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Reconstitution of the active rat liver 60 S ribosomal subunit from different preparations of core particles and split proteins

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Proteins extracted from the 60 S rat liver ribosomal subunit with 50% ethanol/0.5 M KCl produced only a partial reactivation of the corresponding core particles. In contrast, the same split proteins were able to reactivate the core particles prepared with dimethyl-maleic anhydride (DMMA) to the same level as that observed using the DMMA-split proteins, i.e. 60–80% of the control according to the catalytic activities tested. Comparative analysis of the two split protein fractions showed only four common proteins: P1-P2, which alone restored part of the activities, especially the EF-2-dependent GTPase one, and L10a, L12, which must be responsible for the additional reactivation. The poor ability of the ethanol/KCl core particles to be reactivated was shown to be probably related to a conformational alteration which destabilized the 5 S RNA-protein complex. Proteins present in the ethanol/KCl wash of *Saccharomyces cerevisiae* 60 S subunits were found to be partly active in subunit reconstitution using rat liver DMMA core particles.

Ribosome; 60 S Subunit; Reconstitution; (Rat liver)

1. INTRODUCTION

Bacterial ribosomal subunits can be totally reconstituted, thus enabling the structure-function relationship for their individual RNA-protein components to be established [1]. Cytoplasmic ribosomes of eucaryotes, structurally much more complicated than those of bacteria, have been only partially reconstituted and even successful experiments are rare. Two reagents have been used in these experiments: 2,3-dimethyl-maleic acid anhydride (DMMA) in the reconstitution of yeast ribosomal subunits [2] and ethanol at relatively high-salt concentrations [3–5]. The latter method has yielded partially reactivatable core particles from 80 S ribosomes and yeast 60 S subunits, but not from mammalian 60 S subunits, which remains unexplained. Moreover very few authors have attempted to activate core particles with split-protein fractions derived from different organisms in order to gain insight into the evolution of the translational apparatus. It has been shown that a high-salt/ethanol extract from rat liver 80 S ribosomes (containing proteins P1-P2, L12 and S25) can substitute to a small extent the liquid wash obtained from yeast 80 S ribosomes in the reconstitution of EF-2 dependent activities (GDP binding and GTP hydrolysis) using the remaining particles from yeast 80 S ribosomes [6]. The reciprocal experiments have not been performed.

We recently showed that rat liver 60 S subunits can be partially reconstituted from DMMA-core particles specifically deprived of proteins: L10a, L12, L22, A33, X and P1-P2. The phosphoacidic proteins P1-P2 which are extracted selectively, in the presence of ethanol at low-salt concentration, partly replace the DMMA-split protein fraction in the reconstitution process. P1-P2 alone restore most of the EF-2-dependent GTPase activity but only half the protein synthesis ability recovered when using the DMMA-split protein fraction [7,8]. This indicates that split proteins other than P1-P2 are needed for the interaction of the reconstituted

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large subunits with elongation factor EF-1 and/or 40 S subunits and/or aminoacyl-tRNA. Direct identification of these additional proteins is difficult because eucaryotic ribosomal proteins inactivate during any known standard purification procedure.

Here we examined the possibility of activating DMMA residual core particles from rat liver 60 S subunits, with different high-salt/ethanol washes prepared from rat liver 60 S subunits and polysomes or from *Saccharomyces cerevisiae* 60 S subunits. The main aim of this study was to identify proteins in the rat liver DMMA wash which were responsible for the catalytic activities and to explain why the core particles obtained by ethanol/KCl treatment of the rat liver 60 S subunit were poorly reactivatable.

2. MATERIALS AND METHODS

2.1. *Materials*

The following buffers were used. Buffer A: 50 mM triethanolamine/HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 20 mM 2-mercaptoethanol. Buffer B: 50 mM K^+/Hepes, pH 8.2, 25 mM KCl, 1.5 mM $MgCl_2$, 20 mM 2-mercaptoethanol. Buffer C: 20 mM sodium cacodylate, pH 6.0, 25 mM KCl, 1.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol. Buffer D: 20 mM Tris-HCl, pH 7.4, 30 mM KCl, 8 mM $MgCl_2$, 5 mM 2-mercaptoethanol. DMMA was purchased from Sigma, [^{14}C]phenylalanine (7.4–18.3 GBq/mmol) and [γ-^{32}P]GTP (962–1110 GBq/mmol) were obtained from the CEA (France) and Amersham (England), respectively.

