Missense mutations associated with Diamond–Blackfan anemia affect the assembly of ribosomal protein S19 into the ribosome

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RPS19 has been identified as the first gene associated with Diamond–Blackfan anemia (DBA), a rare congenital hypoplastic anemia that includes variable physical malformations. It is mutated in ~25% of the patients although doubts remain as to whether DBA clinical phenotype depends on the ribosomal function of RPS19 or on an extra-ribosomal role or on both. RPS19 mRNAs with mutations that introduce premature stop codons or eliminate it are rapidly turned over by the surveillance mechanisms possibly causing a decrease in the RPS19 protein level. A decrease in RPS19 level has been shown to cause a defect in the maturation of 18S ribosomal RNA. Less clear is the effect of missense mutations in RPS19. With the aim of analyzing the functional features of mutated RPS19, we prepared cDNA constructs expressing RPS19 containing 11 missense mutations and a trinucleotide insertion found in DBA patients. After transfection, we analyzed the following properties of the mutated proteins: (i) protein stability, (ii) subcellular localization and (iii) assembly into ribosomes. Our results indicate that some RPS19 mutations alter the capacity of the protein to localize in nucleolar structure and these mutated RPS19 are very unstable. Moreover, none of the mutated RPS19 analyzed in this study, including those proteins that appear localized into the nucleolus, is able to be assembled into mature ribosome.

INTRODUCTION

Diamond–Blackfan anemia (DBA, MIM##s 105650, 205900) is the first human disease associated with mutations in a ribosomal structural protein. In fact, following the finding that ribosomal protein (RP) S19 (MIM #603474) is involved in this disorder (1), causal mutations have been characterized in 24% of 217 DBA patients (2–4). The major clinical feature of DBA is a congenital erythroblastopenia characterized by absent or decreased erythroid precursors. Approximately 30% of affected children present a variety of associated physical anomalies, which are predominantly craniofacial but also include thumb, cardiac and urogenital malformations (5,6). Clinical features associated with the mutations in the RPS19 gene show a wide range of variability, even for the same mutation in the same family (3). The lack of correlation between clinical presentation and genotype suggests that other factors may modulate the expression of the genetic defect.

Since all DBA patients with RPS19 mutations appear to have a normal allele, it can be assumed that the disease is caused by haploinsufficiency and/or dominant negative effect of the mutated proteins. It is not clear how alterations in the synthesis and/or functions of RP could influence normal development. Possible hypotheses are (i) ribosome function is critical during erythropoiesis and any decrease in activity could compromise the process and (ii) RPS19 has an extraribosomal-specific function necessary for erythroid differentiation. Alternatively (or in addition), RPS19 could play a specific role within the ribosome, e.g. (i) it could be a target for ribosome-binding factors or (ii) it could be necessary for the proper translation of particular mRNAs (7).
An extraribosomal role of RPS19 dimer as monocyte chemotactic factor has been described by Yamamoto et al. (8). However, the physiologic relevance of this finding is not clear, and we have never found the dimer in the analyzed cultured cells. On the other hand, the recent finding that ~2% of DBA cases show mutations in the RPS24 gene (9), supports the hypothesis of an alteration of a ribosomal function in the disease, although such function could still be both general and hemopoiesis specific. Consistent with the involvement of ribosome synthesis in DBA, we have recently defined the RPS19 interactome and showed that it interacts with multiple proteins involved in the ribosome biogenesis and function (10). Moreover, gene products mutated in some bone marrow failure syndromes are predicted to be involved in ribosome biogenesis (11). The general translation efficiency plays a role in DBA, which is also suggested by the observation that lymphocytes from DBA patients with or without RPS19 mutations have reduced the translation activity (12). We have observed an interaction between RPS19 and PIM1 (13), a serine threonine kinase controlled by erythroid growth factors known as phosphorylate translational regulators (14,15).

The attempt to generate an animal model for studying DBA produced puzzling results. In fact in mice, complete loss of RPS19 results in early embryonic lethality whereas RPS19<sup>+/−</sup> mice are viable and without major abnormalities, even in the hematopoietic system (16). The authors propose that the loss of one RPS19 allele is fully compensated for at the mRNA level with preservation of erythropoiesis. Suppression of erythroid differentiation and cell growth has been observed in a human leukemic cell line expressing siRNA against RPS19 (17). This suggests that RPS19 may function in the regulation of cell proliferation and differentiation. The specific role of RPS19 in ribosome synthesis and function has been recently addressed in yeast (18,19). Both reports showed that the deletion of one copy of the two RPS19 genes causes a defect in the maturation of 18S ribosomal RNA (rRNA). A similar alteration has been shown in cells from DBA patients and in cell lines depleted of RPS19 by specific siRNA (17,20,21). However, how this alteration correlates with the clinical features of DBA remains unclear.

