moreover, since ribosomal RNA (rRNA), transfer RNA (tRNA), and messenger RNA† (mRNA) were until relatively recently thought to be the only cellular classes of RNA, and since these RNAs presumably functioned in the synthesis of protein only in the cytoplasm, there was no reason to suspect that intact RNA molecules return from the cytoplasm to the nucleus.

Recent evidence from this laboratory2 showed that RNA does indeed migrate from cytoplasm to nucleus. This was demonstrated by grafting a [3H]RNA nucleus into an unlabeled cell; several hours later the radioactivity of grafted nucleus, host cell nucleus, and host cytoplasm was analyzed. The analysis showed that [3H]RNA not only had moved into the host cell nucleus, but also had accumulated in that nucleus to a concentration on the order of 12 times the cytoplasmic concentration of [3H]RNA. This movement against a concentration gradient occurred even in the presence of concentrations of actinomycin D (AMD) that inhibited nucleoside incorporation into RNA by 99%, indicating
that the nuclear acquisition of \(^{3}H\)RNA could not be due entirely to reincorporation of the breakdown products of cytoplasmic \(^{3}H\)RNA. In fact, in the presence of such concentrations of AMD, the host cell nucleus acquired \(^{3}H\)RNA to a concentration about eight times that of the cytoplasm.

We report here that there are at least three classes of RNA, distinguishable by sucrose gradient centrifugation analyses, that migrate from cytoplasm to nucleus. In addition, the experiments show the existence of a class of nuclear RNA (nRNA) that does not migrate from nucleus to cytoplasm—and, thus, almost certainly does not move from cytoplasm to nucleus either, at least during the interphase of the cell cycle.

Materials and Methods. Cells and culture methods: Amoeba proteus was grown as described by Prescott and Carrier.\(^5\) Stock cultures of Tetrahymena pyriformis were grown in 2% proteose-peptone solutions. For labeling purposes tetrahymena were grown as described below.

Cell labeling: Amebae were labeled by feeding on radioactive tetrahymena. To promote uniformity, several hundred dividing amebea were picked up with a pipet from a stock culture and fed radioactive tetrahymena for several days. (The results reported here were essentially the same regardless of whether cells were given labeled food for less than or more than one cell generation.) The labeled amebea were fed unlabeled food (chased) for at least 1 day before use in an experiment.

To obtain \(^{3}H\)RNA in tetrahymena (which gave rise to \(^{3}H\)RNA amebea) a drop of late log phase, proteose-peptone grown, culture was added to 1 ml of defined growth medium,\(^4\) less proteose-peptone and pyrimidines, and to which 50 \(\mu\)Ci of [\(^5\)H]uridine (26.1 Ci/mmol, New England Nuclear Corp.) had been added. The cultures were kept at 29°C. About 16 hr later, and every 4 hr during the day for the next 2 days, an additional 50 \(\mu\)Ci of [\(^5\)H]uridine was added to give a final total of 400–500 \(\mu\)Ci added before the tetrahymena were harvested in the usual way and fed to amebea as needed.

To obtain \(^{3}H\)DNA, folic acid-free defined medium was inoculated as above and 100 \(\mu\)Ci of [methyl-\(^3\)H]thymidine (12 Ci/mmol, New England Nuclear Corp.) was added. On each of the next 2 days, another 100 \(\mu\)Ci of labeled thymidine was added to the culture, which was kept at 29°C. Tetrahymena were fed to amebea for 2 weeks before the amebea were used in an experiment.

Actinomycin D treatment: To inhibit RNA synthesis, amebea were placed in ameba medium that contained 0.5 mg/ml AMD, a concentration that inhibits 99% of the \(^{3}H\)uridine incorporation by amebea.

Transplantation of nuclei: Ameba nuclei were transplanted by the method of Jeon and Lorcher.\(^6\)

Nuclear isolation: Nuclei were isolated by the method of Prescott et al.\(^4\) Amebea were lysed at 0°C in a solution of 0.5 ml Triton X-100 (Rohm and Haas)–4 mg spermidine phosphate per 100 ml H\(_2\)O at pH 6.7–6.8. Nuclei were passed through three consecutive Triton-spermidine solutions to remove traces of cytoplasm, then placed directly into the RNA extraction medium or onto a planchet for assay of radioactivity.

