

## DISTRIBUTION OF NEWLY FORMED RIBOSOMAL PROTEINS IN HELA CELL FRACTIONS

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### ABSTRACT

The distribution of newly formed ribosomal proteins between cytoplasmic, nucleoplasmic, and nucleolar fractions of HeLa cells was determined. All but a few of the newly formed ribosomal proteins were concentrated 10- to 50-fold in the nucleolus and two- to fivefold in the nucleoplasm. Nevertheless, substantial amounts were found in the cytoplasm. Pretreatment of cells with actinomycin D to deplete the nucleolar pool of ribosomal precursor RNA had no effect on the concentration of newly formed ribosomal proteins in the nucleus, but did lead to an increased amount in the nucleoplasm at the expense of the nucleolus.

**KEY WORDS** ribosomal protein · nucleus · nucleoplasm · nucleolus · actinomycin D · cell fractionation · HeLa cells

The geography of a eukaryotic cell poses severe problems of compartmentation. Protein synthesis occurs in the cytoplasm, yet the nucleus has a distinct set of proteins. How does such segregation occur? A number of years ago, Goldstein and Prescott (7), by transplanting nuclei between amoebae, demonstrated that many proteins, although highly concentrated in the nucleus, were in equilibrium with the cytoplasm and could pass from one nucleus to another through the cytoplasm. More recently, it has been shown that homologous nuclear proteins injected into the cytoplasm of *Xenopus* oocytes (3) or hepatocytes (20) are highly concentrated in the nucleus. Furthermore, when nuclei are manually disrupted, most nuclear proteins remain in the nuclear remnant, suggesting that the nuclear membrane does not play a major role in sequestering the nuclear proteins (6). These results suggest that there is some element of the structure of the nuclear proteins which is responsible for their concentration in the nucleus. However, they do not distinguish between a cytoplasmic machinery responsible for transferring the protein to the nucleus and

the selective binding of newly formed nuclear proteins to intranuclear structures.

Ribosomal proteins, synthesized in the cytoplasm (2), migrate through the nuclear envelope and into the nucleolus (19) where they are assembled with ribosomal precursor RNA into ribonucleoproteins (9, 18) which are ultimately destined to migrate back to the cytoplasm as mature ribosomal subunits.

Using recently developed analytical techniques (8, 17), we have determined quantitatively the distribution of newly formed ribosomal proteins in cell fractions and have asked whether the distribution of newly formed ribosomal proteins is altered in cells depleted of ribosomal precursor RNA by treatment with actinomycin.

### MATERIALS AND METHODS

#### *Growth and Labeling of Cells*

HeLa (S3) cells were grown in spinner culture in Eagle's (4) medium containing 5% fetal calf serum. Pulse and long-term labeling was carried out as previously described (17).

#### *Preparation of Cell Extracts*

Nuclei and nucleoli were prepared by a combination of the techniques of Bombik et al. (1) and Muramatsu and Onishi (11). A culture containing  $5 \times 10^7$  cells was

harvested by adding frozen Earle's solution (5). The cells were collected by centrifugation and washed twice with Earle's solution. They were suspended in 2.5 ml of buffer A (10 mM Tris, pH 8.0; 3 mM CaCl<sub>2</sub>; 2 mM MgCl<sub>2</sub>; 0.5 mM dithiothreitol) (1). After 10 min the cells were disrupted with 10 strokes of a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.), followed by addition of 0.02 vol of 10% (wt/vol) Triton X-100 and brief vortexing. The extract was centrifuged for 5 min at 1,000 rpm. The supernate was considered the cytoplasmic fraction. The crude nuclei were suspended in 10 ml of 10 mM Tris-Ac, pH 7.4, 3.3 mM MgCl<sub>2</sub>, 0.25 M sucrose (1), and recentrifuged for 5 min at 1,000 rpm. The supernate was discarded. The nuclei were suspended in 2.5 ml of 10 mM MgCl<sub>2</sub>, 0.25 M sucrose (11) and layered over an equal volume of 0.5 mM MgCl<sub>2</sub>, 0.88 M sucrose. After centrifugation for 10 min at 2,500 rpm, the supernate was discarded and the purified nuclei were suspended in 2.5 ml of 0.05 mM MgCl<sub>2</sub>, 0.35 M sucrose (11). At this point they appear free of cytoplasmic tabs and membranous material. To prepare nucleoli, the solution was subjected to two to four 15-s treatments with a Branson Sonifier, equipped with a microprobe, checking microscopically to determine the extent of nuclear breakage. The sonicate was layered over 2.5 ml of 0.5 mM MgCl<sub>2</sub>, 0.88 M sucrose, and centrifuged for 10 min at 2,500 rpm. The upper two-thirds was considered the "nuclear supernate." The pellet consisted almost entirely of irregularly shaped, highly refractile nucleoli. There was a small amount of chromatin fibers and, occasionally, what appeared to be a resealed nuclear membrane enclosing several nucleoli and little else.