On the basis of the results obtained by us and by other researchers (22,23), RPS19 mutations found in DBA can be divided into (at least) two groups: (i) mutations that cause a decrease in the amount of RPS19 mRNA, i.e. insertions, deletions, splice site and nonsense mutations which alter the position of the termination codon and therefore cause mRNA degradation by surveillance mechanisms and (ii) missense mutations which presumably affect the function(s) of the protein. The first class of mutations is likely to cause a decrease in the synthesis of the protein and, at least in some cases, this is associated with alteration of rRNA maturation (21). The second class probably includes subclasses with alterations in different functions. In fact, RPS19 missense mutations could cause a defect at different steps in the process of ribosome assembly. Alternatively, the altered protein could be integrated into the ribosomal subunit and generate malfunctioning in the translation process. It has been shown that at least two missense mutations can alter nucleolar localization of RPS19 (24). However, other mutations do not cause this alteration; therefore, it is not clear if or how much mutated RPS19 can proceed in the pathway of ribosome biogenesis. To investigate this issue, we prepared cDNA constructs expressing Flag-RPS19 that contains 11 missense mutations and 1 insertion found in DBA patients. We used these constructs in transfection experiments into cultured cells to study the functional capacity of the differently mutated RPS19. The results indicate that the mutations analyzed can affect protein stability and nucleolar localization in different ways. In all cases, however, the altered proteins fail to be assembled into ribosome.

### RESULTS

**Expression and stability of mutated RPS19**

Eleven missense mutations and one trinucleotide insertion after codon 19 identified in DBA patients were introduced into Flag-tagged RPS19 cDNA by site-directed mutagenesis. The cDNAs were then inserted into a eukaryotic expression vector and used in transient transfection experiments into human embryonic kidney 293 (HEK293) cells. The expression of the mutated RPS19 proteins, distinguishable from the endogenous by size, was monitored by western blot analysis using Flag- and RPS19-specific monoclonal antibodies (mAb). As a control for transfection efficiency, we used the product of the neomycin phosphotransferase II (NPT) gene present in the cloning vector (pcDNA3) and therefore expressed at a level proportional to the amount of plasmids inserted into the cells. An example of the western analysis is reported in Fig. 1A. The intensity of the signals shows an evident variability not justified by transfection efficiency. In particular, the mutated RPS19 proteins V15F, L18P, A57P, A61E, G127E, and INS are barely detectable (indicated by

![Figure 1](https://academic.oup.com/hmg/article-abstract/16/14/1720/2356025)
an asterisk), whereas the remaining mutations show an intensity comparable with the wild type (WT) RPS19 protein used as control. The difference in the level of the transfected Flagged RPS19 proteins could be because of a differential stability of the mRNAs or, more likely, of the mutated RPS19 proteins. To investigate on the turnover of the mutated RPS19, we isolated HEK293 clones stably expressing Flag-tagged proteins. Surprisingly, compared with the levels of WT Flag-RPS19, those of mutated Flag-RPS19 were very low in all the clones analyzed (at least three for each cDNA construct, data not shown). To assess whether the level of the transfected proteins was affected by rapid turnover, clones stably expressing R62W, R101H and WT RPS19 proteins, respectively, were treated with the translation inhibitor cycloheximide and analyzed by western blot with an mAb specific for RPS19. The mAb can detect both endogenous and Flag-tagged transfected RPS19, the two of them being distinguishable by size. The results of this analysis (Fig. 1B) indicate that both mutated Flag-RPS19 (R62W and R101H) are degraded more rapidly compared with unmutated Flag-RPS19 that exhibits stability comparable with the endogenous RPS19. Further confirmation of the increased turnover of the mutated RPS19 was obtained by treating the cells with the proteasome inhibitor MG132. In this case, as reported in Fig. 1C, the signal relative to R62W- and R101H-mutated RPS19 shows a clear increase compared with WT Flag-RPS19 and endogenous RPS19. This again suggests that the two mutated proteins are degraded (at least partly by the proteasome) more rapidly than WT Flag-RPS19.

