

Heterogeneity of the Native Ribosomal Subunit Fraction in HeLa Cells

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The cytoplasm of growing HeLa cells contains a certain quantity of *native* ribosomal subunits. They constitute a heterogeneous fraction consisting of at least two categories of particles.

The first category comprises the *newly emerging native* 40S subunits which do not form 80S couples under any *in vitro* condition tried. It is postulated that these *native* subunits carry an *anti-association factor* which cannot be stripped off by 0.5 M KCl treatment.

Transiently native subunits of the second category reassociate to 80S ribosomes only in the presence of an *association factor*.

Introduction

Postmitochondrial supernatants of HeLa cells contain besides polyribosomes, monoribosomes and vacant¹ single 80S ribosomes a small quantity of *native* subunits. These are revealed generally by zonal centrifugation in sucrose gradients.

We have previously reported that single 80S ribosomes are in equilibrium with their subunits². This phenomenon — also found by others³ — is observed when the KCl or NaCl concentration in the lysis buffer is raised from 10 mM to 500 mM. However, in low salt concentrations the equilibrium between ribosomes and subunits is entirely shifted to the 80S ribosomes state². Since *native* subunits, which are discovered under low salt conditions, obviously do not participate in the equilibrium reaction, they must differ in their properties from subunits which readily form 80S ribosomes.

We have looked closer into this *native* subunits fraction, and found that we are dealing with at least two categories of particles. The first of these comprises what is known as *newly emerging native* subunits⁴. These are 40S particles recently synthesized in and released from the nucleus, containing 18S ribosomal RNA. According to PERRY and KELLEY⁴ *newly emerging native* subunits can be distinguished by their densities from other *native* subunits or from derived

subunits obtained by high salt or EDTA treatment of 80S ribosomes. These authors conclude that *newly emerging native* subunits contain additional proteins.

As we shall report in this paper, there exists a second category of *native* subunits (at 10 mM KCl) which will associate to 80S ribosomes provided a protein fraction from ribosomes is added. We wondered under what conditions *native* ribosomal subunits of either category form 80S couples *in vitro*, and participate in protein synthesis.

Materials and Methods

1. Buffers

Tris buffered saline: One liter contains 8 g NaCl, 0.38 g KCl, 0.1 g Na₂HPO₄, 3 g Tris-HCl (pH 7.4), 0.1 g MgCl₂ · 6 H₂O, 0.1 g CaCl₂.
Lysis buffer: 10 mM Tris-HCl pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 14 mM mercaptoethanol, 0.01% macaloid as RNase inhibitor.

2. Cell cultures

HeLa (S 3) cells were grown in suspension cultures at 37 °C in Eagle's MEM (made up from powder medium F-14 from Grand Island Biological Co.) containing 5% calf serum.

3. Cell fractionation procedure

All preparations were done at 0 ° or 4 °C. 500 ml of a cell suspension (0.5 · 10⁶ cells/ml) were poured rapidly on to a frozen shell of 100 ml Tris buffered saline (−70 °C); the resulting suspension was immediately centrifuged. The cell pellet was washed once in cold lysis buffer, centrifuged, and resuspended in the same buffer. The cells were allowed to swell for 10 min and afterwards broken up with 15 strokes in a Dounce homogenizer. The cell nuclei, unbroken cells, membranes, and mitochondria

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dria were removed by centrifugation. The post-mitochondrial supernatant was layered on a sucrose gradient (15%–30% w/w) in lysis buffer, and centrifuged for 12 hours at 22 000 rpm (Spinco L 2, rotor SW 41). At the end of the run, fractions were collected after passage through a flow-cell equipped Isco spectrophotometer, and readings were taken at 254 nm.

The fraction containing the *native* subunits and the 80S ribosomes were separately concentrated by centrifugation (Spinco L 2, rotor 65, 17 to 25 hours at 22 000 rpm, 4 °C). The pellets were thereafter resuspended in the desired buffers, and used in the experiments.

