*NASCENT RIBOSOMES FROM HELA CELLS**

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In recent years several stages in the synthesis of mammalian ribosomes have been resolved. (*a*) The 45*S* precursor RNA is synthesized in the nucleolus as a single polynucleotide chain.[1] (*b*) The 45*S* RNA is cleaved in a number of steps to 32*S* RNA and 18*S* RNA.[1-3] (*c*) While still in the nucleolus, the 32*S* RNA is further cleaved to 28*S* RNA,[3] to which is bonded noncovalently a small, 7*S*, piece of RNA.[4] 7*S* RNA may be released by heat, dimethyl sulfoxide, or urea. (*d*) The mature 28*S* and 18*S* RNA's can be detected transiently in ribonucleoprotein particles in the nucleus.[5] (*e*) The individual subribosomal particles pass quickly to the cytoplasm where they become incorporated into polyribosomes and subsequently equilibrate with single ribosomes.[6, 7]

A major point of interest is the process by which ribosomal protein is assembled with ribosomal RNA, which probably occurs in the nucleolus. Since available techniques for nuclear fractionation have not yet permitted a direct approach to this problem, we have attempted to isolate from the nucleolus ribonucleoprotein particles in various stages of development toward cytoplasmic ribosomes. Two types of such particles have been identified, which we have termed "nascent ribosomal particles," for reasons which will become evident. These particles may be similar to those described by Tamaoki and Mueller,[8] and by Yoshikawa-Fukada.[9]

Materials and Methods.—Cells: HeLa S3 cells were grown in spinner culture in Eagle's[10] minimal essential medium (MEM) supplemented with 7% horse serum.

Solutions: RSB: 0.01 *M* NaCl; 0.0015 *M* $MgCl_2$; 0.01 *M* Tris, pH 7.4; HSB: 0.5 *M* NaCl; 0.05 *M* $MgCl_2$; 0.01 *M* Tris, pH 7.4; NEB: 0.01 *M* NaCl; 0.01 *M* EDTA; 0.01 *M* Tris, pH 7.4; NETS: 0.1 *M* NaCl; 0.001 *M* EDTA; 0.01 *M* Tris, pH 7.4; 0.1% SDS.

Radioactive labeling of cells: Cells were labeled with C^{14}- and H^3-uridine as previously described.[6] For labeling with C^{14}-leucine, a culture was centrifuged at 1500 rpm for 2 min, and the resulting cell pellet was resuspended in $^1/_5$ to $^1/_{10}$ the original volume of a modified Eagle's MEM, containing 7% whole serum but no added leucine. C^{14}-leucine was added for a given length of time, followed by dilution of the culture to the original volume in Eagle's complete MEM with serum plus ten times the normal concentration of leucine.

Cell fractionation: Nucleoli were prepared by a modification of the method of Penman.[2] Cells from a 100-ml culture were harvested by centrifugation at 1500 rpm for two min, followed by two washes with cold Earle's[11] solution. All subsequent steps were carried out at 4°. The cells were resuspended in 1.5 ml RSB and were subsequently ruptured with a Dounce homogenizer. The nuclei were collected by centrifugation for 5 min at 1500 rpm and were washed with another 1.5 ml RSB. Ribosomes were prepared from the pooled supernatants by Mg^{++} precipitation as previously described.[12] The nuclei were suspended in 2.0 ml RSB to which was added 0.3 ml of a solution containing 6.7% Tween 40 and 3.3% sodium deoxycholate. The suspension was briefly mixed on a Vortex mixer and centrifuged at 1500 rpm for 5 min. The gelatinous pellet of nuclei was resuspended in 2.5 ml of cold HSB containing 100 μg DNase (Worthington, electrophoretically). The viscosity of this suspension was reduced markedly after vigorous pipetting for several minutes. The crude nucleoli were collected by centrifugation in the cold for 10 min at 10,000 rpm. The nucleolar pellet was resuspended in 1.5 ml of NEB containing 0.01 *M* dithiothreitol and gently stirred for 15 min at room temperature. After centrifugation for 10 min at 15,000 rpm the supernatant, containing the nascent ribosomal particles, was analyzed on a 15–30% w/w sucrose gradient in NEB solution.

Electrophoretic analysis of ribosomal proteins,[12] and low[13] and high[3, 14, 15] molecular weight RNA was carried out as previously described. Radioactivity in gel fractions was counted as described by Maizel.[16]

Radioactive material: C^{14}- and H^3-leucine and C^{14}-uridine were obtained from New England Nuclear Corp., and H^3-uridine from Nuclear-Chicago Corp.