Subcellular localization

To investigate the intracellular localization of the mutated RPS19 proteins, the same cDNA constructs described in the previous section were used in transient transfection experiments in HeLa cells. The cells were then analyzed by immunofluorescence microscopy using mAb against the Flag epitope. As a marker for nucleolar localization, we used a polyclonal antibody against nucleolin (C23). As shown in Fig. 2A, the WT Flag-RPS19 exhibits an evident nucleolar staining as indicated by the co-localization with nucleolin. The same analysis on the 12 mutated RPS19 identifies two classes: (i) proteins with a nucleolar localization similar to WT Flag-RPS19 (P47L, W52R, R56Q, R62W, R62Q and R101H) and (ii) proteins that do not localize into the nucleolus (V15F, L18P, A57P, A61E, G127E and INS). It should be pointed out that even the WT Flag-RPS19 shows a staining pattern quite different from the endogenous RPS19 reported in Fig. 2B as a control. In fact the endogenous protein appears mostly cytoplasmic, whereas the majority of transfected tagged RPS19 is detectable in the nucleus. This could indicate that the transiently transfected Flag-RPS19 accumulates in the nucleus and is not exported in the cytoplasm efficiently. However, the same Flag-RPS19 protein stably expressed in HEK293 cells shows a localization similar to the endogenous RPS19 (Fig. 2A and B). The reason of the different behavior of the Flag-RPS19 in transient versus stable transfection is not clear. One possibility is that transient transfection causes a massive overexpression of the protein with a consequent overloading of the ribosome assembly pathway. This being the case, the excessive Flag-RPS19 would accumulate in the nucleus and only part of it would be processed by the assembly machinery (see below). On the other hand, the Flag-RPS19 produced by the more controlled stable expression would be mostly processed as the endogenous protein. Similarly, Da Costa et al. (24) found that transiently transfected GFP-tagged WT RPS19 localizes into the nucleolus, but not into the cytoplasm. This could indicate that due to overexpression and/or the presence of the GFP tag, the transfected RPS19 is not correctly assembled into ribosomes. In the same study, the authors report a faint signal of endogenous RPS19 in the cytoplasm, in contrast to what we found (Fig. 2B). The discrepancy could be due to the fact that the antibody used by Da Costa et al. does not recognize RPS19 assembled into the ribosome that is the form of RPS19 predominant in the cytoplasm.

Ribosome association

The other property of the mutated Flag-RPS19 proteins we analyzed is their capability to be assembled into functional or aberrant ribosomal subunits. For this purpose, after transient transfection into HEK293 cells, cytoplasmic extracts were separated by sucrose gradient centrifugation. The gradients were then collected into fractions while monitoring the absorbance at 260 nm. As shown in the absorbance profile of Fig. 3A, translating polysomes are localized in fractions 1–5, which were pooled for further analysis. Ribosome monomers (80S) and 60S and 40S ribosomal subunits are also indicated in the absorbance profile. Proteins isolated from the different fractions were subjected to SDS–PAGE and immunoblot analysis with antibodies against Flag, RPS19 and S6K1. The results, relative to the transfection of Flag-RPS19 WT, are reported in Fig. 3A. Endogenous RPS19 is present, as expected, in all the ribosomal fractions including 40S subunits (fraction 1–9) whereas S6K1 is visible only in light cytosolic fractions (fractions 10–12). The Flag-tagged WT RPS19 protein is mostly present in the free cytosolic fractions, but can also be clearly seen to be associated with ribosomes, including polysomes. The experiment was repeated for all RPS19 constructs and, as shown in Fig. 3B, produced essentially the same result: mutated RPS19 is never found associated with ribosomes. To confirm this observation, we tried a slightly different technique, namely fractionation of the cytoplasmic extracts by 100 000 g centrifugation in a ribosomal pellet (including 40S ribosomal subunits) and cytosolic supernatant. The results, reported in Fig. 3C, again show that part of the Flag-RPS19 WT can be found in the pellet, whereas all mutated RPS19 analyzed appear unable to associate with ribosomes. We then analyzed the HEK293 cell lines stably expressing WT Flag-RPS19 cDNAs and the mutated RPS19, A57P, R62W (not shown), R101H and G127E. The cells were also treated with the proteasome inhibitor MG132 to induce an increase in the protein level. The results, reported in Fig. 4, show that the stably expressed WT Flag-RPS19 is mostly present in the ribosomal pellet. In contrast, the mutated proteins, of which only R101H and G127E are shown as an example, are never present in the pellet even in cells treated with the inhibitor.
Our analysis of the ribosomal association of the mutated RPS19 showed that part of the WT and all the mutated Flag-RPS19 proteins are not assembled into ribosomes and are visible as putative-free cytoplasmic proteins. However, this is not consistent with the immunomicroscopy analysis that showed no cytoplasmic staining for the Flag-RPS19 proteins. Therefore, we hypothesized that the experimental conditions used during the preparation of the cytoplasmic extracts were too drastic and caused the leakage of material from the nucleus. To verify the hypothesis we repeated the experiment with the cells transiently expressing WT Flag-RPS19 by using milder conditions to prepare cytoplasmic extracts. This time, as shown in Fig. 5, the amount of protein present in the supernatant was clearly lower than in the previous procedure. This suggests that most of the Flag-RPS19 that appeared as free protein in the previous analyses is probably present in the nucleus.

