THE CELLULAR SITES OF SYNTHESIS OF RIBOSOMAL AND 4S RNA*

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In previous publications,[1–4] we presented evidence which indicates that the major portion of cytoplasmic RNA (C-RNA) is derived from RNA which is synthesized in the nucleolar region of the nucleus (n-RNA). Furthermore, it was proposed that n-RNA and the RNA of the extranucleolar parts of the nucleus (N-RNA) are synthesized independently of each other. These studies, carried out on the level of the individual cell, did not distinguish between the different molecular species of RNA such as ribosomal, amino acid-accepting (transfer), and messenger RNA. A general relationship, such as C-RNA is mostly ribosomal RNA, can readily be inferred but there is as yet no description of RNA synthesis which adequately comprehends both the cytological and molecular terms.

Some progress along these lines has been made by Georgiev and collaborators.[5, 6] These investigators used different phenol extraction procedures to separate partially the various classes of RNA and deduced from an analysis of incorporation kinetics that an RNA, from what they term "the chromosomal-nucleolar apparatus," not extractable by phenol at pH 6.0–0.14 *M* NaCl, is a precursor of "high polymeric" cytoplasmic RNA. Other nuclear fractions were described, one of which is "low polymeric" and capable of an *in vivo* incorporation of P^{32} which is 20 times faster than a similar "low polymeric" cytoplasmic fraction. Unfortunately, no clear distinction is made between nucleolar and chromosomal RNA.

An approach to this problem which allows direct comparison with autoradiographic studies was suggested when it was found that actinomycin D, when used at sufficiently low concentrations, greatly suppresses the incorporation of nucleosides into n- and C-RNA of growing tissue culture cells with little or no depression of the rate of incorporation into N-RNA.[7] Using this agent, together with a radioactively labeled RNA precursor, one may obtain relatively large quantities of cells in which the intracellular pattern of RNA synthesis is altered in a well-defined way. From such cells, one can extract and analyze the newly formed RNA and then correlate the alterations in the different molecular species of RNA with the changes observed in the various cytological classes. One of the advantages of this approach is that it allows a comparison between autoradiographic and biochemical data without risking the pitfalls of cellular fractionation (cf. ref. 8). In addition, it allows conclusions concerning precursor product relationships among the RNA fractions which are stronger than deductions made solely on the basis of kinetic data.

Materials and Methods—General: Exponentially growing L cell fibroblasts, of a strain originally obtained from A. F. Graham, were cultured in Eagle's basal medium with 10 per cent calf serum on Leighton Tube coverslips for autoradiography and in 400 and 800 ml spinner cultures for RNA extraction. Actinomycin D (Merck-56R4094) was used at a concentration of 0.04 μg/ml (3.3×10^{-8} *M*). H^3-cytidine (1.84 C./mmole) was used at 0.2 μc/ml for the coverslip cultures and 0.15 μc/ml for the spinner cultures. Chase incubations were done in media containing unlabeled cytidine at a concentration of 0.1 mg/ml.

Autoradiography: Details of the autoradiographic technique have been described in a previous publication.[2] However, the following important points should be mentioned. (1) After fixation in Carnoy's and before application of the radioautographic emulsion, the cells were treated

with 1 per cent perchloric acid (PCA) for 20 min at 5°C in order to thoroughly remove all of the acid-soluble precursor material. (2) No measurements of the three-dimensional geometry of L cells were made, and therefore it was not possible to obtain grain count data corrected for β-ray self-absorption as was done previously with HeLa cells.[2] Nevertheless, it should be kept in mind that a given amount of radioactivity located in the relatively thin cytoplasm has a greater efficiency in producing grains than if it were located in the nucleus.