Extraction of carrier RNA: The unlabeled carrier, which provided absorbance reference markers in the gradients and facilitated precipitation of \(^{3}H\)RNA, was extracted from amebea fasted for at least 2 days. At least 7 ml of washed and packed (by centrifugation at about 1000 \(\times\) g for 1 min) amebea were homogenized in about 50 ml of a medium composed of 0.5% sodium N-lauroyl sarcosinate (SLS, K & K Laboratories), 0.1 M NaCl, 0.01 M EDTA, and 0.1% mercaptoethanol at pH 7.0 and about 50 ml of medium-saturated phenol (redistilled in the presence of 0.1% 8-hydroxyquinoline). The homogenate was stirred rapidly in an ice bath for 30 min, then the phenol and aqueous phases were separated by centrifugation at 20,000 \(\times\) g for 10 min. The aqueous phase was re-extracted twice with phenol, NaCl was added to a final concentration of 0.5 M, and twice the volume of cold 100% ethanol was added. The precipitated RNA was stored at −20°C under ethanol and samples were removed as needed.
Extraction of [3H]RNA from isolated nuclei: Isolated [3H]RNA nuclei were added directly to DEP buffer (3% diethyl pyrocarbonate, Eastman Organic Chemicals–1% SLS–0.05 M Tris, pH 7.6) and M MgCl at 0°C and homogenized with a Teflon homogenizer. To increase the yield of extracted RNA the mixture was placed at 37°C for 5 min and re-homogenized. After addition of carrier RNA, NaCl was added to a final concentration of 0.5 M and 2.5 times the volume of aqueous homogenate of cold 100% ethanol was added to precipitate the RNA. The method is adapted from that of Fedorovský et al.1

Sucrose gradient centrifugation: The alcohol-precipitated [3H]RNA (kept under alcohol at −20°C for at least 15 hr before further processing) was centrifuged at 20,000 × g for 10 min, the supernatant was decanted, and the precipitate was dried under reduced pressure. The RNA precipitate was resuspended in 0.2–0.3 ml NETS (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris, 0.5% SLS, pH 7.1) and placed atop a 17.5-ml linear 15–30% sucrose gradient (in NETS) in a 10-cm-long SW27 centrifuge tube. The tubes were centrifuged at 27,000 rpm for 22 hr at 4°C in a SW27 rotor in a Spinco L2-65B centrifuge. After centrifugation the gradients were collected by puncturing the bottom of the tube and pumping the contents through a flow cell in a Gilford 2000 recording spectrophotometer, which recorded the absorbance at 260 nm; the gradient was collected in 0.33-ml fractions for radioactivity assay.

Assignment of sedimentation constants: All sedimentation constants assigned to particular fractions were extrapolated from the values of 30 S and 19 S for cytoplasmic ribosomal RNAs reported by Craig and Goldstein.4

Assay of radioactivity: 0.33 ml of cold 20% trichloroacetic acid was added to each of the collected gradient fractions; at least 15 min later each precipitate was collected by aspiration onto a type HA Millipore filter. The filters were dried under an infrared lamp and placed in scintillation vials containing a solution of 0.4% Omnifluor (98% 2,5-diphenyloxazole and 2% p-bis-(O-methylstyryl)-benzene, New England Nuclear Corp.) in toluene. The vials were assayed in a Beckman LS-133 or a Packard 3375 scintillation counter. Because of technical limitations no attempt was made to determine absolute dpm for the samples; instead, our conclusions are based on comparisons of cpm between different parts of each experiment.

For monitoring various steps in our procedures, samples were placed on planchets, spread with formic acid, and assayed in a windowless, low-background, automatic, gas-flow Geiger counter (Nuclear-Chicago Corp.): efficiency for 3H about 20%.

Results. In previous experiments we detected movement of cytoplasmic [3H]RNA into the nucleus by implanting a [3H]RNA nucleus into an unlabeled cell and, sometime later, isolating the grafted nucleus, the host cell nucleus, and the cytoplasm and determining the radioactivity in each compartment. That procedure, for the kind of assays described in this paper, would have been inefficient and very burdensome; thus, we used a much simpler procedure. Uniformly labeled and well-chased cells were enucleated and immediately renucleated with unlabeled nuclei. Next day the nuclei, which had by then acquired radioactive RNA from the cytoplasm, were isolated and pooled for assay of [3H]RNA.

The results of this experiment are illustrated in Fig. 1. The original [3H]RNA cells were given unlabeled food for 3 days before isolation of nuclei; nuclear transplantations were performed after 2 days on unlabeled food. The nuclei for parts A–D were isolated at about the same time.

