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Binding of 16S rRNA to chloroplast 30S ribosomal proteins blotted on nitrocellulose

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ABSTRACT

Protein-RNA associations were studied by a method using proteins blotted on a nitrocellulose sheet. This method was assayed with Escherichia Coli 30S ribosomal components. In stringent conditions (300 mM NaCl or 20° C) only 9 E. coli ribosomal proteins strongly bound to the 16S rRNA: S4, S5, S7, S9, S12, S13, S14, S19, S20. 8 of these proteins have been previously found to bind independently to the 16S rRNA. The same method was applied to determine protein-RNA interactions in spinach chloroplast 30S ribosomal subunits. A set of only 7 proteins was bound to chloroplast rRNA in stringent conditions: chloroplast S6, S10, S11, S14, S15, S17 and S22. They also bound to E. coli 16S rRNA. This set includes 4 chloroplast-synthesized proteins: S6, S11, S15 and S22. The core particles obtained after treatment by LiCl of chloroplast 30S ribosomal subunit contained 3 proteins (S6, S10 and S14) which are included in the set of 7 binding proteins. This set of proteins probably play a part in the early steps of the assembly of the chloroplast 30S ribosomal subunit.

INTRODUCTION

Chloroplast ribosomes present the peculiarity that they are of prokaryotic type (1,2,3) but that many of their proteins are encoded in the nucleus and are synthesized in the cytoplasm. Therefore the assembly of all the chloroplast ribosomal components raises fundamental problems. One approach consists in the study of RNA-protein interactions.

A great amount of work has been done on the protein-RNA interactions in Escherichia coli ribosomes (4,5,6,7,8). Concerning the 30S ribosomal subunit it has been shown by Nomura's group and it is generally admitted, that 7 ribosomal proteins (S4, S7, S8, S13, S15, S17, S20) are independently and specifically bound to the 16S rRNA (4). Cooperative interactions between ribosomal proteins and 16S rRNA have also been found (9). These results provide the basis for the assembly of the 30S ribosomal subunits in E. coli.

Several methods were used to determine which proteins bind to the 16S rRNA (10). These last years, a new technique for the study of protein-nucleic acid interactions have been developed, using blots of proteins on

nitrocellulose sheets (11). This technique is attractive since one blot containing electrophoretically separated ribosomal proteins can be used to determine interactions with rRNA and after washing, can be re-used with different conditions of binding. The protein-blotting method is particularly useful for the study of RNA-protein interactions in the case of chloroplast ribosomes because the preparation of all the isolated chloroplast ribosomal proteins, not yet achieved at the present time and needed by other methods, can be avoided.

In the present study, the protein-blotting method was assayed with E. coli 30S ribosomal components and results are compared with known protein-16S rRNA interactions. Binding of chloroplast 30S ribosomal proteins with chloroplast 16S rRNA was also determined, giving for the first time an insight on the protein-RNA interactions in the chloroplast 30S ribosomal subunit. It is shown that chloroplast 16S rRNA strongly binds to 7 chloroplast 30S ribosomal proteins in stringent conditions, and that 3 out of these 7 proteins are found in the LiCl washed 30S ribosomal subunit (core particle).

MATERIALS AND METHODS

Preparation of ribosomal subunits

Chloroplasts were isolated from spinach leaves (Spinacia oleracera, L., var. Géant d'hiver) purchased from a local market. Ribosomal subunits were isolated as previously described (3), but 10 µg/ml of heparin were added to the osmotically disrupting medium.

Escherichia Coli (K12 strain) were grown in the LB (Luria-Bertrani) medium. The bacteria were collected in the exponential phase of growth, washed with a medium containing 10 mM Tris-HCl, pH 7.6, 1 mM Mg acetate, 5 mM dithiothreitol and stocked at -20° C. Frozen bacteria were ground 10 min in presence of carborundum (silicium carbure) in a medium containing 10 mM Tris HCl, pH 7.6, 1 mM Mg acetate, 5 mM dithiothreitol. After the disruption of the bacteria, carborundum and cell debris were removed by a 20 min centrifugation at 30,000 g. The pellets were reextracted in the same manner. Supernatants containing the 70S ribosomes were pooled and layered on 10-43% sucrose gradients containing 1 mM Mg^{2+} for the obtention of the ribosomal subunits.