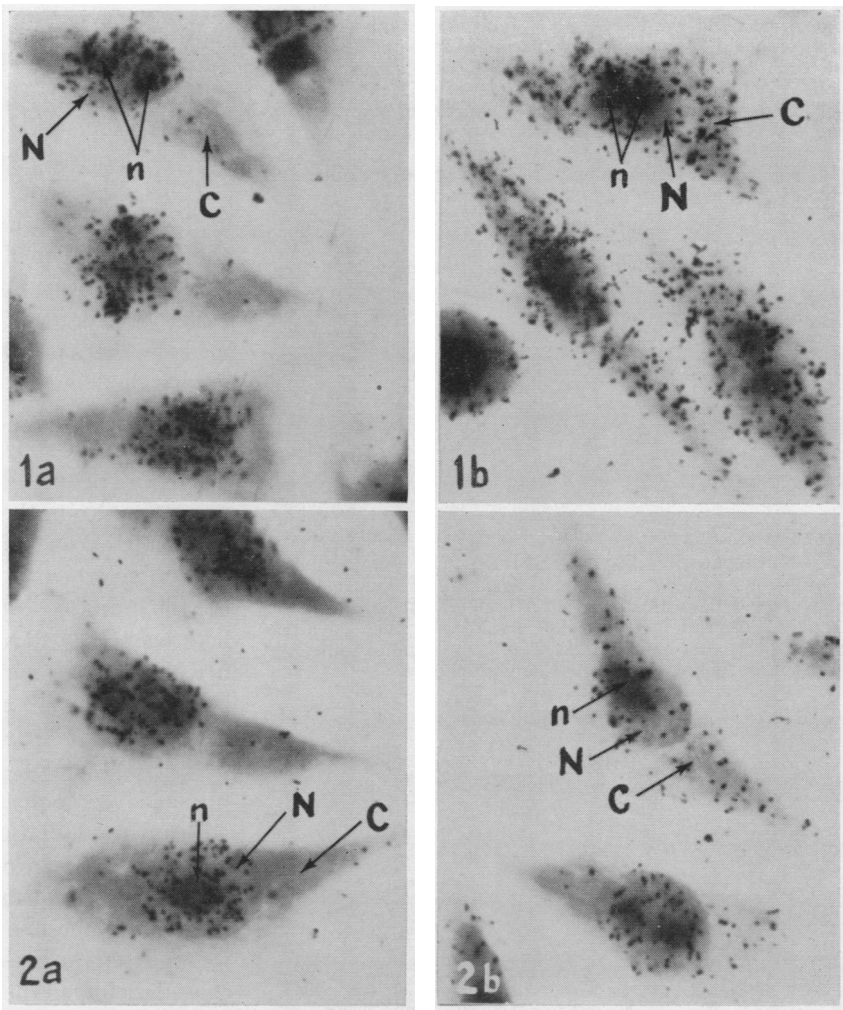
Extraction procedures: At the completion of the labeling or chase period, the spinner culture cells at concentrations of roughly 4×10^5 cells/ml were harvested by centrifugation and rapidly frozen at −70°C. The frozen pellets were stored at −35°C until used for analysis. An extraction procedure similar to that described by Scherrer and Darnell[9] was used. The cell pellets were suspended at 0°C in 3.3 ml of 0.01 *M* acetate buffer, pH 5.0, containing 0.5 per cent recrystallized sodium dodecyl sulfate (SDS), 0.1 *M* NaCl, and 10^{-3} *M* Mg^{++}. The viscous extracts were shaken for 3 min at 60°C with 3 ml of freshly distilled, water-saturated phenol, quickly chilled, and centrifuged 1 min at 20,000 × *g*. The aqueous phase was removed and twice more extracted with phenol at 60°C. The final extract was shaken with ether to remove the phenol and then the ether was removed by bubbling nitrogen through the preparation. The RNA was precipitated with 2 vol of ethanol at −35°C. Conventional phenol extractions, identical to the above except that they were carried out at 20–24°C with the SDS omitted from the acetate buffer, were also made.

Sedimentation analysis of RNA: The RNA precipitate was dissolved in 2 ml of acetate-NaCl-Mg^{++} buffer and passed through a Sephadex G25 column (30 × 200 mm).[10] The rapidly moving front, containing all the RNA, was eluted in 6–9 ml, and the RNA was again precipitated at −35°C with 2 vol ethanol. The RNA was redissolved in 1 ml of the acetate-NaCl-Mg^{++} buffer and applied to a 5–20 per cent sucrose gradient according to the method of Britten and Roberts.[11] The preparations were then centrifuged at 25,000 rpm for 10 hr at 5°C in a Spinco SW 25 swinging bucket rotor. After completion of the centrifugation, a hole was punched in the bottom of each tube and 31 to 33 one-ml samples were collected. Absorbances at 260 mμ were determined, and measured volumes of each sample (0.6 or 0.7 ml) were counted in a scintillation counter. The scintillation liquid was made according to the method of Bray.[12]

RNA prepared in this manner exhibits three distinct optical density peaks (solid curves, Figs. 3 and 4). According to Graham,[13] who has carried out similar experiments with L cells, these peaks correspond roughly to 32S, 18S, and 4S components. Since it is well established that the two larger components are the major RNA components of ribosomes,[14] we will frequently refer to them as "ribosomal RNA."

