Evidence for the Existence of Several Molecular Species in the "45S Fraction" of Mammalian Ribosomal Precursor RNA

(gel electrophoresis/actinomycin D/L5178Y cells/HeLa cells)

PIERRE TIOLLAIS, FRANCIS GALIBERT, AND MICHEL BOIRON

Laboratoire d'Hématologie Experimentale, Hôpital St. Louis, 40 rue Bichat, Paris 10e, France

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ABSTRACT Analysis of RNA (after 5 or 60 min of labeling with [3H]uridine) from L5178Y cells by electrophoresis in 1.7% polyacrylamide gels demonstrates that the ribosomal RNA "45S fraction" is not homogeneous but consists of at least three molecular species, conventionally designated 47S RNA, 46S RNA, and 45S RNA. Alternatives to the classical scheme for the biosynthesis of ribosomal RNA in mammalian cells are discussed.

The 28S and 18S ribosomal RNA in eucaryotic cells are believed to be derived from a common precursor. This precursor would contain the sequences of one 28S molecule and one 18S molecule, associated with a nonribosomal sequence that is not conserved during the conversion of this precursor into mature RNA (1, 2). Therefore, the biosynthesis of ribosomal RNA in eucaryotes appears to differ completely from the biosynthesis of 23S and 16S bacterial ribosomal RNA, which are synthesized independently (2). This concept arose with the discovery in L and HeLa cells of a polynucleotide called "45S RNA", which appeared from kinetic studies and actinomycin D "chase" experiments to be the precursor of both 18S and 32S RNA, the latter giving rise to the 28S RNA (3, 4).

The possibility that the 45S RNA fraction may consist of two distinct molecular species of similar molecular weight, one being the precursor of 28S RNA and the other the precursor of 18S RNA, seems to have been excluded by the following two observations. (a) The number of methyl residues contained in a 45S molecule has been measured and found to be equal to the sum of the methyl residues contained in a 28S and an 18S molecule (5, 6). Furthermore, most ribosomal RNA methylation occurs on the 45S molecule (7, 8) without any apparent loss of methyl residues during maturation (9).

Other studies indicate that there is, at most, one sequence of 28S RNA and one 18S RNA in one molecule of 45S RNA (10). Taken together, these results seem to be compatible only with the existence of one common precursor molecule. (b) During hybridization of 45S RNA with DNA, 18S RNA and 28S RNA effectively compete with 45S RNA. The competition rates are 13% and 35%, respectively (11). Taking into account the molecular weight of the 45S, 28S, and 18S RNA, this is again an argument in favor of the single-precursor hypothesis.

However, certain facts remain difficult to explain under this hypothesis. (a) In the presence of cycloheximide, toyocamycin, or thioacetamide, some synthesis of 32S RNA persists, whereas the synthesis of 18S RNA is completely suppressed (12-14). On the other hand, during poliovirus infection, or in the presence of cordycepin, some synthesis of 18S RNA proceeds, although that of 28S is completely suppressed (6, 15). The results have been interpreted as a consequence of a preferential degradation of 28S or 18S RNA, but without experimental basis, and only to fit these results with the polycistronic scheme of ribosomal RNA synthesis. (b) Because of their molecular weights, the 32S and 18S RNA are believed to originate from the 41S RNA, and the existence of 36S RNA can only be explained by an aberrant cleavage of the 41S RNA (6). (c) The analogy of the base compositions, the partial sequences, and the methylation patterns, although excellent for the 45S, 32S, and 28S RNA, are not satisfactory for the 45S and 18S RNA (5, 10).

Finally, if the arguments in favor of the existence of a common precursor appear stronger than those suggesting separate origins, it is mostly because the "45S fraction" appears as a single molecular species. In this paper we report the analysis of "45S fraction" by gel electrophoresis; we find that this fraction is heterogeneous and contains several molecular species.

MATERIALS AND METHODS

The cells utilized were the L5178Y line, which grows in suspension without shaking (16), and the HeLa-type S line, which grows in spinner culture (17). The methods of cell culture, labeling, extraction of RNA, and analysis by polyacrylamide gel electrophoresis, have been described (18).

RESULTS

Coelectrophoresis (in 1.9% polyacrylamide gel) of RNA from two LY cell cultures, one labeled with [3H]uridine for 5 min and the other labeled with [3C] uridine for 60 min shows that the "45S fraction" consists of two peaks of RNA, separated by a distance of 2 mm (Fig. 1). The 5-min peak (3H) migrates slightly slower than the peak in the preparation labeled for 60 min (3C), and corresponds to a shoulder in the 60-min peak. Therefore, the use of two different labeling times reveals the heterogeneous nature of the "45S fraction", which appears to consist of at least two different molecular species.