### Preparation of Cell Fractions for Acrylamide Gel Analysis

The two-dimensional acrylamide gel analysis of ribosomal proteins requires a sample nearly free of nucleic acid, particularly RNA. It is possible to recover 90-95% of the protein with negligible nucleic acid contamination by adding to the extract 0.1 vol of 1 M MgCl<sub>2</sub>, 0.1 vol of 0.1 M dithiothreitol, and 2 vol of glacial acetic acid (17). After stirring on ice for 30 min, followed by centrifugation at 12,000 g for 15 min, the supernate is dialyzed against 1% acetic acid, lyophilized, and subjected to analysis on two-dimensional polyacrylamide gels run at pH 5 in the first dimension and in the presence of SDS in the second dimension (8, 17).

## RESULTS

The distribution of protein in the subcellular fractions prepared as described in Materials and Methods is shown in the first line of Table I. Fig. 1 shows an acrylamide gel analysis of the cytoplasmic and nucleolar fractions. This gel system is designed for basic proteins; in the first dimension,

most of the cellular proteins remain near the origin or migrate toward the anode. The ribosomal proteins are clearly evident in the cytoplasmic fraction, and several are numbered in accordance with the scheme presented earlier (17).<sup>1</sup> Many of the ribosomal proteins are also visible in the nucleoli, along with the histones, which are the predominant proteins of the nuclear supernate. The ribosomal proteins in the nucleolus, while clearly present, comprise only a small fraction of the ribosomal proteins in the cell, since the material in Fig. 1*b* represents roughly 35 times as many cells as does that in Fig. 1*a*.

### Distribution of Newly Synthesized Protein

When cells are labeled briefly with [<sup>3</sup>H]leucine, the distribution of newly synthesized protein is similar to that of total protein (Table I). To determine the distribution of individual ribosomal proteins, each fraction was analyzed on a two-dimensional polyacrylamide gel in the presence of whole cell proteins labeled uniformly with [<sup>14</sup>C]leucine. Each spot was cut from the gel and its <sup>3</sup>H/<sup>14</sup>C ratio determined. From this value, the <sup>3</sup>H/<sup>14</sup>C ratio of the sample applied to the gel and the distribution of <sup>3</sup>H among the cell fractions, one can calculate two parameters with respect to newly synthesized proteins: (a) the relative distribution of each protein, i.e., its concentration in each subcellular fraction with respect to the total <sup>3</sup>H in that fraction; (b) the absolute distribution of each protein, i.e., the amount in each subcellular fraction as a percent of the total amount of that protein.

The relative distribution of 21 representative ribosomal proteins, two histones, and five nonribosomal proteins is presented in Table II. It is clear that newly formed ribosomal proteins are highly concentrated in the nucleus. The degree of concentration is not uniform for all the ribosomal proteins, however, varying, with a few exceptions, from 5- to 15-fold. The differences among the ribosomal proteins are reproducible from one experiment to another. Proteins 1, 2, 4, 18, and 43 are consistently among the most highly concentrated in the nuclear fractions, while proteins 21 and 35 are less so. Proteins 6 and 12 are clearly

<sup>1</sup> The numbering system used in this paper is not in accordance with the standard numbering system adopted by McConkey et al. (10), since the latter is based on the Kalthschmidt-Wittman gel system.