Comment on extraction methods: Other workers[9, 15] have reported that the phenol-SDS method is capable of extracting all the cellular RNA, including the rapidly labeled nuclear fractions which are not extractable by phenol alone.[3, 16] In preliminary experiments, we confirmed this result by comparing specific activities of phenol- and phenol-SDS extracted material with specific activities of RNA extracted by 10 per cent PCA at 80°C. We found that whereas only 11 per cent of the RNA labeled during a 10-min pulse is extracted by phenol,[17] 100 per cent is extracted by the phenol-SDS method. Hence, the RNA observed on the autoradiographs is extracted unselectively by phenol-SDS, and therefore one may validly compare the data from these two different types of experiments.

Results and Conclusions.—Controls: Figures 1 and 3 illustrate the autoradiographs and sedimentation diagrams for cells which were incubated for 30 min with H^3-cytidine (pulse) and for cells which were incubated for 30 min in H^3-cytidine and then further incubated for 4 hr with excess unlabeled cytidine (chase). At 30 min (Fig. 1*a*), more than 95 per cent of the cytidine incorporation is confined to n- and N-RNA. The sedimentation behavior of this RNA (Fig. 3) indicates that it is composed of 4S RNA plus numerous high-molecular-weight components, some of which sediment faster than the largest ribosomal RNA component. Sedimentation diagrams from cells labeled for 10 min with H^3-cytidine are indistinguishable from these except for a lesser amount of the 4S component. Such sedimentation behavior of newly formed RNA is basically similar to that described by Scherrer and Darnell.[9]



FIGS. 1, 2.—Autoradiographs of L cell fibroblasts. (*a*) Cells pulsed for 30 min with H^3-cytidine. (*b*) Cells pulsed for 30 min with H^3-cytidine and further incubated for 4 hr in a chase medium containing excess unlabeled cytidine. 1. Controls. 2. Actinomycin D added 30 min before pulse labeling. n designates nucleoli; N, an extranucleolar part of the nucleus; C, the cytoplasm. Note that the dense nucleolar labeling, clearly seen in the controls, is absent in the cells treated with actinomycin. On the other hand, no marked differences are observed in the labeling of the extranucleolar portion of the nucleus. Note also the marked reduction in cytoplasmic label seen in the actinomycin-treated cells.

However, we do not obtain a distinct peak of the high-molecular-weight RNA as was obtained in their experiments. Perhaps this is related in some way to their use of polyvinyl sulfate.

After a 4-hr chase incubation, roughly $^2/_3$ of the labeled RNA is in the cytoplasm (Fig. 1*b*). Sedimentation analysis (Fig. 3) shows that at this time the bulk of the labeled RNA is distributed in the three peaks characteristic of ribosomal and 4S RNA. The specific activity of the total RNA increases appreciably between the beginning and end of the chase incubation. This effect, which is most likely caused by a continued uptake from nonexchangeable precursor pools, is also observed on

Note for Figs. 3 and 4: Except for a relatively small difference in total RNA yield, the curves for 260 mμ absorbance are essentially all the same. Therefore, for ease of comparison we have plotted both the pulse and the pulse-chase experiments for each series together with the single absorbance curve for the pulse experiment. Where appropriate, the ordinates of the radioactivity curves were shifted slightly so as to correspond to the same total yield of RNA. Plotted in this way, the cpm values for pulse and pulse-chase experiments are immediately comparable.