Treatment of chloroplast 30S ribosomal subunits by LiCl

About 10 A_{260} units of chloroplast 30S ribosomal subunits were treated with 1M or 2M of LiCl in the conditions described by Homann and Nierhaus (12). Core particles were pelleted at 220,000 g for 3 hrs at 4° C. Proteins contained in the supernatant were precipitated by 10% TCA at room temperature and were prepared for electrophoresis as indicated below.

Isolation of ribosomal proteins and two-dimensional electrophoresis

The isolation of ribosomal proteins and their electrophoretic separation were done as previously described (2). The main steps were the following: ribosomal proteins were extracted by the acetic acid method of Hardy et al. , (13). They were concentrated by acetone (14). Iodoacetamide was added just before electrophoresis according to the method of Welfle et al. , (15). The electrophoresis was done according to Madjar et al. , (16), using system I (acidic-SDS) or system III (basic-acidic). Gel slabs had the following dimensions: 1.5x160x180 mm. Reference proteins were the following: phosphorylase (Mr = 94,000) ; bovine serum albumin (67,000) ; ovalbumin (43,000) ; carbonic anhydrase (30,000) ; soybean trypsin inhibitor (20,100) ; lactalbumin (14,400). They were used in the second dimension. The nomenclature of spinach chloroplast ribosomal proteins has been published previously (2,3).

^{32}P-labelling and extraction of rRNA

E. coli rRNA was labelled in vivo by the addition of 2 mCi of ^{32}P-orthophosphoric acid (Amersham) in 40 ml of culture medium. The 30S ribosomal subunits were isolated as indicated above and the 16S rRNA was extracted by phenol according to Dyer and Bowman (17). The RNA was precipitated from the aqueous layer by the addition of two volumes of ethanol containing 10 mM Mg acetate. Several ethanol-Mg^{2+} precipitations were done in order to eliminate traces of phenol. The rRNA specific activity was ca 5×10^4 cpm/µg. The purity and integrity of isolated 16S rRNA were checked by gel electrophoresis. 16S rRNA isolated either from E. coli or from chloroplasts by the phenol procedure indicated above was in vitro 5' labelled by $[\gamma^{32}P]$-ATP using the method of Schlief et al. (18). But the heating RNA step was done during 5 min instead of 30 min. Nucleotides and small labelled fragments were eliminated by chromatography on a G50 Sephadex column. The specific activity of the rRNA was ca 10^6 cpm/µg.

^{32}P-labelled non-ribosomal RNA was obtained by the in vitro transcription of pBR 322 DNA using E. coli RNA polymerase and $[\alpha^{32}P]$-GTP in the conditions described by Blanc et al. (19).

Blotting of ribosomal proteins and RNA-binding

Protein blotting and RNA-binding were made by diffusion according to Bowen et al. (11). The transfer of all ribosomal proteins was verified after the binding experiments, using 0.1% amido black in 43% methanol and 10% acetic acid. According to Bowen et al. (11) the efficiency of the diffusion method is of 75% and the renaturation of proteins occured during the transfer by diffusion. 10^6 cpm of labelled RNA were used for each assay in 10 ml of

binding buffer. The standard binding buffer contained 10 mM Tris-HCl, pH 7.0, 1 mM Na-EDTA, 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll and 50 mM NaCl. The various binding conditions (temperature, pH, ionic strength and Mg^{2+} concentration) were as described in the Results. After the binding process, the nitrocellulose sheet was systematically washed in 200 ml of standard binding buffer for 30 min with a constant agitation at room temperature. In some experiments a supplementary 30 min-washing was done at ordinary temperature using the same standard binding buffer but in the presence of 0.5 M or 1 M NaCl instead of 50 mM. After washing, the presence of all ribosomal proteins was checked by amido black staining. The nitrocellulose sheet was dried at room temperature and was exposed 24 h for autoradiography on Kodak X-Omat AR films and Dupont Quanta II intensifying screens. Identification of the RNA-protein binding complexes was done by superposition with the Coomassie blue stained ribosomal proteins remaining on the slab gel after the transfer to the nitrocellulose sheet. After one experiment, the nitrocellulose sheet was overwashed with 4M NaCl and the disappearance of all the radioactivity was checked by autoradiography. The nitrocellulose sheet was then used for other experiments. Each series of experiments was repeated at least twice, independently.