To obtain *newly emerging native* subunits, HeLa cells were labeled with ^3H -uridine (2 $\mu\text{Ci}/\text{ml}$) for 40 min before subjecting them to the fractionation procedure as indicated above.

To determine the radioactivity, the fractions were precipitated with 5% trichloroacetic acid (TCA) after addition of 100 μg albumin. The precipitates were absorbed to glass fibre filter discs (Schleicher und Schüll, Dassel, Germany) and the radioactivity measured in a Packard Liquid scintillation counter with 40% eff. The sedimentation constants were calculated using McEwen's Tables⁵.

4. Preparation of the ribosomal wash

Poly- and monoribosomes were resuspended in 10 mM KCl and 1.5 mM MgCl_2 . Then, they were layered on 1.5 ml of 10% sucrose in this same buffer. After centrifugation at 50 000 rpm for 140 min with the rotor SW 50 the upper third was carefully taken off. This ribosomal wash was then added to the experiment.

5. Amino acid incorporation experiments

For each assay the incubation mixture (100 μl) contains: 1 μM ole phosphoenolpyruvate; 5 μg pyruvate kinase; 0.1 μM ole ATP; 0.1 μM ole GTP; 0.25 μM oles of each 19 amino acids except phenylalanine; 0.1 $\mu\text{Ci}/\text{mmole}$ of radioactive phenylalanine; 0.02 ml cytoplasmic supernatant 5 times concentrated, and ribosomes as indicated in the legends of the Tables. The mixtures were incubated at 37 °C for 5 min only. They were spotted on Whatman 3 MM paper squares⁶. The radioactivity was determined in a Packard Liquid scintillation counter.

Results

Evidence for the heterogeneity of the fractions containing *native* subunits

The *native* 60S and 40S ribosomal subunits as obtained by sucrose gradient centrifugation in 10 mM KCl-buffer are a distinct group of ribosomal particles

which do not associate to form 80S couples spontaneously. Partial association of *native* subunits occurs however in the presence of the *ribosomal wash* (Figs 1 a and b, full lines). Furthermore, the percent asso-

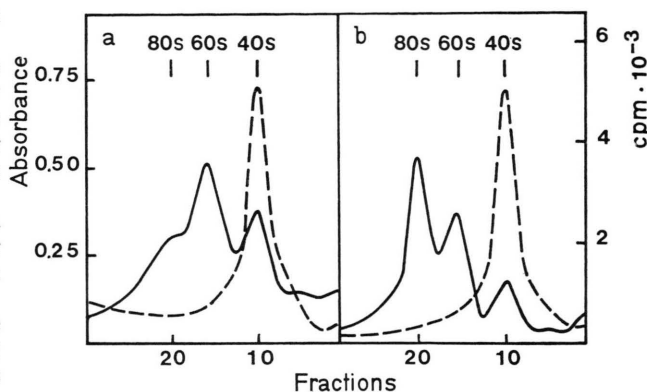


Fig. 1. Evidence for heterogeneity of the *native* subunits fraction. HeLa cells were labeled with ^3H -uridine (2 $\mu\text{Ci}/\text{ml}$) for 40 min and *native* subunits were prepared as indicated in the section "Materials and Methods". The postmitochondrial supernatant was centrifuged through a 15%–30% linear sucrose gradient in lysis buffer. The fractions containing the two *native* subunits and the fractions containing mono- and polyribosomes were pelleted by centrifugation (Spinco L 2, rotor 65, 17 to 25 hours at 22 000 rpm, 4 °C).