Results.—Isolation of nascent particles: In Figure 1 are shown sucrose gradient analyses of nucleolar extracts, prepared as described in *Materials and Methods*. The extract in Figure 1*A* was from cells labeled for 20 minutes with C^{14}-leucine, while that in Figure 1*B* was from cells labeled for 20 hours with C^{14}-uridine. It is clear that the extracts contain two distinct classes of ribonucleoprotein particles, which can be well resolved from other protein or nucleic acid components of the nucleolus.

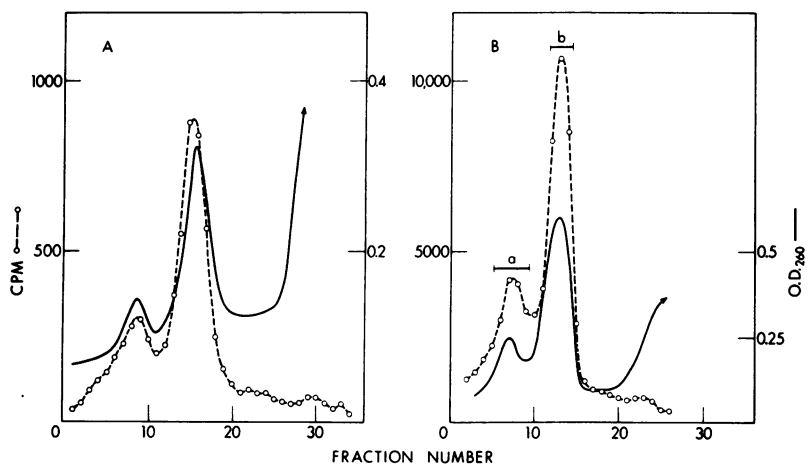


FIG. 1.—Sucrose gradient analyses of nucleolar extracts. (*A*) A culture containing 10^8 cells was labeled for 20 min with 5 μc C^{14}-leucine and "chased" as described in *Materials and Methods* for 5 min before harvesting. (*B*) A culture containing 4×10 cells was labeled for 20 hr with C^{14}-uridine (10^{-5} *M*) and then harvested. In each case, nucleolar extract was prepared as described in *Materials and Methods* and analyzed in a 28-ml 15–30% w/w sucrose gradient in NEB solution. Centrifugation was for 17 hr at 22,000 rpm at 5°.

In order to determine their approximate size, a preparation of nascent particles, labeled with C^{14}-leucine, was mixed with a preparation of cytoplasmic ribosomal subunits labeled with H^3-leucine, and the mixture analyzed on a sucrose gradient. The smaller nascent particle sediments approximately 10 per cent faster than the 50*S* cytoplasmic particle (Fig. 2). From the data in Figure 1 and 2 we assign values of 55*S* and 80*S* to the smaller and larger nascent particles, respectively.

RNA content of nascent particles: In order to characterize the RNA contained in the nascent ribosomal particles, the regions marked *a* and *b* in Figure 1*B* were treated with 1% SDS, and the RNA precipitated by the addition of two volumes of ethanol, redissolved in a buffer containing EDTA and SDS (NETS), and analyzed on sucrose gradients in NETS. As shown in Figure 3*A* and *B*, the 80*S* particle contains 45*S* RNA as well as some 32*S* RNA; the 55*S* particle appears to contain exclusively 32*S* RNA. As it is difficult to distinguish 32*S* RNA from 28*S* RNA on a sucrose gradient, a similar sample prepared from the 55*S* particle was analyzed on a dilute

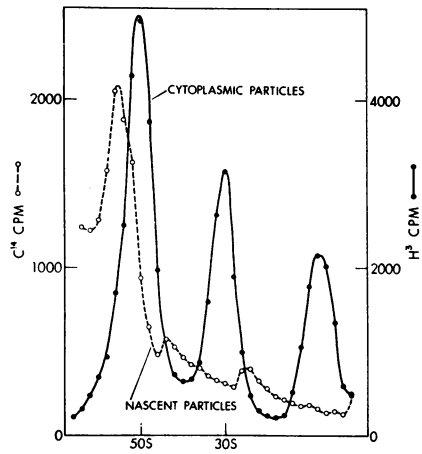


FIG. 2.—A comparison of sedimentation values of nascent and cytoplasmic particles. A nucleolar extract was prepared from cells labeled with C^{14}-uridine for 75 min. Cytoplasmic ribosomes were prepared from cells labeled with H^3-uridine for 20 hr. The two preparations were mixed and analyzed in a 28-ml 15–30% w/w sucrose gradient in NEB solution. Centrifugation: 17 hr at 25,000 rpm at 5°. The 80*S* particles have reached the bottom of the tube.

polyacrylamide gel according to the method of Weinberg *et al.*,[3] as modified by Bellamy *et al.*[15] The main peak of RNA was found to migrate in coincidence with marker 32*S* RNA, and less than 5 per cent was 28*S* RNA (Fig. 4). The 80*S* and 55*S* particles contained about 80 per cent of the nucleolar 32*S* RNA and 50 per cent of the nucleolar 45*S* RNA.