2.2. *Preparations*

Rat liver 60 S ribosomal subunits were prepared by zonal centrifugation as previously described using free polysomes [9]. *Saccharomyces cerevisiae* 60 S ribosomal subunits were prepared from 80 S ribosomes according to [6]. Both 60 S subunits were kept in buffer A. 95% pure rat liver EF-2 was prepared according to a method adapted from [10]. 80% pure EF-1 from calf brain was kindly given by Dr Parmeggiani.

2.3. *Treatment of the ribosomal subunits with DMMA*

Rat liver 60 S ribosomal subunits in buffer B were treated with DMMA as previously described [7] at a reagent molar excess of 15000 (1 h/20°C). The pH had to be maintained at 8.2 by addition of 0.5 M KOH otherwise we observed that almost no protein was removed (a slight amount of P1-P2 only) and the subunits kept about 85% of their initial activity. The preparation was centrifuged over a 10% sucrose cushion in the same buffer. The upper layer of the supernatant containing the split proteins (S_{LDMMA}) and the sediment (C_{DMMA}), representing 80% of the initial subunits, were dialyzed separately against buffer C and used for either subunit reconstitution or electrophoresis analysis.

2.4. *Extraction of subunits or polysomes with ethanol/KCl*

Rat liver 60 S subunits in buffer A were adjusted to 50% ethanol and either 0.08 M or 0.5 M KCl, using a 4 M solution of KCl in buffer A. The extraction, 30 min at 4°C, was repeated twice. It yielded $C_{0.08}$, $C_{0.5}$ particles and $S_{L0.08}$, $S_{L0.5}$ split proteins that were dialyzed against buffer C. We also extracted, under the same conditions, rat liver polysomes and *Saccharomyces cerevisiae* 60 S subunits that yielded the $S_{LP0.5}$ $S_{Y0.5}$ split-protein fractions, respectively. In some experiments rat liver 60 S subunits treated with 50% ethanol/0.5 M KCl (30 min at 4°C) were directly electrophoresed (see below).

2.5. *Reconstitution of active particles*

In these experiments we only used the inactive C_{DMMA} and $C_{0.5}$ residual core particles; $C_{0.08}$ core which was only partly depleted of P1-P2 was not utilized. In each case untreated 60 S control subunits and C_{DMMA}, $C_{0.5}$ core particles were subjected to the same treatment as the reconstituted subunits. Mixtures of core particles plus split proteins, in buffer C (see table 1), were added to one quarter volume of 100 mM K^+/Hepes, pH 7.4, 1.5 M KCl, 75 mM $MgCl_2$, 30 mM 2-mercaptoethanol, incubated 1 h at 37°C and then dialyzed against buffer D, as previously described [8].

2.6. *Assays of activity*

Poly(U)-directed polyphenylalanine synthesis and EF-2-dependent GTPase assays were carried out as previously described [7]. Enzymatic Phe-tRNA binding was assayed according to [11]. In each case we used limiting amounts of 60 S subunits or particles derived from them (1.65, 9.9 and 25 pmol, respectively).

3. RESULTS AND DISCUSSION

60 S subunits were subjected to either ethanol/KCl or DMMA treatment and the core-particle and split-protein fractions isolated (see section 2). The reconstitution systems obtained by several combinations of these cores and saturating amounts of split proteins (determined from the

Fig.1. Analysis of split and core particle proteins from rat liver 60 S ribosomal subunits. Proteins from the $S_{L0.5}$ and S_{LDMMA} washes and the corresponding $C_{0.5}$ and C_{DMMA} residual core particles (b, c, d, e, respectively) were electrophoresed in the acidic SDS system [12] along with proteins from control 60 S subunits (a). The proteins were extracted using the acetic acid/Mg^{2+} procedure [13] from 1 A_{260} unit of 60 S subunit or core particle in (a) and (e), 2 A_{260} units of core particles in (d) and from 3.6 and 5.5 A_{260} units of starting 60 S subunits in (b) and (c), respectively. Some faint spots not numbered correspond to contaminating 40 S proteins. Two acidic components (F) correspond to ferritin. The arrows in (d) and (e) indicate the position of the proteins that have been removed. The code used for numbering the proteins is that of McConkey et al. [14].