**DISCUSSION**

Recent publications have provided support to the hypothesis that DBA may be caused by alteration of a ribosomal function (9,11). Ribosome biogenesis is a complex process that requires the transfer of multiple proteins in and out of the nucleolus. Maturation of the rRNA and its assembly into ribosomal subunits involves >150 accessory proteins and about as many as small nucleolar ribonucleoprotein particles (25,26). The role of the RPs in the process is mostly obscure. A recent systematic analysis of yeast RPs of the small subunit revealed that different RPs control distinct steps of nuclear and cytoplasmic pre-18S rRNA processing (18). Depletion of RPS19, as observed also by Leger-Silvestre *et al.* (19), causes a defect in the 18S rRNA processing in the nucleus and blocks maturation of 40S subunits. Similar defects have been observed in human cells after RPS19 knock-down by siRNA (21,27).
Therefore, the mutations in the RPS19 gene that cause a decrease in the level of the protein are expected to induce ribosome biogenesis defects. It is not clear, however, what is the effect on protein function of those mutations affecting the structure rather than the level of RPS19.

To analyze the functional characteristics of mutated RPS19 found in DBA patients, we expressed tagged cDNA in human cells. We used RPS19 cDNA containing 11 missense mutations and a trinucleotide insertion. The first observation we made is that in transient transfection experiments into HEK 293 cells, the level of the mutated RPS19, after normalization for transfection efficiency, is variable among the different proteins. In a first set of experiments, we could distinguish two classes on the basis of the apparent stability of the proteins: (i) comparable with WT RPS19 and (ii) clearly less stable. The analysis of the same mutated proteins by stable transfection further confirmed that all the analyzed mutations were able to induce protein instability. In fact, even the R62W and the R101H mutations, which in transient transfection were comparable with WT, showed a half-life much shorter than that of WT Flag-RPS19. This was also confirmed by the treatment with proteasome inhibitor. The reason of the difference in the two experimental approaches could be that the transient transfection causes a massive overexpression of the exogenous protein with a consequent overloading of the degradation machinery. In such a situation of saturation, only the largest differences were evident. The relatively more controlled stable expression allowed the detection of minor differences. In any case, the two classes of mutations identified on the basis of protein stability showed a correlation with the immunofluorescence localization data. In fact all the more stable RPS19 proteins (including the WT) showed a clear nucleolar localization, whereas the less stable ones were not detectable in the nucleoli (Table 1). Although the correlation between the two analyses is evident, the possible causal relationship between the two observations is less clear. In fact, we favor the hypothesis that the lack of nucleolar localization, as a result of the incapability of mutated RPS19 to interact with other components of the ribosome biogenesis pathway, would cause rapid degradation of the protein. In the case of nucleolar localization, the interaction with at least some component of the ribosome biosynthesis machinery would cause a partial stabilization of the protein. However, it could also be that it is the stability of the protein that determines whether they are visible in the nucleolus or not. The maximum stability of the transfected RPS19 (comparable with the endogenous RPS19) is observed only when the transfected protein successfully participates in ribosome assembly and is exported into the cytoplasm. This is verified only in the case of WT Flag-RPS19. In fact, our analysis by two different experimental approaches of transient and stable transfectants failed to detect that any of the mutated RPS19 analyzed was associated with ribosomes or 40S subunits.
Table 1. List of the constructs and summary of the results