RESULTS

E. coli 16S rRNA binding proteins

In order to test the validity of the protein blotting method for the detection of the rRNA binding proteins, experiments were done using the components of *E. coli* 30S ribosomal subunit. Several experimental conditions of binding were tested. When the binding experiments were made with the standard binding buffer at 37° C and at low ionic strength (50 mM NaCl) a large number of RNA-protein complexes were obtained: only S6, S10 (and reference proteins) did not form a complex (figure 1A,B). After a second washing in the presence of 1M NaCl only five ribosomal proteins remained strongly bound to the 16S rRNA (S4, S7, S9, S13 and S14) and three ribosomal proteins (S5, S19 and S20) were weakly bound (figure 1C). Results were irrespective of whether 16S RNA was labelled *in vitro* with γ^{32}P -ATP or *in vivo* with ^{32}P. The temperature of the binding step at 37° C or 30° C had little influence, if any. At 20° C the number of complexes was smaller than at 30°: the same complexes as those obtained after washing with 1M NaCl were obtained *plus* a weakly bound complex with protein S11 (not shown).

A high ionic strength (330 mM KCl), a relatively high concentration of magnesium ions (20 mM $MgCl_2$) and a temperature of 37° C have been found to be

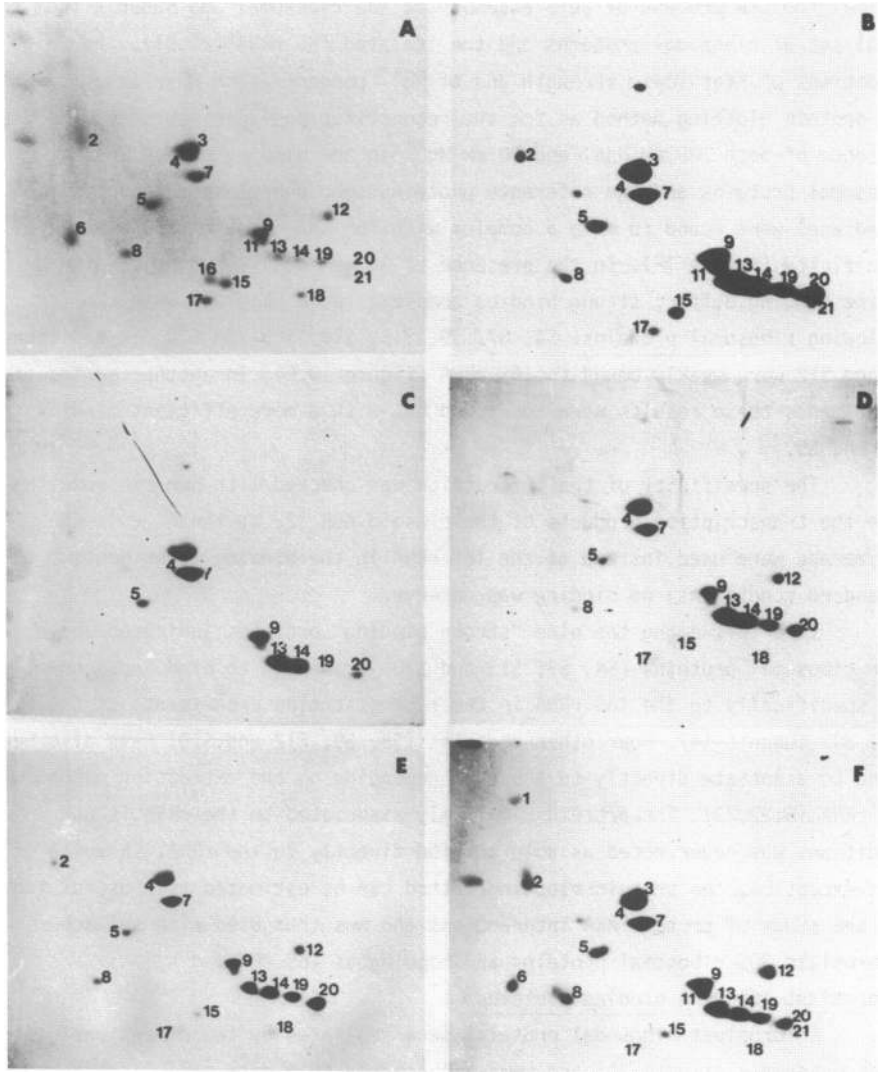

Figure 1. Binding of E. coli 16S rRNA to blotted E. coli 30S ribosomal proteins. The 16S rRNA was ^{32}P-labelled, in vivo. The ribosomal proteins were separated by two-dimensional gel electrophoresis and blotted on a nitrocellulose filter. (A), slab gel with stained ribosomal proteins. The photograph was used to determine the binding proteins by superposition with autoradiographs. The reference proteins on the left of the gel (downward) are those indicated in Materials and Methods. (B) to (F), autoradiographs of filters after the binding experiments. (B), binding at 37° C or 30° C in the standard binding buffer (50 mM NaCl). (C), as in (B) but after 1M NaCl washing. (D), binding at 37° C in the binding buffer containing 300 mM NaCl. (E), as in (D) but in the presence of 3 mM Mg acetate, (F), as in (D) but in presence of 20 mM Mg acetate.