The important conclusions to be drawn from the experiment of Fig. 1 are: (1) the kinds of RNA in the original, well-chased, [3H]RNA nucleus (1A) and the second nucleus (1B), in essentially equivalent cytoplasm, are similar in most respects; (2) one certain difference between the two kinds of rRNA is the presence of a relatively higher proportion of material in the 4–6S region in the orig-
Fig. 1. Sucrose density patterns of: A, nuclei from cells labeled with $^3$H]uridine, then chased with unlabeled food for 3 days; B, nuclei grafted unlabeled into enucleate $^3$H]uridine cytoplasms from same population as cells in A; C, same as cells in A but incubated in 0.5 mg/ml AMD for 24 hr before isolation of nuclei; D, same as B but incubated in 0.5 mg/ml AMD while the nuclei were in $^3$H]uridine cytoplasms. Nuclei for B and D were in labeled cytoplasm for 24 hr. All nuclei were isolated at about the same time. The cpm for each part cannot be compared directly because different numbers of nuclei were used. The recovered cpm/nucleus were:

- A = 119 (85 nuclei);
- B = 72 (91 nuclei);
- C = 42 (176 nuclei);
- D = 10 (240 nuclei).

The absorbancy peaks at 260 nm of the three major fractions in the carrier RNA are indicated by arrows.

inal $^3$H]RNA nucleus; (3) the second conclusion is substantiated by the finding that incubation in AMD amplified this difference (1C vs. 1D); (4) the material in the 30S, 19S, and 4–6S regions must, at least in part, migrate from cytoplasm to nucleus, since it appears in the second nucleus in the presence of AMD; (5) it may be that the tritium counts in 39S and 16S material reflect reutilization of $^3$H]RNA breakdown products since they are found only in the absence of AMD, but the possibility that AMD blocks the nuclear ingress of the larger molecules cannot be excluded; (6) in general, the proportion of 39S RNA is greater in the second nucleus.

(The reality of a distinct 16S fraction was made evident by centrifugation of the material for 42 hr rather than the usual 22 hr.)
That essentially all of the labeled material of the sucrose gradients is RNA is shown by Fig. 2. We see that the material labeled with [3H]uridine is alkali-labile; the material labeled with [3H]thymidine (to indicate presence of DNA) is distributed differently than the [3H]uridine-labeled material. Other experiments have shown that at least 95% of the material labeled with [3H]uridine by methods identical to those employed here is hydrolyzed by ribonuclease.

The difference between Fig. 1A and B in the 4–6 S region suggested the possibility that much of that material does not move from nucleus to cytoplasm. This notion is fortified by a comparison of the patterns of Fig. 1A and C, in which it is seen that the 4–6S material becomes predominant in the presence of AMD. If much of the 4–6S RNA does not leave the nucleus, passage of a [3H]RNA nucleus through successive unlabeled cytoplasm would dilute out those nuclear [3H]RNAs that did migrate, and would result in 4–6S [3H]RNA becoming the predominantly labeled RNA. Such dilution was effected by transplanting [3H]RNA nuclei into unlabeled cytoplasms, repeating the transplantations to fresh, unlabeled cytoplasms the next day, and the following day isolating the nuclei and extracting the RNA. A comparison was made with nontransplanted [3H]RNA nuclei from the same cell population that provided the original [3H]RNA nuclei but taken from cells fed unlabeled food during the period of the sequential transplantations. The results, in Fig. 3, clearly show that our prediction is fulfilled: 4–6S [3H]RNA became predominant. From these data we estimate that approximately 10% of the [3H]RNA (essentially all in the 4–6S region) of a well-chased nucleus does not leave the nucleus during interphase.
Fig. 3. Sucrose density gradient patterns of: Left, nuclei isolated directly from cells labeled with \(^{3}H\)uridine, then chased with unlabeled food for 3 days; Right, nuclei from the same cell population but passed through unlabeled cytoplasm twice in a 24 hr period, after 2 days of chase, then isolated. Recovered cpm/nucleus were: Left = 149 (60 nuclei); Right = 46 (159 nuclei).

Discussion. The data given here demonstrate that RNAs with estimated sedimentation constants of 30 S, 19 S, and 4-6 S migrate from cytoplasm to nucleus. Since the other two nRNA classes, 39S and 16S, do not appear when AMD is present, an interpretation is that they are molecules synthesized from products of \(^{3}H\)RNA breakdown. While this explanation seems plausible for the 16S material, which is rapidly labeled, the mechanism for the nuclear accumulation of 39S RNA, which is slowly labeled, is still to be determined. Since some RNA egress from, and probably ingress into, the nucleus is inhibited by AMD, we should be prepared to accept any of a variety of possible effects of AMD on the behavior of RNA molecules. We should note also that because 39S nRNA is labeled slowly, it is unlikely to be a precursor of the cytoplasmic 30S and 19S rRNA subunits—thus, the 39S nRNA seen in our experiments may be a heretofore unrecognized nRNA that migrates from cytoplasm to nucleus as an intact molecule.

The assigned sedimentation values for the unambiguously migrating RNAs, 30 S, 19 S, and 4-6 S, suggest that they may be identical to the predominant cytoplasmic species: the two ribosomal RNA subunits and the tRNAs. Such a conclusion is unwarranted until more evidence, including more accurate estimations of sedimentation constants, is available. Other kinds of evidence are also needed, since identity of S values might be only coincidental. Indeed, such caution is indicated by preliminary evidence that the nuclear 30S RNA contains material that is precursor of cytoplasmic 19S RNA.

