COMPLETION OF NASCENT HELA RIBOSOMAL PROTEINS IN A CELL-FREE SYSTEM

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Received May 14, 1970

SUMMARY. HeLa cell ^{3}H-labeled proteins completed *in vitro* and ^{14}C-labeled 60S subunit ribosomal proteins isolated from cells labeled *in vivo* were processed together and applied to a carboxymethyl-cellulose column. A linear gradient of NaCl was used to elute the adsorbed proteins. Prominent peaks of both isotopes eluted together at 0.1M and from 0.175M to 0.25M NaCl. Polyacrylamide gel electrophoresis of the doubly-labeled proteins confirmed the identity of the ribosomal proteins completed *in vitro* with the ribosomal proteins labeled *in vivo*.

We wish to determine the intracellular site of ribosomal protein (rP) synthesis. Although it is well known that ribosomal RNA (rRNA) is synthesized in the nucleolus (1), and that rP's join rRNA precursor molecules before the RNA's leave the nucleolus (2,3), the actual site of rP synthesis is unknown. The extent and even the existence of protein synthesis within the nuclei of most types of cells is still controversial (4); but it is virtually certain that at least some nuclear proteins - the histones - are synthesized on cytoplasmic polysomes in HeLa cells (5,6). Gallwitz and Mueller (6,7) have reported a cell-free system that permits the completion of nascent histone polypeptides. We have adapted their system to our needs. In this communication we report preliminary evidence for the cell-free completion of nascent HeLa rP's.

METHODS. HeLa cells were grown in suspension culture in Eagle's Minimum Essential Medium (8) supplemented with 5% calf serum.

To prepare marker rP's, a cell culture was diluted 1:1 with amino acid-free medium, to a final concentration of 2×10^5 cells/ml and a mixture of 15

^{14}C-amino acids (uniformly labeled, New England Nuclear) was added to 0.75 μCi/ml final concentration. After 24 hours the cells were harvested; ribosomes and ribosomal subunits were prepared by the procedures of McConkey, Linsky, Malville and Eldridge (9) and Linsky and McConkey (10).

The cells were swollen in buffer (A)[1], and broken in an Omni-Mixer (Sorvall). KCl was added to a final concentration of 0.3M and the nuclei and mitochondria were pelleted at 15,000 g for 15 minutes. The supernatant from this low-speed spin was layered over buffer (B)[1] and the ribosomes were pelleted by overnight centrifugation. These were resuspended in buffer (C)[1] and clumps and dirt were removed by centrifugation at 10,000 g for 10 minutes. Ribosomes were then incubated at 37° for 30 minutes in buffer (C) with puromycin added to a final concentration of 5×10^{-4} M. Next, $MgCl_2$ and KCl were added to the concentration in buffer (D)[1] and the subunits were separated on isokinetic gradients of 10 to 30% sucrose in buffer (D).

To complete nascent rP's *in vitro*, the procedures of Gallwitz and Mueller (6,7) were closely followed. However, an Omni-Mixer was used to break the cells in the incorporation buffer made with 0.001M Dithiothreitol (DTT). Up to 2.2 mg of microsomal protein per 0.5 ml and 0.6 to 0.8 mg pH 5.0 fraction per 0.5 ml were used (measured by a modification of Lowry, 11). Incubation was for 20 minutes at 37°. ^{3}H-amino acids (randomly labeled mixture, New England Nuclear) at 150 μCi per 0.5 ml were used as label. Charged tRNA was hydrolyzed with 1.0M NaOH for 15 minutes at room temperature before samples were counted for radioactivity.