H^3-uridine-labeled nascent particles taken from regions similar to *a* and *b* in Figure 1*B* were examined for the presence of low-molecular-weight RNA species. The RNA extracted as above was mixed with C^{14}-uridine-labeled RNA isolated from cytoplasmic polysomes, and the mixture heated for five minutes at 50° in order to release 7*S* RNA.[4] The gel analysis (Fig. 5) shows that the 55*S* nascent particles contain 5*S* RNA, but no 4*S* or 7*S* RNA. The nascent particles are thus related to the mature cytoplasmic 50*S* particles, which contain a single 5*S* molecule.[13] Furthermore, the absence of the 7*S* RNA demonstrates that the nascent particles are distinct from the recently described particles[5] in the nuclear supernatant fraction which do contain 7*S* RNA.[4]

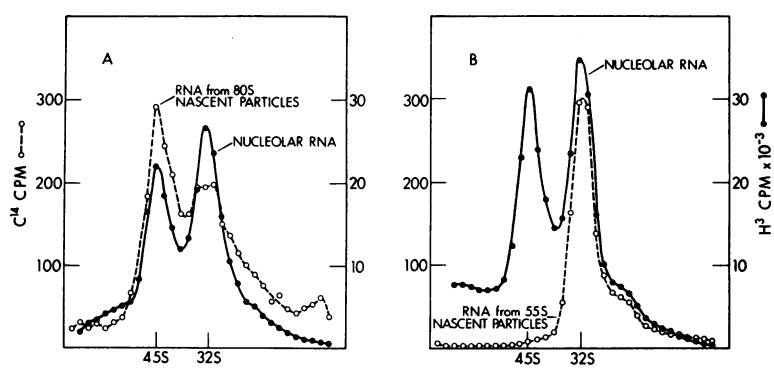


FIG. 3.—Analysis of RNA of nascent particles. Fractions from regions *a* and *b* of Fig. 1*B* were pooled and treated with 1% SDS to release the RNA. The RNA was precipitated with ethanol and redissolved in NETS. A sample of each was mixed with nucleolar RNA labeled with C^{14}-uridine and the mixture analyzed in a 28-ml 15–30% w/w sucrose gradient in NETS solution. Centrifugation: 16 hr at 19,000 rpm at 25°C.

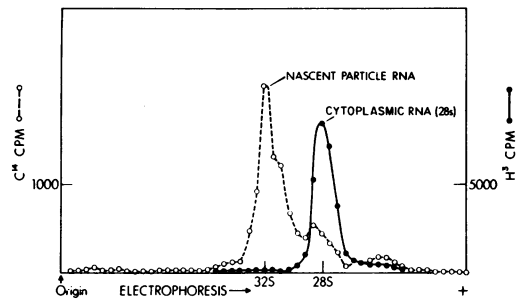
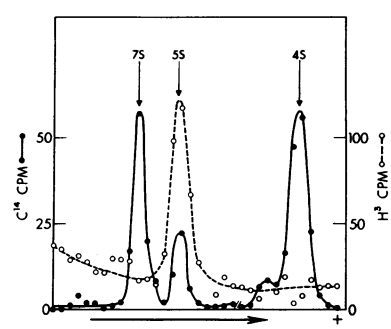


FIG. 4.—Analysis of high-molecular-weight RNA from the 55*S* particle by gel electrophoresis. A sample of RNA region *b* (Fig. 1*B*) was mixed with C^{14}-uridine labeled cytoplasmic 28S RNA and analyzed by polyacrylamide gel electrophoresis. Conditions of electrophoresis: gel concentration 2.4%, 11 ma/gel, 16 hr at room temperature.

Protein content of nascent particles: Girard and Baltimore[17] have found that purified ribosomal RNA can nonspecifically adsorb enough proteins to sediment as if it were part of an intact ribosomal particle. In any study of putative ribosomal particles it is therefore important to show that the protein component is true ribosomal protein, and specific for the RNA in question. We have examined the proteins of the 55*S* particle on polyacrylamide gels and compared them with those of 50*S* cytoplasmic particles[12] (see Fig. 6). The similarity between the two patterns is striking. Most of the proteins associated with 28*S* RNA in the 50*S* cytoplasmic ribosome are also associated with 32*S* RNA in the 55*S* nascent particle. An exception indicated by the arrow in Figure 6 corresponds to one of the proteins which does not take part in the *de novo* construction of the ribosome, but becomes associated with the 50*S* particle in the cytoplasm.[12] This protein is absent from the nascent particle (see Fig. 6 inset).