Even should subsequent investigations show that the 30S, 19S, and some of the 4-6S nRNAs are identical to their cytoplasmic counterparts, the migration of these molecules into the nucleus could not have a trivial basis (such as simple diffusion of cytoplasmic RNAs through nuclear envelope pores) for the following
reasons: (1) even in the presence of AMD, the RNAs migrate from cytoplasm to nucleus against a concentration gradient; (2) in well-chased cells the proportion of 30S RNA to 19S RNA generally is greater in nucleus than in cytoplasm—thus arguing against the ingress of complete ribosomes; (3) also arguing against ingress of complete ribosomes, as well as intact ribosomal 60S and 40S subunits, is the evidence that the amount of rRNA in the nucleus is proportionately much greater than the amount of nuclear ribosomal protein. [Ribosomes attached to the outer nuclear membrane play no role here since electron microscopic observations show that the outer nuclear envelope is removed by the isolation medium we use that contains Triton X-100 (K. Murti, personal communication).] That the RNA molecules entering the nucleus from the cytoplasm are from a select group, not simply a random sample of all the kinds in the cytoplasm, also is indicated by the finding that, after the implantation of an [3H]RNA nucleus into an unlabeled cell, the host cell nucleus acquires 20% of the label that enters the cytoplasm, whereas an unlabeled nucleus acquires less than 2% of the cytoplasmic [3H]RNA when implanted into cytoplasm uniformly labeled in the usual manner.

The low molecular weight nRNAs, of which our 4–6S fraction must be representative, have received much attention recently. Our studies of the 4–6S material are reminiscent of those of Weinberg and Penman who report the existence in HeLa cells of nine nRNA species with sedimentation constants below 10 S. All but one of these nine species seem to be absent from the cytoplasm, but the evidence is not conclusive. The conclusion of exclusive nuclear localization is based on the finding that after incubation for one hour in radioactive precursor there is no detectable labeled cytoplasmic RNA equivalent to eight of the nine nuclear species. This seems an unsatisfactory procedure if, as Weinberg and Penman report, these low molecular weight RNAs have relatively slow turnover rates. Nevertheless, our data suggest that some nuclear 4–6S molecules are not represented in the cytoplasm, primarily because they apparently do not leave the nucleus during interphase.

Although these 4–6S RNAs do leave the nucleus during mitosis, they return, essentially in their entirety, immediately after division is complete (L. Goldstein and M. Beeson, unpublished data). When a [3H]RNA nucleus is grafted into an unlabeled, enucleate cell and the cell is then fed unlabeled food, just before the cell enters mitosis the labeled nuclear 4–6S material contributes about 10% to the cell’s radioactivity. This amount of radioactivity, if associated with the mitotic chromosomes, would be readily detectable radioautographically, but we can detect no concentration of label anywhere in the mitotic cell under these conditions. Thus, although it is impossible to state that no RNA remains associated with nuclear materials at mitosis, we find that very little 4–6S RNA can be so associated. Yet, essentially all the nRNA liberated to the cytoplasm during mitosis returns to the post-mitotic nuclei immediately after division is complete. We conclude, therefore, that the non-migrating nuclear 4–6S RNA does appear in the cytoplasm briefly during the mitotic period of the cell cycle.

The nonmigrating nuclear 4–6S RNA of A. proteus is estimated to be about 0.5% of the cell total, which compares with 0.4%, or somewhat more, estimated for
the low molecular weight nRNAs of HeLa cells. We caution, however, that we can account in our sucrose gradients for only 25–50% of the labeled RNA present in the isolated nuclei. This suggests the possibility of nonrandom extraction of RNA molecules, but Prescott et al.10 have shown that the patterns seen in our controls resemble the patterns they obtain under conditions where essentially all the [3H]RNA of isolated nuclei is accounted for in sucrose gradients.

Since we have no precise measurement of the size of the molecules in the region we have called 4–6 S, we cannot judge the relationship of this material to so-called chromosomal RNA14—but that is a possible relationship worth exploring.

We are indebted to David M. Prescott and his associates whose early experiments smoothed the path for our efforts, and to Mary Beeson who provided assistance for the latter part of the experiments.

Abbreviations: AMD, actinomycin D; nRNA, nuclear RNA; rRNA, ribosomal RNA.

* This research was supported by a U.S. Public Health Service research grant 5 RO1 GM15156 and U.S.P.H.S. program project grant HD 02282.

† Contrary to common usage, we insist that the proper term should be “message RNA.”