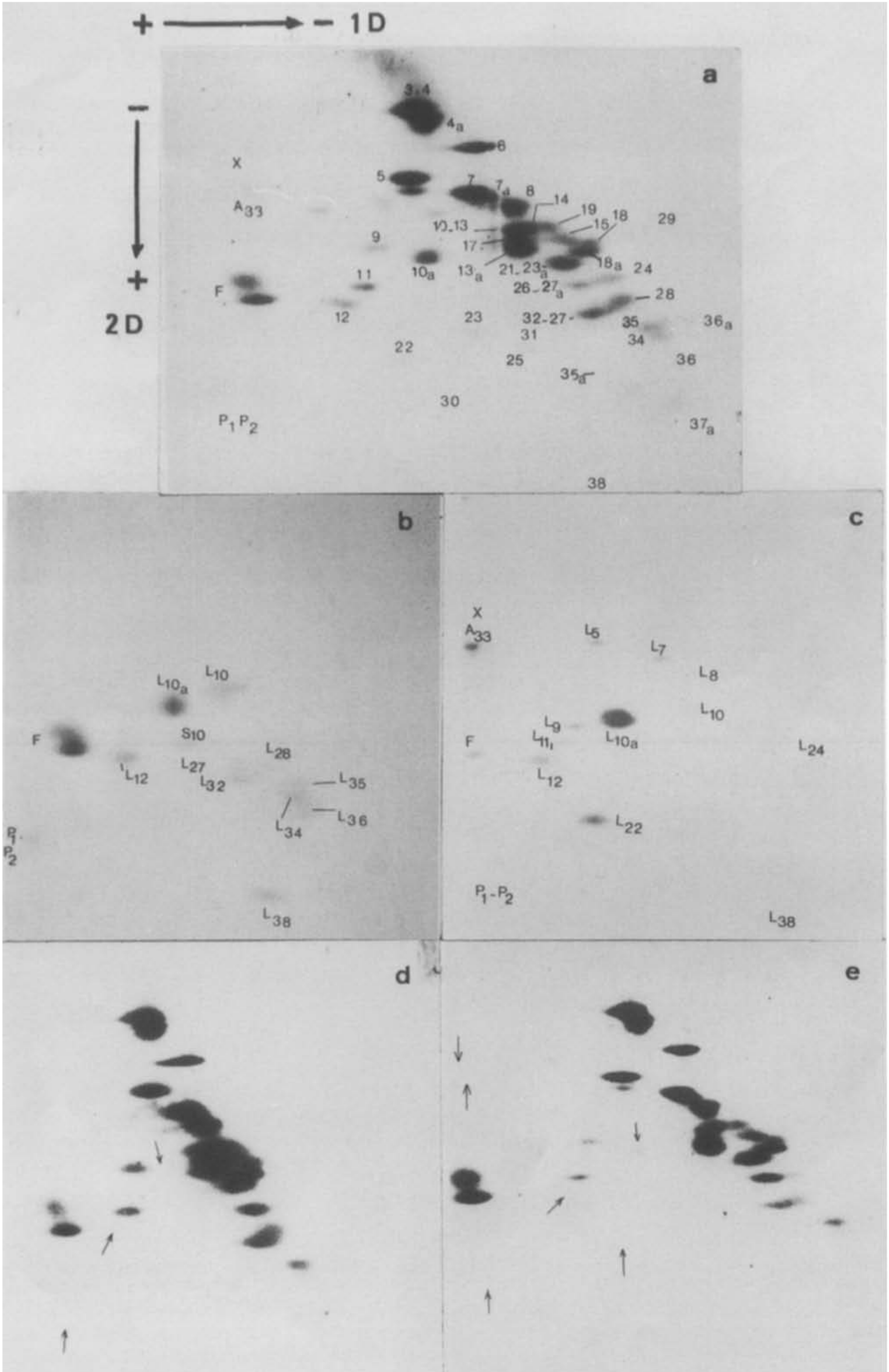
1D
2D
a
b
c
d
e

Table 1

Reconstitution of active 60 S subunits from rat liver core particles and different preparations of ribosomal proteins