optimal for the process of self assembly of the ribosomal 30S subunit from the total set of ribosomal proteins and the isolated 16S rRNA (20,21). The conditions of high ionic strength and of Mg^{2+} concentration were assayed with the protein blotting method as for the reconstitution experiments. In the presence of both 300 mM NaCl and 20 mM Mg^{2+} in the binding buffer, almost all ribosomal proteins and two reference proteins (phosphorylase and carbonic anhydrase) were found to make a complex with 16S RNA, showing the absence of specificity (figure 1F). In the presence of 300 mM NaCl and 0 mM or 3 mM Mg in the binding buffer, strong binding complexes were observed with the following ribosomal proteins: S4, S7, S9, S13, S14, S19 and S20. In addition S5 and S12 were weakly bound to 16S rRNA (figure 1D,E). In another series of experiments these results were confirmed but with a more efficient binding to S12 and S5.

The specificity of the interaction was checked with non-ribosomal RNA. When the transcription products of the plasmid pBR 322 by the E. coli RNA polymerase were used instead of the 16S rRNA in the binding experiments (standard conditions) no binding was observed.

In brief, among the nine "strong binding" proteins indicated above, four ribosomal proteins (S4, S7, S13 and S20) are known to bind independently and specifically to the 16S rRNA in the reconstitution experiments of the E. coli 30S subunit (4). Four other proteins (S5, S9, S12 and S19) have also been found to associate directly to the rRNA depending on the extraction method of the rRNA (5,22,23). The protein S14 firmly associated to the rRNA in our conditions was never noted as able to bind directly to the rRNA. In spite of this exception, the protein-blotting method can be estimated as a useful tool for the study of protein-RNA interactions and was thus used with spinach chloroplast 30S ribosomal proteins and homologous 16S rRNA.

Chloroplast 16S rRNA binding proteins

Chloroplast ribosomal proteins were separated by two dimensional electrophoresis (figure 2A) and were transferred to a nitrocellulose sheet. Binding experiments were done at 37° C with the standard buffer and in the same buffer without NaCl using in vitro labelled chloroplast 16S rRNA. Nearly all ribosomal proteins were bound to 16S rRNA (figure 2B,F). These protein-RNA complexes were specific since reference proteins added in the second dimension (SDS) of electrophoresis and transferred on nitrocellulose were not bound to the rRNA in all binding conditions assayed. When more stringent conditions were used, only 7 binding proteins (S6, S10, S11, S14, S15, S17, S22) were observed: after a binding in the standard buffer (50 mM NaCl) but at 20° C or