The mixture of ^{3}H-labeled proteins completed *in vitro* and the ^{14}C-labeled ribosomal subunits was precipitated in 10% trichloroacetic acid for 30 minutes. The pellet was washed twice with ether, incubated for 60 minutes at 37° in

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[1] (A) 0.01M Tris-HCl pH 7.4, 0.001M $MgCl_2$, 0.01M KCl and 0.001M Dithiothreitol (DTT).
(B) 1.75M sucrose in 0.05M Tris-HCl pH 7.4, 0.001M $MgCl_2$, 0.08M KCl and 0.001M DTT.
(C) 0.05M Tris-HCl pH 7.4, 0.001M $MgCl_2$, 0.025M KCl and 0.001M DTT.
(D) 0.05M Tris-HCl pH 7.4, 0.01M $MgCl_2$, 1.0M KCl and 0.001M DTT.

ethanol-ether (1:1) and this pellet was again washed twice with ether. The pellet was resuspended and left in 6.0M guanidinium chloride (GuCl), pH 5.6, for several hours at 0°. The insoluble material was removed at 15,000 g for 15 minutes. The supernatant was layered over 4.75M CsCl and spun at 40,000 rpm for 60 hours to separate the nucleic acids from the protein. The fractions with $OD_{280}/OD_{260} > 1.2$ were pooled, and dialyzed against the column buffer (6.0M urea, charcoal filtered (12), 0.01M sodium acetate buffer, 0.1% methylamine (12) and 0.1% 2-mercaptoethanol at pH 5.6) for 24 hours.

The mixture of labeled proteins was fractionated on a 0.9 x 13 cm column of carboxymethyl-cellulose (CM-cellulose; CM 52, preswollen, Whatman). The adsorbed proteins were eluted with a 1 liter linear gradient of 0 to 0.35M NaCl in column buffer. Fractions were collected every 10 minutes; the flow was kept constant at 12 ml/hr with a peristaltic pump.

For polyacrylamide gel electrophoresis, peak fractions from the column were pooled, 200 µg of bovine serum albumin (BSA) was added to each and they were precipitated with 10% trichloroacetic acid for 30 minutes. The precipitate was collected on a 0.45 µ pore size Millipore filter. The filter was cut into small pieces and the protein was eluted with 0.25 ml of 8.0M urea with 0.05M sodium acetate buffer, pH 5.0. Each eluted fraction was run on a polyacrylamide gel at pH 4.5 (13,14) or pH 8.7 (15) toward the cathode. Details appear in the legend of Figure 2.

The gels were crushed with a Savant gel crusher at 2 mm per sample with 1.0 ml of distilled water as diluent. Thirty percent H_2O_2 was added to the samples to a final concentration of 3%; samples were incubated overnight at 37° in tightly capped vials. The fractions were counted in EGME-butyl PBD[2] in a Beckman LS-133 scintillation counter.

RESULTS AND DISCUSSION. To avoid uncertainties about the release of completed polypeptide chains from polysomes, we developed a method for preparing the total proteins of the incorporation mixture for CM-cellulose

[2] 2 ethoxyethanol: 2-(4-tert-Butylphenyl)-5-(4-Biphenylyl-1,3,4-Oxadiazole (8 gm/liter in toluene) [1:1]

chromatography. Initially, we tried the acetic acid extraction of Hardy, Kurland, Voynow and Mora (12) which works well for HeLa rP's (9). We were never able to recover more than 25% of the radioactivity from CM-cellulose columns, however. The optical density 280/260 ratio of these acetic acid-extracted proteins indicated the presence of nucleic acid contaminants ($\geq$ 3.5% by weight), but neither RNase nor DNase removed the contaminants. In addition, removal of lipids with ethanol-ether and careful efforts to prevent disulfide bridge formation failed to improve recovery from the columns.