TABLE I  
Distribution of Total and Newly Synthesized Protein  
in Cell Fractions

	Cytoplasm	Nuclear super- nate	Nucleoli
		%	
Total protein	81.7	15.9	2.4
Newly synthesized pro- tein	82.8	15.2	2.4
Newly synthesized pro- tein in the presence of actinomycin	84.8	13.8	1.6

For total protein,  $4 \times 10^6$  HeLa cells were fractionated as described in Materials and Methods, and protein was determined. For newly synthesized protein, a culture of  $4 \times 10^7$  cells was divided in two equal parts, one of which was given  $0.1 \mu\text{g/ml}$  of actinomycin D. After 150 min, each culture was centrifuged and suspended in 50 ml of medium lacking leucine, with or without actinomycin D. After 60 min, each culture was given 2.5 mCi of [ $^3\text{H}$ ]leucine. After 20 min more, each culture was given  $250 \mu\text{g/ml}$  of nonradioactive leucine to chase radioactive nascent peptides. After 5 min more, each culture was harvested on frozen Earle's solution, fractionated as described in Materials Methods, and the distribution of radioactivity was determined. A sample of the nuclei was removed before sonication to analyze further (Table II). The values have been corrected for this.

exceptional; they are distributed uniformly throughout the cell. As controls, the distribution of histones and other nonribosomal proteins was examined (Table II). As expected, the histones are highly concentrated in the nucleus. Some nonribosomal proteins, such as A, are slightly concentrated in the nucleus; others, such as J, are substantially concentrated in the cytoplasm.

The relative distribution of newly formed proteins within the nucleus is also shown in Table II. Clearly, newly formed ribosomal proteins are highly concentrated in the nucleolus, roughly tenfold more concentrated than in the nucleoplasm. The concentration in the whole nucleus, determined independently, is approximately the weighted mean of its two fractions.

While the nucleolus contains the highest concentration of ribosomal proteins, it represents only a small fraction of the cell's protein. If one recalculates the data from Table II to determine the absolute distribution of ribosomal proteins among cell fractions (Table III), it becomes apparent that the nucleolus contains only a portion of

the newly formed ribosomal proteins. A substantial amount of the newly formed ribosomal proteins is in the cytoplasm. This material is presumably on its way to the nucleus since previous work (16) showed that little of the newly formed ribosomal proteins is found on mature ribosomes until 30 min after its synthesis. A significant amount of the newly formed ribosomal proteins is found in the nucleoplasm. It is not unlikely that some of this material was extracted from the nucleolus during sonication and subsequent centrifugation. However, a similar distribution was obtained when the nucleoli were collected after treatment

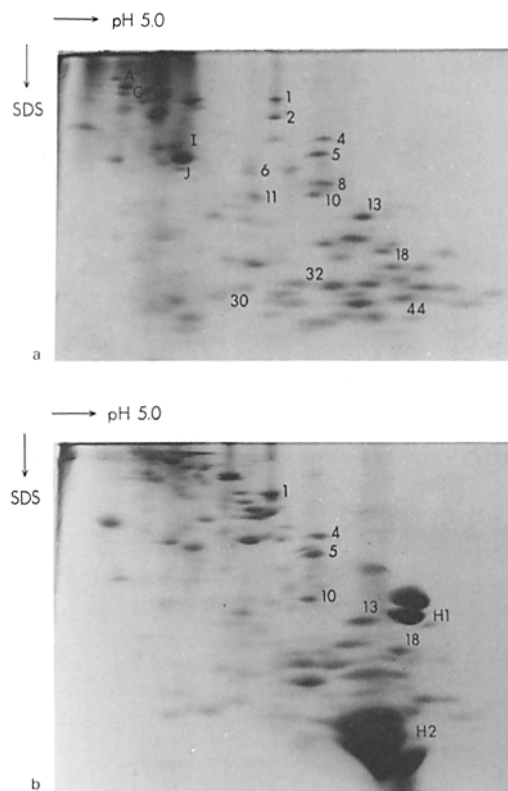


FIGURE 1 Two-dimensional polyacrylamide gel analysis of cell fractions. From the preparation described in the first line of Table I,  $800 \mu\text{g}$  of cytoplasmic protein (a) and  $800 \mu\text{g}$  of nucleolar protein (b) were lyophilized and subjected to two-dimensional polyacrylamide gel electrophoresis (8). The origin is at the upper left. Electrophoresis in the first dimension is from left to right at pH 5 in the presence of 8 M urea. Electrophoresis in the second dimension, 17% acrylamide, is from top to bottom in the presence of SDS. Some of the ribosomal proteins are indicated by numbers (17) (see footnote 1) and nonribosomal proteins by letters.