Expt	Core particles	Protein fraction added	Composition of added protein fraction %	EF-1-dependent Phe-tRNA binding (%)	Poly(Phe) synthesis (%)	EF-2-dependent GTP hydrolysis (%)
1	Control 60 S	–		100	100	100
2	Cores:C_{DMMA}	–		20	13	6
3	C_{DMMA}	$S_{L0.5}$	P1-P2,L10a,L12,L10,L38(L27,L28,L32, L34,L35,L36,S10)	78	75	57
4	C_{DMMA}	$S_{LP0.5}$	P1-P2,L10a,L12,S12,L38(L27,L32,L34, L36,S10,S25)	–	83	–
5	C_{DMMA}	S_{LDMMA}	X,A33,L22,P1-P2,L10a,L12,L10,L38 (L5,L7,L8,L9,L11,L24)	82	79	55
6	C_{DMMA}	$S_{L0.08}$	P1-P2	53	38	45
7	C_{DMMA}	$S_{Y0.5}$	YL44, YL15 (YL4, YL39)	37	27	21
8	Cores:$C_{0.5}$	–		4	15	–
9	$C_{0.5}$	$S_{L0.5}$	(see expt 4)	24	33	–

Reconstitution was obtained by adding to the core particles 3 and 7 times the complementary amount of proteins split with DMMA or EtOH/KCl (see section 2). C_{DMMA} and S_{LDMMA}, cores and split proteins prepared from rat liver 60 S subunits with DMMA. $C_{0.5}$, cores prepared from these subunits with EtOH/0.5 M KCl. $S_{L0.08}$, $S_{L0.5}$, $S_{LP0.5}$ and $S_{Y0.5}$, split proteins prepared from these subunits, polysomes and yeast subunits, respectively, using EtOH/0.08 M KCl or EtOH/0.5 M KCl. Reconstituted subunits were assayed as described in section 2. The 100% values correspond to 4.9 pmol Phe tRNA bound, 6.0 pmol phenylalanine incorporated and 84.0 pmol of GTP hydrolysed. Underlined proteins were present in large amounts, those in parentheses in small amounts. Proteins not underlined were prominent in both split and core fractions

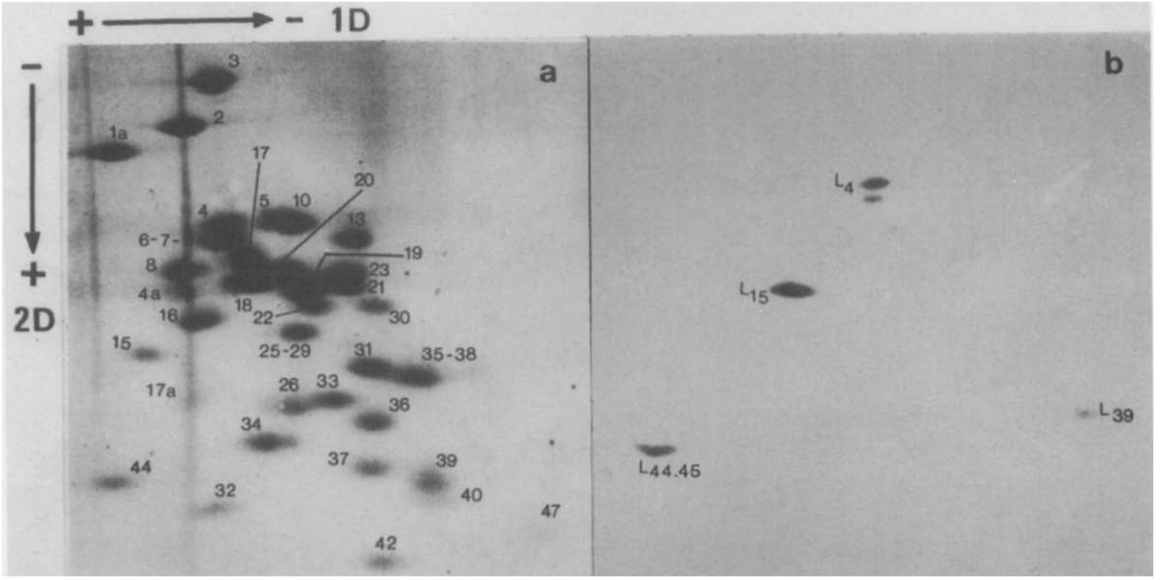

Fig.2. Analysis of split proteins from yeast 60 S ribosomal subunits. Split protein fraction obtained with 50% ethanol/0.5 M KCl (b) was electrophoresed in the acidic SDS system, along with proteins from control 60 S subunits (a). The code for numbering the proteins is that of Bollen et al. [15].