The subunit pellet was resuspended in lysis buffer. Six equal portions were loaded on 15% to 30% sucrose gradients in lysis buffer, with varying quantities of *ribosomal wash* added. (Centrifugation: Rotor SW 41, 12.5 hours at 22 000 rpm, 5 °C). The *ribosomal wash* was prepared from the mono- and polyribosomal pellet by treatment with 0.5 KCl in lysis buffer. 1 mg ribosomes yielded 0.1 ml of *ribosomal wash*.

a. No *ribosomal wash* added; b. 0.5 ml of *ribosomal wash* added. Full lines: Absorbance at 254 nm. Dashed lines: TCA-precipitable radioactive material.

ciation depends on the amount of *ribosomal wash* added; Fig. 2 illustrates this correlation and the final saturation characteristics. As it will be discussed in a forthcoming paper, a stoichiometric relationship exists between *native* subunits, and a factor in the *ribosomal wash*. Pulse-labeled *newly emerging native* subunits, however, do not form ribosomes under these conditions (Figs 1a and b; dashed lines). Even highly concentrated *ribosomal wash* does not cause these *newly emerging native* 40S subunits to associate with any 60S units.

By the same token when adding the *newly emerging native* pulse-labeled 40S subunits to a purified fraction of derived 60S subunits, prepared by mild dissociation of 80S ribosomes in buffer with 300 mM KCl, no couples are found whether or not

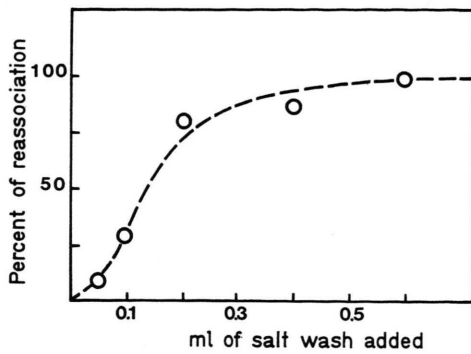


Fig. 2. Dependency of formation of 80S ribosomes from *native* ribosomal subunits on the quantity of *ribosomal wash* added. Six experiments done under conditions identical to the ones described in the legend to Fig. 1.

The percentage of re-association was calculated from the areas under the respective peaks. The maximum of reassociation is assumed to be 100 percent of the reassociable subunits.

ribosomal wash is present. Also, when 80S couples are given instead of 60S subunits, this type of *native* subunits does not exchange with the 40S constituent of the 80S ribosomes.

Assuming that some additional blocking protein⁴ causes the inertia of the *newly emerging native* subunits, we tried the conventional methods of washing ribosomes with high salt buffer. No such treatment renders *newly emerging native* particles incorporable into 80S ribosomes. If, therefore, the blocking is due to additional proteins, they are tightly bound.

Behaviour of native subunits in a cell-free protein synthesizing system

Obviously *newly emerging native* subunits are not in equilibrium with those obtained by dissociation of 80S ribosomes when tested *in vitro* in the cold. A simple temperature rise to 37 °C does not change the results. Knowing that some subunits come together *in vitro* in the initiation step of protein synthesis, we checked the behavior of *native* and *derived* subunits in a cell-free protein synthesizing system. Of the particles obtained by different procedures, the *native* subunits are the most active when polyU is given as messenger (Table I). Equimolar mixtures of the two *native* subunits support polyphenylalanine synthesis, and give monoribosomes with nascent polyphenylalanine (Fig. 3, open circles). In the absence of polyU this is not the case. (Fig. 3 curve represented by +). The same result is recognized, when *native* subunits (labeled with ³H-uridine for 8 hours) are added to

Table I. Incubation of 200 µg of various ribosomal fractions in a cell-free protein synthesizing system (Conditions are indicated in the section "Material and Methods").

Ribosomal Fraction	Without PolyU [cpm]	With 100 µg PolyU [cpm]
polyribosomes	1500	2840
monoribosomes	210	2910
<i>native</i> subunits	255	2390