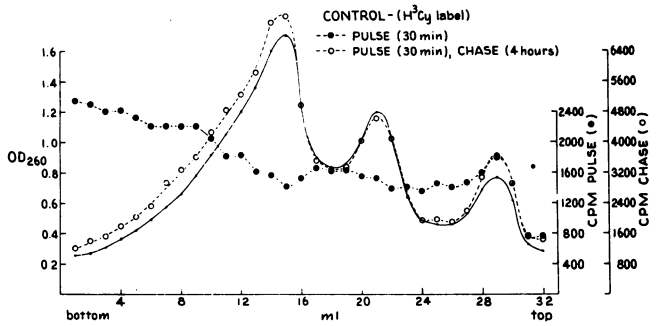


FIG. 3.—Sedimentation diagrams of RNA from cells incubated under conditions similar to those described for Figure 1*a*, *b*. Solid curve represents absorbance at 260 mμ for pulse experiment, dotted curves are radioactivities for pulse and pulse-chase experiments. Left-hand part of diagram is bottom of tube and represents the faster sedimenting RNA components.

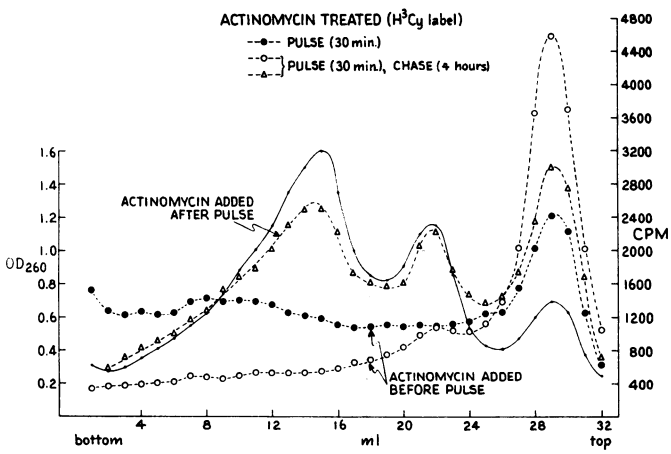


FIG. 4.—(● and ○) Sedimentation diagrams of RNA from cells incubated under conditions similar to those described for Figure 2*a*, *b*. (△) Cells were labeled for 30 min in H³-cytidine, then further incubated for 4 hr in a chase medium containing unlabeled cytidine *plus actinomycin*.

autoradiographs. The analogous experiment performed with H³-uridine as a precursor yields almost identical results.

It is apparent that, concomitant with the change in location of label from exclusively nuclear to predominantly cytoplasmic, there is a change in size distribution from one which is highly polydisperse to one consisting of three discrete components. From these results alone, one cannot determine which portion of the rapidly labeled, heterogeneous RNA is n-RNA and which part is N-RNA, nor can one interpret the relationship between n- and C-RNA previously demonstrated with microirradiation experiments.[1, 3] To obtain additional information concerning these points, we repeated the pulse and chase labeling in the presence of actinomycin.

Actinomycin-treated cells: The L cell cultures were treated with the relatively low concentration of 3.3×10^{-8} *M* actinomycin D as follows: (*a*) incubation for 30 min with actinomycin, H³-cytidine added and cells incubated for additional 30 min; (*b*) treatment as in (*a*), then excess unlabeled cytidine added and cells incubated for additional 4 hr; and (*c*) incubation with H³-cytidine for 30 min, then 4 hours chase in the presence of actinomycin.

Autoradiographs for (*a*) and (*b*) may be seen in Figure 2. Quantitative data are given in Table 1. In agreement with previous results,[7] a marked suppression of n-RNA synthesis is observed under circumstances where the incorporation into

N-RNA is appreciable and essentially indistinguishable from that of control cells. When these cells are given a further incubation in unlabeled medium, one observes, in addition to the decreased incorporation into n-RNA, a very striking diminution of incorporation into C-RNA. The incorporation into C-RNA under these conditions is less than $^1/_4$ of that found in the controls (cf. Table 1).

The sedimentation behavior of RNA from actinomycin-treated cells is illustrated in Figure 4. One notices that an appreciable fraction of the rapidly labeled, fast-sedimenting RNA is absent and that the incorporation into the 4S component is significantly higher than that found in controls. Upon chasing, there is a further diminution in the amount of fast-sedimenting, labeled RNA and a further increase in the amount of labeled 4S component. However, in contrast to the controls, there is absolutely no appearance of label in the peaks characteristic of ribosomal RNA. When H^3-uridine is used as an RNA precursor, the results are basically similar, except that although there is significant incorporation into 4S material, this incorporation does not surpass that of the controls. In accord with results obtained by Homma and Graham,[18] it was found that RNA extracted from actinomycin-treated cells by phenol without SDS exhibits radioactivity *exclusively* in the 4S region.