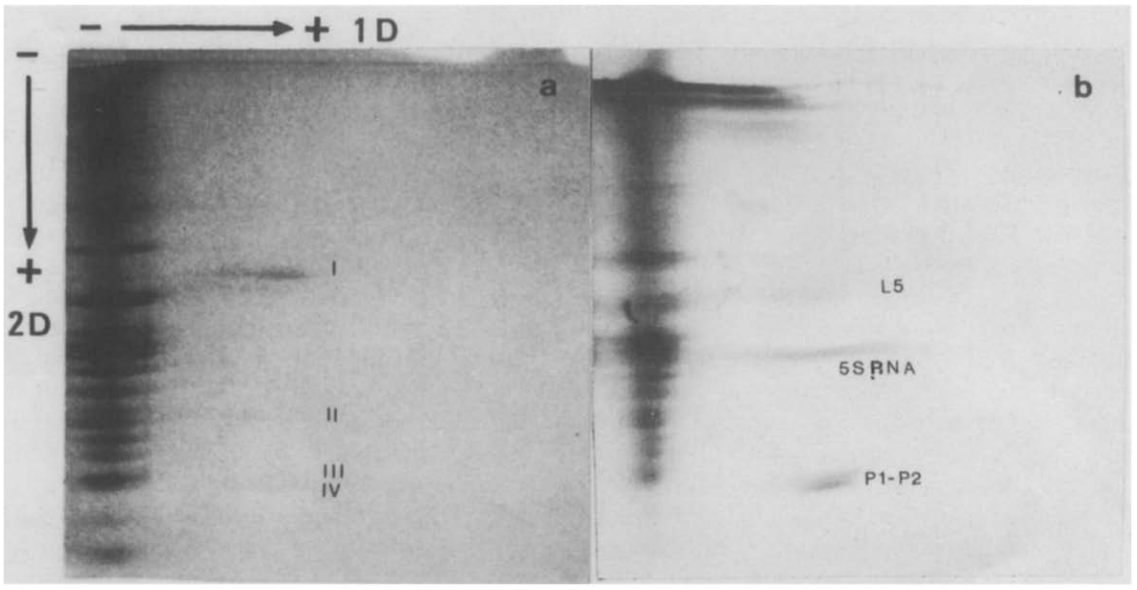

Fig.3. Two-dimensional gel electrophoresis of 60 S subunits treated with ethanol-KCl. 5 A_{260} (a) or 2 A_{260} (b) units of 60 S subunits were treated with 50% ethanol, 0.5 M KCl at 4°C for 30 min and then electrophoresed using a 2.4–7.5% polyacrylamide concentration gradient in the first dimension and 12.5% acrylamide, 0.2% SDS, 6 M urea in the second dimension. One plate (a) was stained with Coomassie brilliant blue and the protein spots were excised and radio-iodinated using the chloramine T method of identification [27]. Another plate was stained with silver under conditions which revealed both RNAs and proteins (b).

plateau of their titration curves) were tested for the different catalytic activities which appear in table 1. Results show that the proteins extracted from the 60 S subunits (or polysomes) with 50% ethanol/0.5 M KCl ($S_{L0.5}$, $S_{LP0.5}$) were as effective as those extracted with DMMA (S_{LDMMA}) to reactivate the DMMA cores (which were almost inactive). In both cases, activities of the reconstituted subunits ranged from about 60 to more than 80% of the controls (table 1, expts 3–5). These values are significantly higher than those obtained with the protein fraction extracted with ethanol at low ionic strength ($S_{L0.08}$), which contained exclusively P1-P2 (expt 6, see also [8]). The ethanol split-protein fraction prepared from yeast 60 S subunits ($S_{Y0.5}$) significantly and reproducibly stimulated the activities of the C_{DMMA} although to a relatively small extent (expt 7). Residual $C_{0.5}$ core particles, which were also inactive, were partially reactivated by the corresponding $S_{L0.5}$, but reactivation remained much lower than that observed with C_{DMMA} (expts 8,9). These results raised two questions: first, are the compositions of the two protein fractions ($S_{L0.5}$ and S_{LDMMA}) identical or different and, in the second hypothesis, which proteins are common to the two fractions? These proteins, or at least some of them, should be essential for the restoration of the catalytic activities. Second, why were the ethanol/KCl core particles poorly reactivatable?