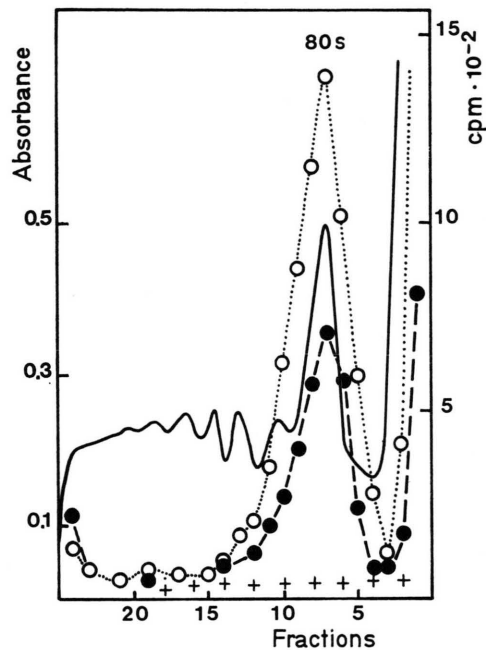


Fig. 3. Behavior of *native* subunits in a cell-free system. *Native* subunits and 80S ribosomes were purified by sucrose gradient centrifugation in lysis buffer. The ribosomal particles were added to a cell-free protein synthesizing system, the details of which are given in the section "Materials and Methods". PolyU was added as messenger. After 5 min of incubation at 37 °C (with ¹⁴C-phenylalanine) the fractions were chilled, and layered on 15% to 30% linear sucrose gradients. They were centrifuged in a rotor SW 41 for 100 min at 40 500 rpm, 4 °C.

— Absorbance at 254 nm (control experiment prior to incubation).
 + + + + Incorporation of ¹⁴C phenylalanine in the presence of 80S ribosomes and *native* subunits but without polyU.
 ●—●—●— Incorporation of ¹⁴C phenylalanine in the presence of 80S ribosomes with polyU.
 ○...○...○ Incorporation of ¹⁴C phenylalanine in the presence of *native* subunits (40S and 60S) with polyU.

the system containing polysomes with endogenous messenger. After a short incubation period labeled *native* subunits are recovered in the monoribosomes fraction (Figs. 4 a and b). This is true for all cases whether *native* 40S or *native* 60S subunits were added to the system individually or both together. Thus, some *native* subunits do participate in polypeptide synthesis *in vitro*⁷. The question remains whether or not the *newly emerging native* 40S particles are involved.

In another set of experiments one batch of *native* ribosomal subunits was labeled with radioactive ³H-uridine for 40 min, the other was labeled for 12 hours, and followed by a two hours chase. The rationale was to label in the first case *newly emerging native* 40S subunits, and in the latter case *transiently native* particles predominantly. These experiments amply demonstrated that no *newly emerging native* 40S subunits entered mono- or polyribosomes (Fig. 5).

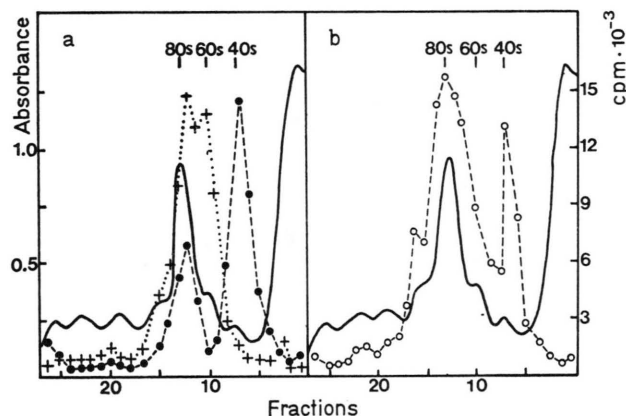


Fig. 4. Incorporation of labeled *native* ribosomal subunits into mono- and polyribosomes in a protein synthesizing system. HeLa cells were incubated for 8 hours with radioactive ³H-uridine in order to label the *native* subunits. They were then purified by sucrose gradient centrifugation (10 mM KCl). The fractions containing the two subunits were collected separately, and pelleted by centrifugation. The labeled subunits were resuspended, and incubated in an *in vitro* system (see "Materials and Methods"). 1.5 mg non-radioactive polyribosomes were added to provide endogenous messenger. After 5 min of incubation the assay tubes were chilled rapidly, and the ribosomal constituents analysed by sucrose gradient centrifugation (rotor SW 27, 14 hours, 17 000 rpm, 4 °C).