<table>
<thead>
<tr>
<th>Construct name</th>
<th>cDNA alteration</th>
<th>Protein stability</th>
<th>Nucleolar association</th>
<th>Ribosome association</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>+</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>V15F</td>
<td>43G &gt; T</td>
<td>–</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>L18P</td>
<td>53T &gt; C</td>
<td>–</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>P47L</td>
<td>140C &gt; T</td>
<td>+/–</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>W52R</td>
<td>154T &gt; C</td>
<td>+/–</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>R56Q</td>
<td>167G &gt; A</td>
<td>+/–</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>A57P</td>
<td>169G &gt; C</td>
<td>–</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>A61E</td>
<td>182C &gt; A</td>
<td>–</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>R62W</td>
<td>184C &gt; T</td>
<td>+/–</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>R62Q</td>
<td>185G &gt; A</td>
<td>+/–</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>R101H</td>
<td>302G &gt; A</td>
<td>+/–</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>G127E</td>
<td>380G &gt; A</td>
<td>–</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>INS</td>
<td>53_54insAGA</td>
<td>–</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Nucleotide number starting from the ATG start codon. Mutation nomenclature according to den Dunnen and Antonarakis (29).

Even the stabilization of the mutated proteins by proteasome inhibitor did not cause an increase in the signal in the ribosomal fractions above the detection level. In the same experimental setup, we were able to show that, partially in transient and more completely in stable transfection, the WT Flag-RPS19 is associated with actively translating ribosomes.

In conclusion, all the RPS19 mutations analyzed in this study cause a decrease in the protein stability, with some mutations having a stronger effect. Some of the mutated RPS19 proteins appear localized in the nucleolus, whereas the others apparently fail to remain associated with nucleolar structures (see Table 1). The failure of the V15F and G127E mutated proteins to localize in the nucleolus is in agreement with previous observations by Da Costa et al. (24). In such study, the authors suggest that the two mutations identify nucleolar localization signals. Our observations may point out to further signals, as indicated by the failed localization of mutated proteins A57P, A61E and INS. However, we think that nucleolar localization depends on the interaction of RPS19 with other nucleolar components. Mutations affecting such interactions would cause a failure of the mutated proteins to be retained in the nucleolus. Interestingly, the mutants that are not visible in the nucleolus appear to have a much shorter half-life. None of the mutated RPS19 is able to be assembled into mature ribosome, not even those proteins which appear localized inside the nucleolus.

These results allow RPS19 missense mutations to be grouped into two classes:

1. mutations that cause a failure in the nucleolar localization and, possibly as a consequence, a drastic decrease in the half-life of protein. Moreover, the mutated proteins are not detectable in association with ribosomes
2. mutations that allow RPS19 to associate with nucleolar structures but not to be assembled into ribosome. In this case, the half-life of the proteins, although shorter than WT RPS19 is, may be longer than that of the mutated RPS19 of the first class.

Implications of our findings for the understanding of the molecular mechanism of DBA should be considered with caution. In fact our experimental setup consists in overexpression of tagged RPS19 constructs in standard cell lines (HEK293, HeLa). Therefore, we cannot assume that the results obtained are also valid in hematopoietic cells. However, considering the constitutive and ubiquitous expression of RPS19, it could be that the effect of mutations on the function of the protein is the same in all cell types. If this is case, our results indicate that missense mutations, in addition to mutations altering RPS19 mRNA level, cause insufficiency of RPS19 in hematopoietic cells of DBA patients. Therefore, the variability of clinical symptoms associated with the disease cannot be due to a differential effect of the mutations but, possibly, to the effect of unknown modifier factor(s). Moreover, our findings highlight the risk of failure in attempting a rescue of the DBA clinical symptoms by inducing overexpression or stabilization of mutated proteins, a method that has been proposed for other diseases (28). In fact, it is highly likely that mutated RPS19 will never be assembled into functional ribosomes.