To answer the first question, we compared the 2D gel electrophoretograms of the proteins released from liver 60 S subunits by ethanol/KCl, by DMMA and of those remaining bound to the corresponding particles (fig.1). Results obtained in four separate experiments, using acidic-sodium dodecylsulfate and in some cases acidic-acidic systems [12] are summarized in table 1. We also analyzed the proteins extracted from liver polysomes and *Saccharomyces cerevisiae* (see table 1 and fig.2).

Fig.1 shows that the dissociation process was relatively specific. Residual $C_{0.5}$ as well as C_{DMMA} core particles were totally depleted of the main proteins found in the corresponding split-protein fractions, which are underlined in table 1 (cf. fig.1b,d with fig.1c,e, respectively). The ethanol/KCl extracts ($S_{L0.5}$ and $S_{LP0.5}$) contained

proteins different from those found in the S_{DMMA}, except four proteins: P1-P2, L10a and L12, plus L38, which was very faint in S_{DMMA} (see fig.1b,c and table 1). As found previously $S_{L0.08}$ contained only P1-P2 [16]. This fraction restored most of the EF-2-dependent GTPase activity and only half of the polyphenylalanine synthetizing activity and half of the EF-1-dependent Phe-tRNA binding capacity restored by $S_{L0.5}$. Therefore P1-P2 should participate in both EF-2 and EF-1 binding sites which must overlap. L10a and/or L12, which are/is most likely responsible for the additional stimulation of Phe-tRNA binding and poly(Phe) synthesis, should participate at least in the EF-1 binding site. Until now, there has been little information on the components of these sites. We recently showed that when EF-2 is complexed with the 60 S subunit in the presence of a non-hydrolysable GTP analog, the major part of the factor binds to the surface area of ribosomal subunits with its ADP ribosylatable region protruding into the interior of the interface region (Lavergne, J.P. et al., unpublished). This might explain the high number of ribosomal components which have been cross-linked to EF-2 within this complex: 8–13 proteins and 5 S RNA [17–19]. From the available data, it seems that P2 interacts directly with EF-2 and L12 with both EF-1 and EF-2 [17,20] which would agree with our results. The fact that $S_{L0.5}$ could be at least partially substituted by $S_{Y0.5}$ is an additional and strong argument for a relationship between some 60 S ribosomal proteins from rat liver and yeast, most likely between those that have been reported to be immunologically related: P1-P2 and YL44-YL45, L12 and YL15 (see fig.2 and [21,22]).

In trying to explain why the ethanol/KCl core particles were poorly reactivatable, we examined the possibility that 50% ethanol/0.5 M KCl washing had irreversibly modified the association between $C_{0.5}$ core particles and 5 S RNA. It is known that alcohols affect the tertiary structure of ribosome-bound rRNAs and that KCl, around 1.0 M, detaches 5 S RNA [23,24]. Ethanol/KCl-treated 60 S subunits were electrophoresed in 2 dimensions, using non-denaturing conditions in the first dimension and SDS in the second [25]. Consistently a small number of proteins migrated together in the first dimension as a complex and were resolved as a heavily stained spot (I) and three faintly stained ones (II–IV) in the second dimension (fig.3a). Protein I, easily identified as L5, was totally extracted from the residual particles that could not penetrate the first dimension and whose proteins were seen at the left of the electrophoretogram. Electrophoretic analysis of radioiodinated spots (I–IV) confirmed this result and indicated that L5 was associated to traces of protein L27-L32 (spot II) and P1-P2 (spots III and IV). Staining a gel plate identical to that of fig.3a with silver under conditions which revealed both RNAs and proteins [26] indicated that high-salt/ethanol treatment of 60 S subunits induced a noticeable release of 5 S RNA (fig.3b). This trailed towards the cathode as protein L5 did, indicating that the 5 S RNA-protein L5 complex was progressively detached during gel electrophoresis. Control experiments have shown that no material penetrated into the gel plates when using either untreated 60 S subunits or total free 60 S subunit proteins (not shown). These results clearly show that 50% ethanol/0.5 M KCl induced a conformational change of the subunit which destabilized the interaction between 5 S RNA-protein complex and the rest of the subunit. This effect was only detectable after electrophoresing ethanol/KCl-treated subunits, otherwise the 5 S RNA-L5 complex precipitated and sedimented with $C_{0.5}$ particles after the ethanol treatment.

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