In HeLa cells, ribosomal protein labeled during a short pulse continues to appear as part of new cytoplasmic ribosomes for two to three hours.[12] Some of this delay is probably due to the presence of pools of precursor ribonucleoprotein particles. If the nascent 55*S* particles are in fact precursors to cytoplasmic ribosomes, then a pulse-chase experiment should show a flow of radioactivity out of the nascent particles and into cytoplasmic particles. Accordingly 65 μc of C^{14}-leucine were added to 5×10^8 cells, followed 20 minutes later by the addition of a large excess of C^{12}-

FIG. 5.—Analysis of low-molecular-weight RNA from the 55*S* particle. RNA was prepared from 55*S* nascent particles from cells labeled with H^3-uridine for 150 min. This was mixed with RNA prepared from 50*S* cytoplasmic particles and the 4*S* RNA from cells labeled with C^{14}-uridine for 150 min. Conditions of electrophoresis: gel concentration 10%, 6 ma/gel, 16 hr at room temperature. The break in the C^{14} tracing indicates an increase of scale by a factor of ten.



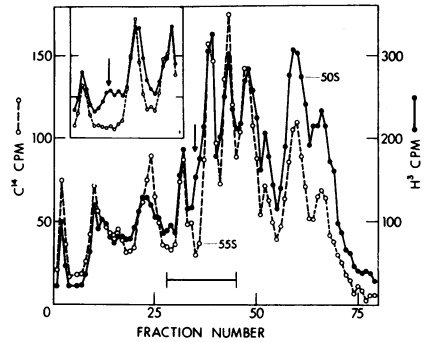


FIG. 6.—Proteins from 55*S* nascent particle. The 55*S* nascent particles were prepared from cells labeled for 50 min with C^{14}-leucine and chased for 10 min before harvesting. The 50*S* ribosomal particles were prepared from cells labeled for one generation with H^3-leucine. In each case, the appropriate tubes from the sucrose gradient were pooled and brought to a final concentration of 5% TCA. The precipitate was collected by centrifugation at 20,000 × *g* for 15 min. After thorough draining it was redissolved in a small volume of 0.01 *M* Na phosphate, pH 7.2, 0.5 *M* urea, 0.5% SDS. Appropriate volumes of the C^{14} and H^3 preparations were mixed and subjected to electrophoretic analysis as previously described.[12] Conditions of electrophoresis: gel concentration 10%, 7 ma/gel, 16 hr at room temperature. The inset shows a portion (corresponding to the mark on the bottom of the main portion of the figure) of a similar analysis in which the electrophoresis was carried out for 24 hr at 7 ma/gel.

leucine. At various times subsequently, a sample of the culture was harvested and both nascent and cytoplasmic particles were prepared from samples of the culture. These were analyzed separately on sucrose gradients and the specific activities compared. As seen in Figure 7, there is a dramatic fall in the specific activity of the nascent particles during the first two hours after the chase, while the specific activity of the cytoplasmic particles rises. After a chase for one generation the specific activity of the nascent particles is in fact less than that of the cytoplasmic particles.

Discussion.—Analyses of the RNA and protein components of the 55*S* nascent particle suggest that it is a precursor to the cytoplasmic 50*S* particle:

(*a*) The major piece of RNA in the 50*S* particle is 28*S*, while that in the nascent particle is 32*S*. Several lines of evidence, such as kinetics of labeling,[1] base composition,[1, 18] and pattern of methylation[19] indicate that 32*S* RNA is a direct precursor to 28*S* RNA. (*b*) Both nascent and cytoplasmic particles contain 5*S* RNA. (*c*) Both particles appear to contain nearly the same complement of proteins. (*d*) After a pulse-chase, radioactive ribosomal proteins decrease in nascent particles and increase in cytoplasmic particles in a manner consistent with a precursor-product relationship. Presumably, this is a two-step process in which the 32*S* RNA of the 55*S* particles is cleaved to form 28*S* RNA, and the resulting 50*S* particles pass through the nucleus into the cytoplasm. Since 45*S* RNA is a precursor to 32*S* RNA, it follows that the 80*S* nascent particle (which contains

45S RNA) is a precursor to the 55S nascent particle. Furthermore, 45S RNA also gives rise to 18S RNA, which is contained in the 30S cytoplasmic particle. One would therefore predict that the 80S, but not the 55S, nascent particle may contain at least some of the proteins specific for the 30S cytoplasmic particle. This possibility is being examined.