TABLE II  
Relative Distribution of Newly Formed Proteins

		Control			Actinomycin		
		Nucleus	Nucleoplasm	Nucleolus	Nucleus	Nucleoplasm	Nucleolus
<i>Concn relative to the cytoplasm</i>							
Ribosomal proteins							
	Subunit						
1	L	8.1	1.9	38	9.7	3.6	59
2	L	12	2.7	46	3.6	2.0	11
4	L	15	3.4	39	13	4.3	24
5	L	7.8	3.0	29	7.9	4.3	18
6	S	2.1	0.7	2.0	1.6	0.9	2.3
7	S	7.1	3.6	21	5.6	3.6	6.6
8	S	5.7	2.6	18	19	13	24
10	L	8.3	6.0	36	9.7	4.5	8.1
12	S	0.6	0.4	0.4	1.5	0.9	2.6
13	L	8.2	3.7	27	7.6	6.6	7.6
16	L	6.6	2.8	32	9.7	5.8	10
18	L	12	5.0	71	23	8.3	87
21	L	5.9	1.7	18	7.4	2.9	12
23	L	12	4.4	38	4.4	2.0	5.3
25	S	4.1	2.0	15	3.5	1.6	12
30	L	1.6	1.3	4.6	2.2	1.3	2.7
32	S	2.5	2.0	3.5	3.9	3.6	6.4
35	S	5.1	3.1	16	8.4	5.5	10
37	L	8.7	4.2	29	6.3	1.9	12
41	L	5.3	3.3	20	14	5.7	19
44	L	7.4	3.6	38	8.8	5.1	15
Nonribosomal proteins							
Histones							
	H1	62	238	43	103	98	44
	H2*	110	102	27	70	50	32
Others							
	A	1.7	1.5	2.3	1.8	1.7	1.3
	C	0.4	0.6	0.2	0.4	0.7	0.3
	I	1.3	1.6	0.8	2.1	2.5	1.1
	J	0.6	0.3	0.1	0.2	0.2	0.2

The radioactive samples described in Table I were extracted with acetic acid, mixed with HeLa protein uniformly labeled with [<sup>14</sup>C]leucine, and with nonradioactive ribosomal proteins, subjected to two-dimensional gel electrophoresis, stained, spots were excised, and the <sup>3</sup>H/<sup>14</sup>C ratio was determined (17). The <sup>14</sup>C in this case is used to correct for losses during electrophoresis and sample preparation. The <sup>3</sup>H/<sup>14</sup>C ratio of the nuclear fractions was compared to that of the cytoplasmic fraction, e.g. [<sup>3</sup>H/<sup>14</sup>C]<sub>nucleus</sub>/[<sup>3</sup>H/<sup>14</sup>C]<sub>cytoplasm</sub>. Thus, the values in the Table represent the concentration of the proteins in the given fraction compared to that in the cytoplasm. Proteins not identified in Fig. 1 can be located by referring to reference 17.

\* This gel system does not resolve H2a and H2b.

of the nuclei with DNase at high ionic strength according to the method of Penman et al. (13). The nucleoli isolated by that method contain all the ribosomal precursor RNA and, when dissociated, yield ribonucleoprotein precursors to ribosomes which contain newly formed ribosomal proteins (18). With the present techniques of nuclear disruption, it is not possible to determine

conclusively the distribution of macromolecules within the intact nucleus.

#### *Distribution of Ribosomal Proteins in the Presence of Actinomycin*

In an attempt to determine whether the high concentration of newly formed ribosomal proteins