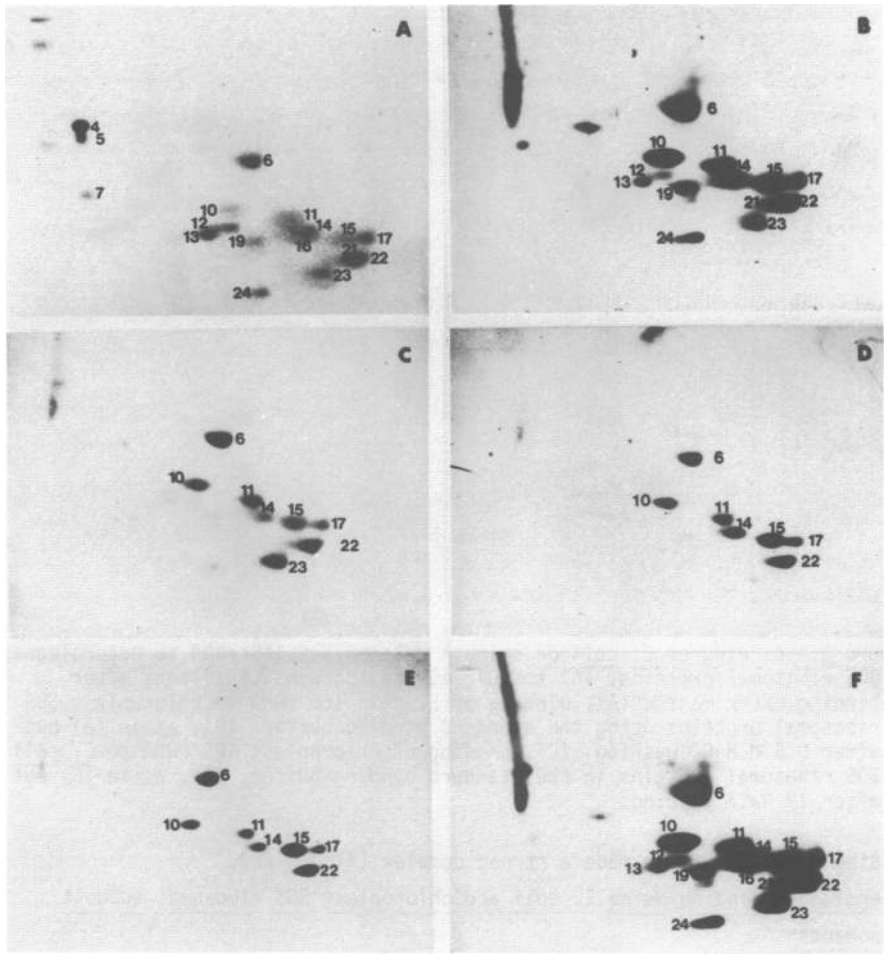

Figure 2. Binding of the chloroplast 16S rRNA to blotted chloroplast 30S ribosomal proteins. The 16S rRNA was 5'-labelled in vitro , with [γ^{32}P] -ATP. Proteins were separated by two-dimensional gel electrophoresis and transferred on a nitrocellulose filter. (A), slab gel with stained ribosomal proteins and reference proteins. The photograph was used as in Figure 1. (B) to (F), autoradiographs of the filters after the binding experiments. (B), binding at 37° C in the standard binding buffer. (C), as in (B) but after 0.5 M NaCl washing. (D), as in (B) but the binding was done at 30° C or 20° C. (E), binding at 37° C in a binding buffer containing 300 mM NaCl. (F), binding at 37° C without NaCl in the binding buffer.

30° C instead of 37° C (figure 2D), in the presence of 300 mM NaCl during the binding process (figure 2E). When a 0.5 M NaCl washing followed a binding in the standard buffer (50 mM NaCl) the protein S23 was added to the set of the 7