The fact that the specific activity of the nascent particles continues to rise for a short time after a C^{14}-leucine pulse-chase (Fig. 7) suggests that there is a pool of labeled ribosomal protein which continues to be utilized in the formation of nascent particles. Preliminary examination of the nucleolar residue after extraction of nascent particles indicates that this is the case.

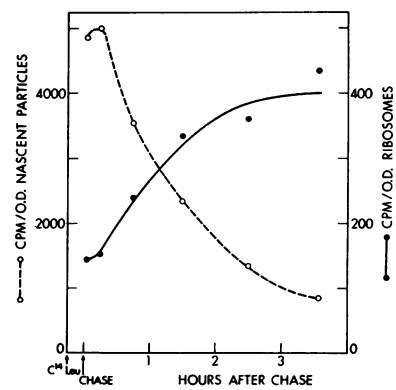


FIG. 7.—Chase of labeled protein from nascent particles. The specific activities of nascent 55S particles and cycloplasmic 50S particles after a pulse-chase of C^{14}-leucine. See text for details. Note difference in scale.

Although the preparation of nucleoli from which the nascent particles are obtained is relatively crude, a variety of both genetic and biochemical experiments demonstrate the central role of the nucleolus in ribosome biosynthesis.[2, 20–23] Furthermore, electron micrographs often show many particles on the periphery of the nucleolus, which may be the nascent particles here described.[24]

Summary.—We have isolated from the nucleoli of HeLa cells ribonucleoprotein particles which contain 45S and 32S ribosomal RNA as well as specific ribosomal proteins. These particles, termed "nascent" ribosomal particles, are precursors to the cytoplasmic ribosomes.

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Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylene diamine tetraacetate.

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[1] Scherrer, K., H. Latham, and J. E. Darnell, these PROCEEDINGS, **49**, 240 (1963).

[2] Penman, S., *J. Mol. Biol.*, **17**, 117 (1966).

[3] Weinberg, R. A., U. Loening, M. Willems, and S. Penman, these PROCEEDINGS, **58**, 1088 (1967).

[4] Pène, J., E. Knight, and J. E. Darnell, submitted for publication.

[5] Vaughan, M., J. Warner, and J. E. Darnell, *J. Mol. Biol.*, **25**, 235 (1967).

[6] Girard, M., H. Latham, S. Penman, and J. E. Darnell, *J. Mol. Biol.*, **11**, 187 (1965).

[7] Perry, R. P., and D. E. Kelley, *J. Mol. Biol.*, **16**, 255 (1966).

[8] Tamaoki, T., and G. C. Mueller, *Biochim. Biophys. Acta*, **108**, 81 (1965).
[9] Yoshikawa-Fukada, M., *Biochim. Biophys. Acta*, in press.
[10] Eagle, H., *Science*, **130**, 432 (1959).
[11] Earle, W. R., *J. Natl. Cancer Inst.*, **4**, 165 (1959).
[12] Warner, J. R., *J. Mol. Biol.*, **19**, 353 (1966).
[13] Knight, E., and J. E. Darnell, *J. Mol. Biol.*, in press.
[14] Loening, U., *Biochem. J.*, **102**, 251 (1967).
[15] Bellamy, A. R., L. Shapiro, J. T. August, W. K. Joklik, *J. Mol. Biol.*, in press.
[16] Maizel, J., *Science*, **151**, 988 (1966).
[17] Girard, M., and D. Baltimore, these PROCEEDINGS, **56**, 999 (1966).
[18] Soeiro, R., C. Birnboim, and J. E. Darnell, *J. Mol. Biol.*, **19**, 362 (1966).
[19] Wagner, E., S. Penman, and V. Ingram, *J. Mol. Biol.*, in press.
[20] Brown, D. D., and J. B. Gurdon, these PROCEEDINGS, **51**, 139 (1964).
[21] Ritossa, F. M., and S. Spiegelman, these PROCEEDINGS, **53**, 737 (1965).
[22] Wallace, H., and M. Birnstiel, *Biochim. Biophys. Acta*, **114**, 296 (1966).
[23] Perry, R., *Natl. Cancer Inst. Monogr.*, **18**, 325 (1965).
[24] Birnstiel, M. L., M. I. H. Chipchase, and B. B. Hyde, *Biochim. Biophys. Acta*, **76**, 454 (1963).