TABLE III  
Total Distribution of Newly Formed Proteins

	Control			Actinomycin		
	Cytoplasm	Nucleoplasm	Nucleolus	Cytoplasm	Nucleoplasm	Nucleolus
	% of Total					
<b>Ribosomal proteins</b>						
1	41	14	45	38	22	40
2	35	18	47	65	21	13
4	36	23	41	47	33	20
5	42	23	35	49	34	16
6	85	11	5	84	12	4
7	44	29	27	59	34	7
8	50	24	26	28	60	12
10	32	35	33	53	39	8
12	92	7	1	83	13	4
13	40	28	32	45	49	6
16	41	21	38	47	44	9
18	25	23	52	25	34	40
21	54	17	29	59	28	13
23	34	28	38	70	23	7
25	56	20	24	68	18	14
30	73	17	10	70	17	4
32	68	25	7	59	34	7
35	49	28	23	48	43	9
37	38	29	32	65	20	14
41	46	28	27	44	41	15
44	36	24	40	48	40	13
<b>Nonribosomal proteins</b>						
<b>Histones</b>						
H1	2	95	3	6	90	5
H2	5	91	4	10	84	6
<b>Others</b>						
A	75	20	5	76	22	2
C	90	10	1	90	10	0.5
I	76	22	2	70	28	1
J	94	5	0.3	97	3	0.3

The data of Table I and of Table II were combined to determine the distribution of each protein among the three cell fractions.

in the nucleolus was due simply to selective binding to newly formed ribosomal precursor RNA, the experiment described in Tables II and III was carried out on cells that had been treated with actinomycin D to inhibit formation of ribosomal RNA. In such cells, the synthesis of most ribosomal proteins continues unabated, although the unused ribosomal proteins are eventually degraded (17).

The results of such an experiment are shown in Tables I, II, and III, to facilitate a comparison with the control experiment. The yield of total radioactivity in the nucleolar fraction was reduced by 30–50% in preparations from cells treated with

actinomycin. This is not surprising, as the nucleoli become more diffuse and fragile in the presence of the drug.

Tables II and III show that most of the newly formed ribosomal proteins are concentrated in the nucleus to the same extent in the presence of actinomycin as in the control. Actinomycin does have an effect on the distribution of ribosomal proteins within the nucleus, the nucleoplasm gaining substantial amounts of ribosomal proteins at the expense of the nucleolus. Whether this is true within the cell or is an artifact caused by sonicating the fragile nucleoli from cells treated with actinomycin D is not known.

## DISCUSSION

It was clear from previous work (2, 18) that newly formed ribosomal proteins are in a dynamic state, flowing from cytoplasm to nucleolus to cytoplasm, finally "fixed" as part of a mature ribosome. The data in this paper represent a single time point in this flow:  $15 \pm 10$  min after the synthesis of the protein. Nevertheless, it is clear that newly formed ribosomal proteins are rapidly and efficiently concentrated within the nucleus. They are even more efficiently concentrated within the nucleolus, as much as 50-fold. This should be considered a minimal value. There is some evidence that the association of newly formed ribosomal proteins with nucleolar ribonucleoprotein precursors to ribosomes is reversible until the later stages of their maturation (12). If so, some of the ribosomal proteins found in the nucleoplasmic fraction could have passed out or been washed out of the nucleolus. This could explain the finding that nearly all the ribosomal proteins in the nucleolus are present in equimolar amounts (14).

Certain proteins found on cytoplasmic ribosomes, e.g., No. 6 and No. 12, are not concentrated in either the nucleus or the nucleolus, suggesting that they become associated with ribosomal subunits only in the cytoplasm.

The concentration of ribosomal proteins in the nucleus is not dependent on the concurrent synthesis of ribosomal precursor RNA. At most, only a fraction of the newly formed ribosomal proteins could be bound to RNA in the nucleus, since, under the conditions of actinomycin treatment described in Table I, the nucleolus has <15% as much ribosomal precursor RNA as the control, all in the form of 32S RNA (data not shown). The nuclear supernate has nearly the same amount of 28S RNA as the control, since actinomycin seems to inhibit the transport of completed 60S subunits to the cytoplasm. Yet these are essentially mature particles, making it unlikely that newly formed ribosomal proteins are bound to them. Furthermore, there seems to be little difference in the behavior of proteins of the large and small subunits, with the exception of No. 6 and No. 12. It seems more likely that within the nucleolus there are binding sites for ribosomal proteins other than ribosomal precursor RNA, such as the "scaffolding" proteins that take part in the assembly of ribosomal precursor particles (9). Alternatively, there is some feature of the structure or the synthesis of newly formed ribosomal proteins that

leads them to the nucleus. It is noteworthy that ribosomal proteins appear not to be reutilized after the degradation of ribosomes since the half-life of the RNA and protein parts is the same (15). In any case, the ability to examine a large number of individual proteins that migrate to the nucleus may provide insight into the means by which cellular proteins become localized.

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