The effectiveness of gel resolution is dependent on the polyacrylamide concentration. Accordingly, different concentrations were tested with RNA extracted from LY cell cultures labeled for 60 min by [3C]uridine (Fig. 2). The concentration of agarose was maintained at 0.5% in all experiments. In a 2.1% polyacrylamide gel (Fig. 2a), the 4S RNA appears

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An L5178Y cell culture, containing $7 \times 10^6$ cells/ml, was concentrated to $1.5 \times 10^6$ cells/ml. 20 ml from this culture was incubated for 5 min with $^3$H uridine (4 μCi/ml; $2 \times 10^{-4}$ mmol/ml) and 20 ml was incubated 60 min with $^{14}$C uridine (0.2 μCi/ml; $2.6 \times 10^{-4}$ mmol/ml). After cooling, cultures were mixed and the RNA was extracted simultaneously from both. An aliquot from this RNA was analyzed by 1.9% polyacrylamide gel electrophoresis (23 × 0.7 cm gels; 4 mA/gel; 180-200 V; 6 hr migration; 1-mm slices). — $^3$H uridine, - - - $^{14}$C uridine.

As a homogeneous peak. At 1.9%, a shoulder is seen on the ascending side of the peak (Fig. 2b). At 1.7%, an additional peak, migrating at a position 3 mm behind the main peak, is observed (Fig. 2c). Moreover, the so-called 36S RNA appears as a double peak. The result is obtained in a 1.6% gel. However, in a 1.4% gel, the separation of the different RNAs begins to deteriorate, and the 45S RNA again appears homogeneous (Fig. 2d). Thus, the limits within which the 45S peak is resolved are very narrow, between 1.8 and 1.6% polyacrylamide. Consequently, an aliquot from the extract whose analysis is shown in Fig. 1 has been analyzed in a 1.7% polyacrylamide gel, as shown in Fig. 3. The heterogeneity of the 45S RNA is much more evident. One can distinguish three different molecular species. The first of these corresponds to the main peak from the 5-min labeling and the secondary peak from the 60-min labeling, the second to the secondary peak from the 5-min labeling. The third corresponds to the main peak from the 60-min labeling, and is considered to be 45S RNA. We found it convenient to designate the two new molecular species as 47S RNA and 46S RNA.

The assertion that 47S and 46S RNA are ribosomal precursors seems to be proven by the following arguments:

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**Fig. 1.** An L5178Y cell culture was concentrated to $1.5 \times 10^6$ cells/ml and was incubated 1 hr with $^{14}$C uridine (0.2 μCi/ml; $2.6 \times 10^{-4}$ mmol/ml). After extraction, the RNA was analyzed by electrophoresis on polyacrylamide gels of different concentrations. The electrophoretic conditions were as described in Fig. 1.
This fraction of 5178Y cells, consists of these results which was more than the min) Hela is completely "chased" into RNA, more. After extraction, the RNA was analyzed in a 1.7% polyacrylamide gel.

(a) The peaks are much better resolved and more heavily labeled than the heterogeneous RNA. (b) After 5 min of labeling, which is sufficient for labeling the 47S and 46S RNA and is insufficient to label the 45S RNA, 0.05 μg/ml of actinomycin D was added and the culture was incubated for 90 min more. As seen in Fig. 4, this dose of actinomycin D completely suppresses any new synthesis of 47S, 46S, and 45S RNA, while the previously incorporated label is "chased" into 28S and 18S RNA.

In order to verify that the existence of 47S and 46S RNA is not a peculiarity of the LY cell line, the "45S fraction" from HeLa cells was analyzed under the same conditions (Fig. 5). The use of two different labeling times (5 min and 60 min) revealed the existence of two peaks. The peak corresponding to the 5-min labeling migrates more slowly than the peak corresponding to the 60-min labeling.

**DISCUSSION**

These results lead to the conclusion that the 45S RNA, which was until now considered to be a homogeneous fraction, consists of at least three distinct ribosomal species, in L 5178Y cells, that can be designated as 47S, 46S, and 45S RNA. This fraction consists of at least two species in HeLa cells, where it appears to be more difficult to demonstrate heterogeneity. This difficulty, and the general use of more concentrated acrylamide gels than we have employed, probably explains why the heterogeneity of the "45S fraction" has been masked until now.

The existence of the 47S and 46S RNA, which are labeled more rapidly than the 45S RNA, demonstrates that the 45S species is not a primary product of gene transcription, but is already a result of ribosomal RNA maturation. Moreover, the existence of several molecular species that are the precursors of the 28S and 18S RNA in the "45S fraction" raises the question of the relationship between these different molecular species. Two possibilities must be considered:

(a) Perhaps these RNAs might be linked by a relationship in which the primary product of transcription of the ribosomal genes is a 47S RNA, which acts as a precursor to 46S RNA, which is then converted to 45S RNA. The 45S RNA would then give rise to both 18S and 28S RNA. This hypothesis assumes that 47S and 46S RNA are larger than 45S RNA, or that the differences in electrophoretic mobility are explained by differences in secondary structures.

(b) Another possibility is that 47S and 46S RNA are both primary products of transcription of ribosomal RNA genes, one leading to 18S and the other to 28S RNA.

Until now, only the first of these hypotheses was seriously considered. This was due to the apparent homogeneity of the 45S peak. The aforementioned data that do not fit well with the common-precursor hypothesis, and the present discovery of the 47S and 46S RNA, compel one to consider both hypotheses equally while awaiting further experimentation.

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