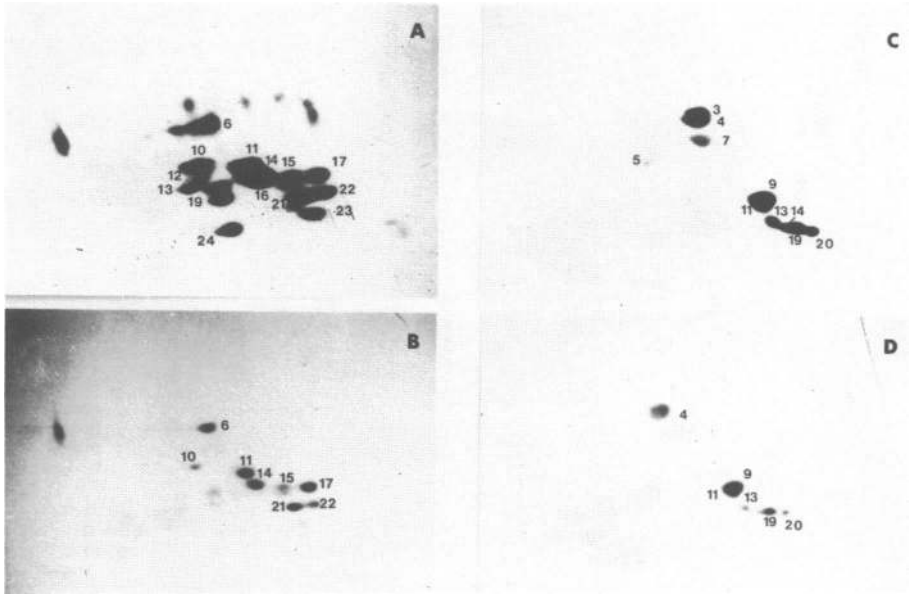

Figure 3. Binding of E. coli or spinach chloroplast 16S rRNA to heterologous 30S ribosomal proteins. (A) to (D), autoradiographs of filters after binding experiments. (A), binding of E. coli 16S rRNA to chloroplast 30S ribosomal proteins using the standard binding buffer. (B), as in (A) but after 0.5 M NaCl washing. (C), binding of chloroplast 16S rRNA to E. coli 30S ribosomal proteins in the standard binding buffer. (D), as in (C) but after 1M NaCl washing.

binding proteins, which made a strong complex (figure 2C).

Heterologous binding using E. coli and chloroplast 30S ribosomal subunit components

Since an important homology had been found between 16S rRNA from E. coli and 16S rRNA from maize chloroplast (1,24), it was interesting to look for heterologous rRNA- protein complexes. E. coli 16S rRNA bound to almost every 30S chloroplast ribosomal protein, at 37° C in the standard binding buffer (figure 3A), exactly as for the homologous chloroplast ribosomal components (figure 2B). When a second washing was made with 1M NaCl, the same group of complexes was found again as for the homologous chloroplast system using selective conditions (figure 3B) with the additional S21 complex. In the other way, chloroplast 16S rRNA bound to a limited number of E. coli ribosomal proteins at 37° C in the standard binding buffer (figure 3C) and after a second washing at 1M NaCl only proteins S4, S9, S11 and S19 remained strongly associated to the spinach chloroplast rRNA (figure 3D).

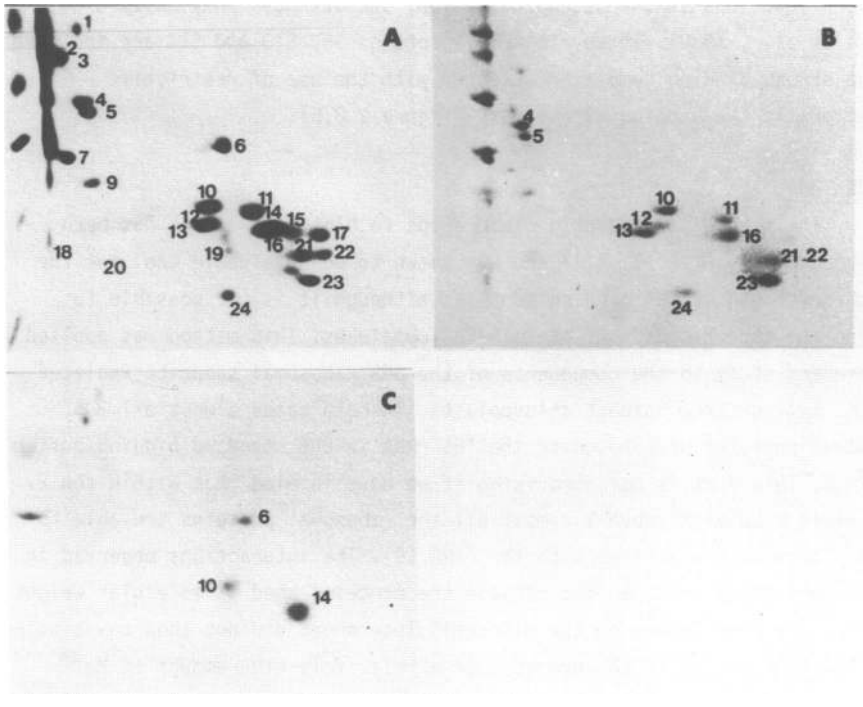

Figure 4. Two-dimensional electrophoretic separation of ribosomal proteins from LiCl treated chloroplast 30S ribosomal subunits. (A), chloroplast 30S ribosomal proteins. (B), proteins of the supernatant of centrifugation of 1M LiCl treated 30S ribosomal subunit (split off proteins). (C), proteins of the core particles.