— Absorbance at 254 nm (profile of a pre-incubation control experiment) given as reference in Figs 5 a and b).

a ●—●—●— 250 µg of *native* 40S subunits added to the system;

+ . . . + . . . + 250 µg of *native* 60S subunits added.

b ○—○—○— 250 µg *native* 40S and 250 µg 60S subunits added.

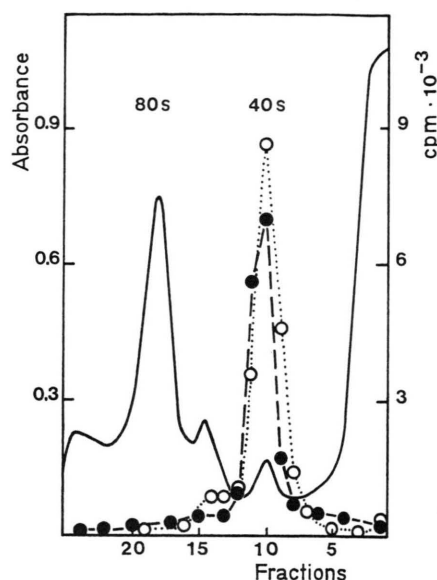


Fig. 5. Evidence for the inactivity of *newly emerging* subunits in a cell-free system. HeLa cells were incubated for 40 min with 1 µCi/ml of ³H-uridine. The *native* subunits were purified by sucrose gradient centrifugation as indicated in the legend of Fig. 5. The fractions containing the *native* 40S subunits were pelleted, resuspended and added to a standard cell-free protein synthesizing system.

After 5 min of incubation the ribosomal particles of the system were analysed by sucrose gradient centrifugation (15% to 30% sucrose in lysis buffer, rotor SW 40, 22 000 rpm for 17 hours at 4 °C).

●—●—●— Experiment with polyU added.

○ . . . ○ . . . ○ Experiment containing unlabeled polyribosomes as source of endogenous messenger.

— Absorbance at 254 nm.

Transiently native particles, however, readily entered the mono- and polyribosomal fraction; this was again observed in all cases no matter whether the small, or the large *native* subunits were added individually or together to a complete *in vitro* system.

Discussion

To our knowledge the term *native* particles has been used by GREEN and HALL⁸ for the first time in their work with ribosomal particles from *E. coli*. The situation in eukaryotes is more complex, as it has been pointed out by PERRY and KELLEY⁴ in their work with L-cells. They introduced the term *newly emerging native* particles; we have here adopted their version.

Our experiments demonstrate that *native* subunits obtained by sucrose gradient centrifugation in 10 mM

KCl are a heterogeneous fraction containing (at least) two categories of particles:

1. *Transiently native subunits readily associable, when a ribosomal wash is added.* These subunits support polypeptide synthesis *in vitro* in the presence of polyU or endogeneous messenger. Our results can be explained, if a association factor is postulated which causes the particles to form couples. Such a factor has been previously found in prokaryotes as well in eukaryotes⁹. This factor is in short supply, and only found with poly- and monoribosomes. Consequently, some subunits remain single. This is supported further by the fact that the S100 cytoplasmic supernatant of the cells does not contain more free *association factor*.

2. *Recently synthesized newly emerging native 40S subunits.* These subunits do not bind to any 60S subunits spontaneously or in a cell-free protein synthesizing system. This could be explained by the presence of an extra blocking protein, an *anti-association factor*, identical with the extra protein found by PERRY and KELLEY⁴.

The fact that this subunit does not participate in the protein synthesis means, that we are most likely dealing with a kind of ribosomal precursor particle, which only after a maturation is incorporated into the polyribosomal fraction. As we have shown earlier, these *native* 40S particles enter into the polysomes before they are found under the 80S peak¹⁰. This *anti-association factor* seems to play a similar physiological role as the factor which inactivates the ribosomes in unfertilized sea urchin eggs¹¹.

Future research has to reveal whether or not these two types of *factors* temporarily associated with ribosomal subunits are specific regulators for protein synthesis in eukaryotes, especially in relations to the initiation process.

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