Hallberg and Brown (16), working with Xenopus laevis eggs, embryos and cultured kidney cells, developed a method for the identification of rP's in the presence of total cell proteins on CM-cellulose columns. A crucial step

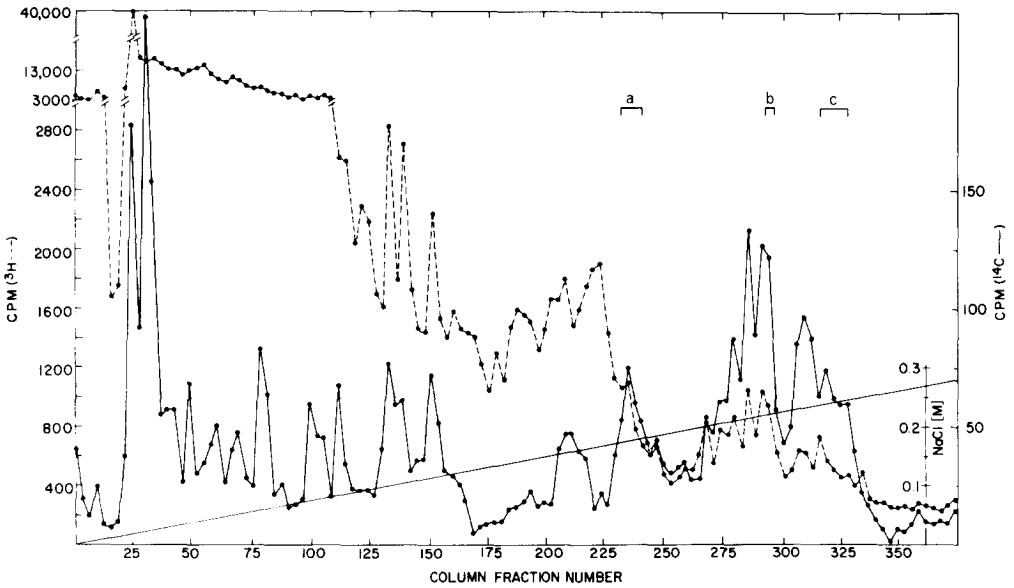

Figure 1. CM-cellulose column chromatography of HeLa cell ^{14}C-labeled rP's and ^{3}H-labeled proteins completed in vitro. Each point on the graph represents cpm of ¼ of every third fraction. The ratio of $^{3}H/^{14}C$ put on the column was 45. Recovery was 75% for the ^{3}H and 50% for the ^{14}C.

in their method was solubilization of phosphorus-containing contaminants by overnight incubation in 10% trichloroacetic acid at 37°. We were unwilling to employ their technique because of the known lability of some peptide bonds, e.g. those involving aspartyl residues (17).

Therefore, we turned to the use of buoyant density separation. After the lipids were removed, the proteins of the incorporation mixture and the 60S subunit were dispersed in GuCl, layered over CsCl as explained in Methods and centrifuged so that the nucleic acids would enter the CsCl. Three or four fractions from the upper 35% of each gradient were pooled; these contained at least 80% of the protein and less than 1.25% nucleic acid contamination.

Figure 1 shows an elution profile of proteins completed in vitro (^{3}H) and rP's from the 60S subunit (^{14}C). All of the rP's were eluted between 0.01M and 0.25M NaCl. The best correspondence of the ^{3}H and ^{14}C labels occurred at 0.1M and from 0.175M to 0.25M NaCl.

To determine whether rP's were actually represented among the ^{3}H-labeled proteins, we pooled and concentrated nine of the peaks with the lowest $^{3}H/^{14}C$ ratio and subjected those proteins to acrylamide gel electrophoresis. Figure 2 presents three of the gel patterns, which provide additional evidence that some of the ^{3}H-labeled proteins and the ^{14}C rP's are identical. In Table 1 we demonstrate that the correspondence of ^{3}H and ^{14}C labels in the gel patterns is not due to a trivial fraction of the ^{3}H in the sample. Apparently, some of the CM-cellulose column fractions contain rP's almost exclusively.

The validity of the ^{14}C marker proteins is attested to by the fact that subunits prepared as described above are active in the poly U-dependent synthesis of polyphenylalanine (10). The use of 1M KCl in the preparation of these subunits minimizes the possibility that non-ribosomal proteins were adsorbed to the ribosomes.