A qualitative correlation of the autoradiographic and sedimentation analyses strongly indicates that a major portion of *newly formed* n-RNA consists of fast-sedimenting heterogeneous components. In the presence of low concentrations of actinomycin, the synthesis of these components is specifically inhibited, with the result that one observes a markedly depressed nucleolar grain count and a concomitant loss of a portion of the radioactivity from the fast-sedimenting part of the gradient. It is also apparent that 4S RNA should be identified with N-RNA. However, one notes that in untreated cells only about $^1/_5$ of the total RNA labeled during a 30-min pulse is of the 4S variety. Inasmuch as this is less than half of the fraction normally attributable to N-RNA, we conclude that N-RNA contains some fast sedimenting components in addition to the 4S component.

The fact that a suppression of the synthesis of n-RNA is followed by a complete inhibition of ribosomal RNA synthesis could be interpreted in two ways. Either n-RNA is a precursor of ribosomal RNA or actinomycin independently inhibits the synthesis of both n-RNA and ribosomal RNA. The previously cited experiments which demonstrated that n-RNA is a precursor of the bulk of the C-RNA[1-3] suggest that the former interpretation is the correct one, because it is now certain that most of the autoradiographically observed C-RNA is equivalent to ribosomal RNA. Furthermore, if the latter interpretation were correct, one

TABLE 1

EFFECT OF ACTINOMYCIN ON THE INTRACELLULAR DISTRIBUTION OF LABEL (grains/30 cells, not corrected for self-absorption)

Labeling conditions (H^3-cytidine)	Cell part	Control	Actinomycin-treated*	Actinomycin/control
30-min pulse	n	984	384	0.4
	N	757	689	0.9
	C	95	—	—
30-min pulse, 4-hr chase	n	660	218	0.3
	N	906	1,163	1.3
	C	3,211	747	0.2

* Actinomycin added to a final concentration of $3.3 \cdot 10^{-8}$ *M*, 30 min before pulse labeling.

might expect that if the cell were allowed to synthesize n-RNA during a pulse labeling period (as in Figs. 1 and 3) and then were chased in the presence of actinomycin, no ribosomal RNA would be formed. A sedimentation diagram from an experiment performed in exactly this way is included in Figure 4. It is seen that there is a considerable amount of labeled ribosomal RNA after 4-hr incubation in a chase medium containing actinomycin. Thus, we see that once the n-RNA is formed, actinomycin cannot block the appearance of ribosomal RNA.

This experiment demonstrates that the rapidly synthesized, heterogeneous RNA of the nucleolus is an obligatory precursor of ribosomal RNA. The specific activity of the total RNA after a chase incubation with actinomycin is slightly less than the specific activity measured before the chase. One may suppose that the 2-fold increase in specific activity which is observed when control cultures are chased for 4 hr does not occur in the presence of actinomycin because there is no further incorporation into the precursor of ribosomal RNA from acid-soluble pools. In an analogous autoradiographic experiment, actinomycin, when added before the pulse, was found to reduce the amount of labeled C-RNA more than twice as much as when it was added after the pulse.[7]

Discussion.—The nature of the RNA synthesized in the extranucleolar part of the nucleus (N): The preceding results indicate that rapidly labeled N-RNA is of at least two distinct types: a 4S variety and a fast-sedimenting, heterogeneous material. Even if all of the RNA which we have designated as 4S were not transfer RNA, it is reasonable to suppose that a significant portion of the 4S component has this function. On the further assumption that some of the incorporation of label represents true polynucleotide synthesis and not terminal addition, it may be concluded that the primary site for transfer RNA synthesis is in N, the part of the nucleus containing the major portion of the chromosomes. However, the fact that most of this RNA has the property of being extractable by phenol at room temperature, without the aid of a detergent such as SDS, leads one to believe that its physical association with the chromosomes—if such exists—is probably a very weak one. In fact, Georgiev and Samarina[6] interpret this property to mean localization in the "nuclear sap" as opposed to localization on the chromosome.

In view of the lack of information concerning the base composition of the fast-sedimenting component of N-RNA, it is perhaps premature to attempt to give it a more functional significance. Nevertheless, its rapidity of labeling, its lability, as demonstrated by its disappearance upon chasing *in the absence of ribosomal RNA synthesis*, and its synthesis in the part of the cell occupied by the chromosomes make it a likely candidate for messenger RNA.