LiCl treatment of the chloroplast 30S subunits

Core particles containing rRNA and about the half of the E. coli 30S ribosomal proteins have been obtained after washing 30S ribosomal subunits with a solution containing 1M LiCl (12). The proteins of these core particles are those of Nomura's classic group of independently binding proteins (S4, S7, S8, S13, S15, S17, S20) plus six additional proteins (S1, S2, S5, S6, S16, S19). The same kind of LiCl washed core particles have been prepared from chloroplast 30S ribosomal subunits in order to know if the proteins of the core particles belong to the group of the strong binding proteins found in the restrictive binding experiments of the present study. Three chloroplast ribosomal proteins, S6, S10 and S14, were found in the core particles obtained after the treatment of 30S subunits with either 1M or 2M LiCl (figure 4A,B,C). The identity of these three ribosomal proteins was verified with the two-dimensional electrophoretic system III (basic in the first dimension and

acidic without SDS in the second dimension, see Methods) previously used (Mache et al. , 1980). These ribosomal proteins S6, S10 and S14 are included in the strong binding complexes detected with the use of restrictive conditions in the binding experiments (figure 2 D,E).

DISCUSSION

The method of binding nucleic acids to blotted proteins has been developed by Bowen et al. (11) and was shown to be a valuable tool for the detection of DNA or RNA binding proteins although it is not possible to measure the stoechiometry of protein-RNA complexes. This method was applied in the present study to the components of the 30S ribosomal subunits isolated from E. coli or from spinach chloroplasts. In both cases almost all 30S ribosomal proteins were bound to the 16S rRNA in the standard binding buffer at 37° C. This fact is not surprising if we have in mind that within the E. coli small ribosomal subunit almost all the ribosomal proteins are able to develop direct interactions with the rRNA (9). The interactions observed in the present study are specific because the proteins used as molecular weight markers, and transferred on the nitrocellulose sheet did not show any binding with the rRNA except in the presence of a relatively high amount of Mg^{2+} (figure 1F). In addition, no binding was observed when non-ribosomal RNA have been used instead of 16S rRNA (see Results). The use of restrictive binding conditions allows a selection of protein-RNA complexes according to their binding strength (figures 1D,2E).

In the case of E. coli the classic group of ribosomal proteins which bind independently and specifically to 16S rRNA i.e. S4, S7, S8, S13, S15, S17 and S20 (4,5,8) were present in the binding experiment made at 30° C and 37° C in 50 mM NaCl (figure 1B). S8, S15 and S17 showed a lower affinity for RNA than the other proteins in the experiment made at 300 mM NaCl, in the absence of Mg^{2+} (figure 1D). Five RNA-protein complexes which involved S5, S9, S12, S14 and S19, are formed in the presence of 300 mM NaCl and are not included in the classic group of the 7 independently bound proteins (see above). Indeed, this observation is not surprising since it has been reported that S5, S9, S12 and S19 can form a complex with 16S rRNA depending on the conditions of preparation of either the ribosomal proteins or the rRNA (5,22,23). As a conclusion, the protein-blotting method gives interesting informations on the RNA-protein interactions especially at a relatively high ionic strength.
A set of 7 chloroplast 30S ribosomal proteins (S6, S10, S11, S14, S15, S17 and S22) showed a strong affinity for both chloroplast and E. coli 16S rRNA

(figures 2E,3B). Three ribosomal proteins (S6, S10 and S14) of this set remain in the ribosomal core-particle obtained after washing subunits with 1M of 2M of LiCl (figure 4). These results strongly suggest that the 7 chloroplast binding proteins have a structural role in the assembly of the chloroplast 30S ribosomal subunits. It is interesting to notice that at least 4 of this set of binding proteins (S6, S11, S15 and S22) are synthesized within the chloroplast (3).

Sequence analysis of fragments of chloroplast DNA have shown a strong homology with the E. coli S4 and S19 genes (25,26). The binding experiments using heterologous components have shown that the two E. coli ribosomal proteins S4 and S19 are strongly bound to the chloroplast 16S rRNA (figure 3D) suggesting that the binding sites of S4 and S19 are conserved on the chloroplast 16S rRNA. It is also suggested that the 7 chloroplast ribosomal proteins which are strongly bound to both E. coli and chloroplast 16S rRNA (figures 2E,3B) have individually an homology with E. coli ribosomal proteins.

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