MATERIALS AND METHODS

Cell culture and transfection

HEK293 (ATCC #CRL-11268) and HeLa cells were cultured in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum at 37°C with 5% CO2. For transient transfection, cells were plated at 90% of confluence and transfected with Lipofectamine 2000 (Invitrogen, Milan, Italy) according to the manufacturer’s instructions. After 24 h, cells were analyzed. For stable transfections, 2 x 10^6 cells per 100-mm dish, seeded the day before, were transfected, using the standard DNA–calcium phosphate coprecipitation method, with 10 μg of RPS19 construct (in the cloning vector pcDNA 3, Invitrogen). Transfected cell clones were selected in the presence of 800 μg/ml of G418 (Invitrogen).

For translation inhibition, cells were treated with 30 μg/ml of cycloheximide. For proteasome inhibition, cells were treated with 10 μM MG132 (Sigma) for 12 h.
DNA constructs

RPS19 expression plasmids were constructed by inserting RT–PCR products into pcDNA3 (Invitrogen) downstream from the sequence coding for the FLAG-tag (pFLAG-RPS).

The RPS19 natural mutants R62Q, R62W, R101H and in-frame insertion (53_54insAGA, which is expected to insert an arginine after residue 18) were prepared by RT–PCR from peripheral blood lymphocytes of DBA patients after informed consent. The following primers, which include EcoRI and XhoI restriction sites, were used:

Forward: 5'-GTGAATTCATGCCTGGAGTTACTGTAAGAAG-3'.
Reverse: 5'-GTCTCGAGCCAGCATGGTTGTTCTAA-3'.

All the other mutants were obtained by PCR-dependent mutagenesis using WT RPS19.

Western blot analysis

For western blot analysis, cells were washed twice with phosphate-buffered saline (150 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4 and 1.4 mM KH2PO4) and were treated with lysis buffer (10 mM NaCl, 10 mM MgCl2, 10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 1% sodium deoxycholate, Aprotinin 1 µg/ml, Leupeptins 1 µg/ml, Pepstatin A 1 µg/ml, PMSF 100 µg/ml). After 1 min of incubation on ice, the extract was centrifuged for 1 min at maximum speed in a microcentrifuge at 4°C. Where indicated, to preserve nucleic integrity and minimize the presence of nuclear material in the extract, the detergents in the lysis buffer (1% Triton X-100, 1% sodium deoxycholate) were replaced by 0.05% NP-40.

Proteins were separated on 12% SDS–PAGE, transferred on PVDF membrane and incubated with a mouse mAb specific for RPS19 (10), mouse anti-Flag (Sigma, F3165), rabbit anti-S6K1 (Santa Cruz, sc-230) and rabbit anti-NPT II (Upstate, 06-747). Detection of immunoblot was carried out with SuperSignal reagent (Pierce).

Immunofluorescence microscopy

Cells were fixed with paraformaldehyde 4%, permeabilized for 5 min in 0.1% Triton X-100 and incubated with anti-Flag, anti-RPS19 and anti-nucleolin (C23) (Santa Cruz, sc-13057) antibodies. Secondary antibody was FITC and TRITC conjugated, and nuclei were stained with Hoechst 33258 (Sigma, not shown). The images were acquired using a confocal laser scanning microscope (LSM 510 Zeiss).

Extract fractionation

For sucrose gradient fractionation, cytoplasmatic extracts, prepared as described above, were layered onto 5–65% linear gradient containing 30 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 10 mM MgCl2 and centrifuged for 3 h at 37 000 rpm in a Beckman SW41 rotor. Twelve fractions were collected while monitoring the absorbance at 260 nm. Proteins from each fraction were precipitated with 10% TCA. The pellet was washed with acetone, dried and resuspended in SDS–PAGE loading buffer [63 mM Tris–HCl (pH 6.8), 5% Glycerol, 1% SDS and 2.5% bromophenol-blue].

The first five fraction (polysomes) were pooled and loaded entirely on a single-well, whereas only part (one-tenth) of fraction 11 and 12 was loaded on the gel.

For ribosome isolation 1 ml of cytoplasmatic extract was layered onto 1 ml of 15% sucrose, 30 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl2 and centrifuged in a Beckman type 70.1 rotor for 90 min at 100 000g. The pellet (P, ribosomal fraction) was resuspended directly in SDS–PAGE loading buffer. The supernatant (S, free cytoplasmatic proteins) was precipitated with 10% TCA and the pellet, washed with acetone, was resuspended in SDS–PAGE loading buffer. The proteins were then analyzed by western blot.

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Conflict of Interest statement. None declared.

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