The nature of the RNA synthesized in the nucleolar region of the nucleus (n): In view of their closely related response to actinomycin treatment, there is little doubt that n-RNA and a major portion of the fast-sedimenting, heterogeneous RNA component are one and the same. Perhaps the most striking property of this n-RNA is that it is in some way an obligatory precursor of ribosomal RNA and yet it is not confined to the two discrete sedimentation bands which characterize ribosomal RNA. There are two possibilities for this. One is that the newly formed n-RNA has molecular weights (polymer lengths) which are actually different from those of ribosomal RNA and that by some as yet unknown maturation process these heterogeneous components are broken down or built up into ribosomal RNA.

It is unlikely that they are broken down to units as small as mononucleotides, however; otherwise, the radioactive tracer should have been diluted by the pool of precursor material accumulated during the chase incubation.[19] The other possibility is that the newly formed RNA consists only of one or two discrete components which are the direct precursors of ribosomal RNA but that under the conditions of our extraction and sedimentation procedures this RNA undergoes a variety of configurational changes which lead to the heterogeneous array of sedimentation values. This latter possibility can only be entertained if one assumes that the newly formed n-RNA has certain properties such as flexibility and solvent-binding capacity which are lost when it becomes ribosomal RNA.

It is necessary to emphasize, as we have done previously,[2] that the entity which we designate as nucleolus must be considered to comprise the elements of chromosome known cytologically as the "nucleolus-associated chromatin" or genetically as the "nucleolar organizing regions." In the tissue culture systems presently under study, one cannot expect to distinguish these chromosomal elements as separate entities, especially if they happen to be diffused throughout the body of the nucleolus as has been observed in some electron micrographs.[20] Indeed, experiments with Dipteran salivary gland giant chromosomes point to the "associated chromatin" as the ultimate site of n-RNA synthesis.[21] It is thus quite plausible that the nucleolar regions of the chromosomes are those few discrete regions which are devoted to the production of ribosomes. If the mechanism of actinomycin inhibition is related to its DNA-complexing ability,[22] then one might suppose that its preferential action on n-RNA synthesis arises because it has a particularly high affinity for the DNA of the nucleolar regions of the chromosomes.

The nature of cytoplasmic RNA: Cytoplasmic RNA consists of the two ribosomal components, plus 4S RNA. As in the case of N-RNA, one may assume that a significant portion of the 4S component is transfer RNA. From a comparison of cytoplasmic and 4S activity in actinomycin-treated cells, it is calculated that roughly 20–25 per cent of the amount of labeled cytidine which appears in the cytoplasm after a 4-hr chase incubation is incorporated into the 4S component. The other 75–80 per cent is incorporated into ribosomal RNA. It should be remembered that these values pertain to rapidly proliferating cells which must double their ribosomes during a generation time of about 24 hr. In nongrowing systems, the percentage of activity which is associated with the 4S component may be much higher. Our present experiments offer no clue as to whether this 4S material is derived from the 4S RNA which at an earlier time is demonstrable exclusively in the nucleus. Arguments which insist that all C-RNA is of a nuclear origin[1, 23] would of course favor such an hypothesis.

Summary.—Parallel autoradiographic and sedimentation experiments were performed on tissue cultures of L strain fibroblasts. A comparison of data from normal and actinomycin-inhibited systems indicates the following: (1) The rapidly labeled nucleolar RNA consists of fast-sedimenting, heterogeneous components, some of which sediment appreciably faster than the heaviest stable ribosomal component (i.e., > 32S). (2) This heterogeneous, nucleolar RNA is an obligatory precursor of the ribosomal components of cytoplasmic RNA. (3) A heterogeneous, labile component is also synthesized in the extranucleolar (chromatin) part of the nucleus. (4) 4S RNA is first synthesized in the chromatin region of the nucleus

and later appears in the cytoplasm.

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[19] An exception to this statement would be the situation where the mononucleotide breakdown products of n-RNA are confined to a pool which is available for synthesis of ribosomal RNA, but *unavailable* for equilibration with the major endogeneous pool of mononucleotides. Such a situation would not obviate the use of the term "precursor" for n-RNA.

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