We note that the elution profile of the 60S rP's in Figure 1 does not closely resemble the patterns published by Hallberg and Brown (16) for X. laevis rP's or the patterns published by Kanai et al. (18) for rat liver and

TABLE 1

Gel	Input ^{3}H cpm/^{14}C cpm; ratio	Recovery ^{3}H cpm/^{14}C cpm; ratio
A	6860/1030 ; 6.66	4867/939 ; 5.2
B	1920/570 ; 3.37	1380/396 ; 3.48
C	5060/1680 ; 3.01	2689/1258 ; 2.14

Recovery of ^{3}H and ^{14}C from acrylamide gel electrophoresis of selected CM-cellulose column fractions. For the input determinations, a 20 µl sample of each column fraction placed on each gel was mixed with 2 mm of blank gel and 1 ml of diluent and incubated in the same manner as the gel fractions.

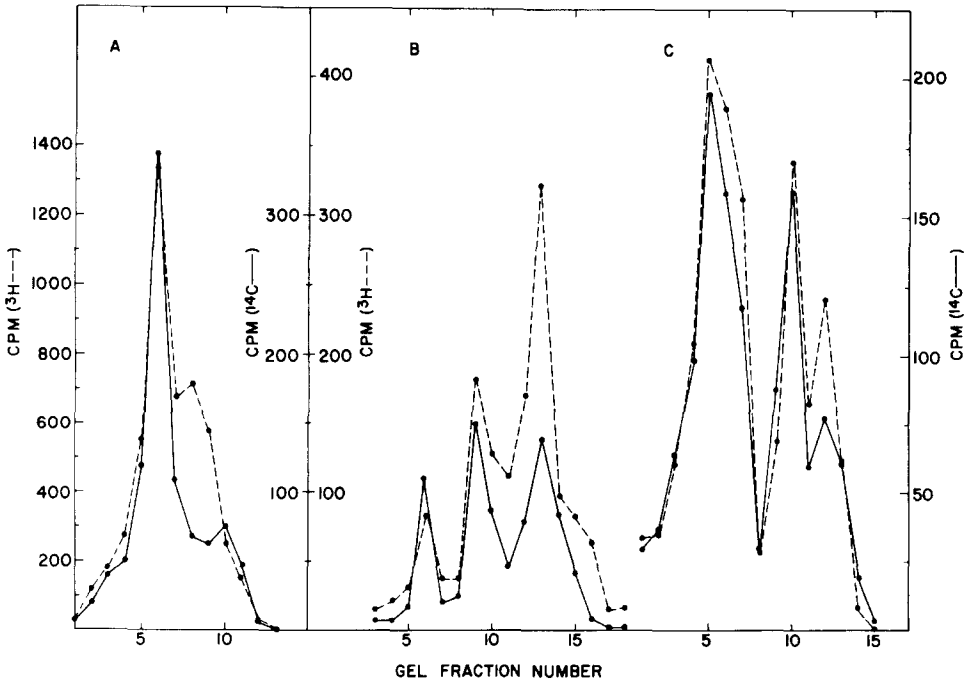

Figure 2. Gel electrophoresis of regions (a), (b) and (c) of Figure 1. (a) and (c) were run at pH 8.7 toward the cathode for 6½ hours at 3.0 ma/gel. (b) was run at pH 4.5 toward the cathode for 3 hours at 2.5 ma/gel. All of the fractions not appearing on the graphs were at background level for both isotopes.

skeletal muscle rP's. This could be due to differences in technique.

Finally, our results can be interpreted as tentative evidence for the cytoplasmic localization of rP synthesis. This matter is now being investigated more thoroughly.

ACKNOWLEDGEMENTS

This research was primarily supported by NSF GB-8009, and in part by NIH HD-02282. J.E.H. is the recipient of an NSF predoctoral traineeship.

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