

Identification of a Ribosomal Protein Essential for Peptidyl Transferase Activity

(reconstitution/fragment reaction/carboxymethyl-cellulose/lithium chloride split proteins)

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ABSTRACT Extraction with 2 M lithium chloride removes a group of proteins (LiCl SP) from 50S ribosomal subunits. Both the LiCl SP and the resulting cores, which contain the remaining proteins as well as 5S and 23S RNA, lack peptidyl transferase activity, as measured by the "fragment reaction." Activity can be restored to the LiCl cores by reconstitution with LiCl SP under conditions of high temperature and high ionic strength. The LiCl SP proteins were fractionated by carboxymethyl-cellulose and Sephadex G-100, and the individual fractions were tested by this reconstitution system. Of the 18 ribosomal proteins found in the LiCl SP, only L16 is essential for reconstitution of peptidyl transferase activity.

The discovery that peptidyl transferase is an integral part of ribosomal structure was of particular importance in focusing attention on the ribosome as an active participant in the translation process (1, 2). Since that time, the view has gradually emerged that many aspects of protein synthesis, such as translocation (3, 4), although mediated by nonribosomal factors, are intrinsic properties of the ribosome itself.

In order to understand the peptidyl transferase reaction in terms of simple chemical mechanisms, it is essential to identify the ribosomal components involved, as well as the extra-ribosomal ligands with which they interact. Monro and co-workers have shown that under the conditions of the "fragment reaction," peptide bond formation is not dependent on the presence of the 30S ribosomal subunit, mRNA, intact tRNA, supernatant protein factors, or GTP (5). Although little is known about the identity of the ribosomal components involved in this function, it has been found that removal of a subset of proteins from the 50S subunit gives rise to inactive, protein-depleted "core" particles, and that reconstitution of the split proteins (SP) with the core particles restores activity (6). The latter observation suggested that one or more proteins that can be easily removed from the 50S subunit are essential for the peptidyl transferase function and presented a system that could be used to identify these proteins. An important step toward a molecular description of peptidyl transferase is the identification of these essential split protein components.

Abbreviations: LiCl SP, the split protein fraction obtained from lithium chloride extraction of 50S ribosomal subunits; LiCl cores, core particles obtained by the same method; CM-cellulose, carboxymethyl-cellulose.

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Using a partial reconstitution procedure based on lithium chloride extraction of split proteins from 50S ribosomal subunits, we have tested purified split proteins for their ability to restore activity to the inactive core particles. We report here that, of the ribosomal proteins found exclusively in the split fraction, only one of these, L16, is essential for reconstitution of peptidyl transferase activity.

MATERIALS AND METHODS

Solutions. Activation buffer contains 5 mM Tris·HCl (pH 7.5), 5 mM MgCl₂, and 50 mM NH₄Cl. SP buffer contains 0.4 M NH₄Cl, 20 mM Tris·HCl (pH 7.5), and 10 mM MgCl₂. Reconstitution buffer contains 0.35 M NH₄Cl, 20 mM Tris·HCl (pH 7.5), 20 mM MgCl₂, 2 mM 2-mercaptoethanol. Core buffer contains 0.2 M NH₄Cl, 20 mM Tris·HCl (pH 7.5), and 10 mM MgCl₂.

Preparation of LiCl Cores and Split Proteins. Ribosomal subunits were prepared as described (7). Cores and split proteins were derived from 50S subunits essentially by the method of Marcot-Queiroz and Monier (8). The 50S subunits were suspended at a concentration of 25 mg/ml in a buffer containing 50 mM Tris·HCl (pH 7.5), 0.05 mM MgCl₂, and 60 mM NH₄Cl and heated for 10 min at 40°. An equal volume of 4 M LiCl was added, and the mixture was gently shaken at 0° for 15-16 hr. Cores were pelleted by centrifugation for 5 hr at 50,000 rpm (A-321 rotor, IEC B-60 ultracentrifuge). The supernatant containing the split proteins (LiCl SP) was dialyzed at 6° against four changes of SP buffer. Pelleted core particles (LiCl cores) were resuspended in core buffer and dialyzed against four changes of the same buffer at 6°. Dialyzed LiCl SP and LiCl cores were frozen in dry ice/acetone and stored at -80° if not used on the same day. LiCl cores used in reconstitution experiments were thawed no more than once, and stored frozen no longer than 6 weeks.

Reconstitution of particles from LiCl cores and various split protein preparations was done essentially by the method described by Staehelin and Maglott (9) for reconstitution of particles from cesium chloride cores and split proteins. LiCl cores (7.5 A₂₆₀ units) were incubated with split proteins for 90 min at 50° in reconstitution buffer. The total volume of the reconstitution reaction and the amount of split protein fraction varied, and are indicated in the legends to Tables 1 and 2. A typical reconstitution mixture contained 7.5 A₂₆₀ units of LiCl cores and 60 μg of LiCl SP in a total volume of 1 ml of reconstitution buffer. Particles were recovered by precipitation with an equal volume of ethanol at 0°, followed by centrifuga-

tion for 30 min at 15,000 rpm (SS-34 rotor, Sorvall RC-2B centrifuge). Pellets were resuspended in activation buffer at a concentration of about 50 A_{260} units/ml.

Peptidyl Transferase Assay. The peptidyl transferase activity of particles was assayed by the method of Monro (10), after the particles were heated for 10 min at 40° in activation buffer. Assay mixtures contained 6000 cpm of the C-A-C-C-A-(acetyl-[³H]Leu) fragment and 1.5 A_{260} units of 50S subunits or reconstituted particle in a final volume of 100 μ l. The reaction was initiated by the addition of 50 μ l of methanol (0°), and incubation was continued for 1 hr. The amount of radioactivity incorporated into ethyl acetate-extractable material was measured by scintillation counting in 10 ml of a solution containing 0.84% 2-(4'-*t*-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole (butyl PBD) and 10% Bio-Solv BBS-3 (Beckman) in toluene.

Fractionation of LiCl SP. All operations were carried out at 6°. LiCl SP in SP buffer were dialyzed into 6 M urea, 10 mM KCl, 10 mM Tris·HCl (pH 7.5), applied to a CM-cellulose column, and fractionated as described in Fig. 2. Fractions were monitored by measuring absorbance at 230 nm and by one-dimensional and two-dimensional gel electrophoresis. Fractions were pooled according to their protein composition, diluted with an equal volume of 6 M urea, and concentrated by adsorption on a small column (0.5 ml) of CM-cellulose followed by elution with 1.5 ml of a solution containing 6 M urea, 10 mM Tris (pH 7.5), and 0.5 M KCl. Material in peak V was subjected to further purification by direct application of the concentrated protein sample to a column of Sephadex G-100, as described in Fig. 2 (insert).

Concentrated solutions of purified proteins were dialyzed against SP buffer, and final protein concentrations were determined by the Lowry assay (11). Protein solutions were frozen in dry ice/acetone and stored at -80° until needed.

Gel Electrophoresis. Total 50S and LiCl core proteins were prepared by the ribonuclease/EDTA method as described (12). Prior to electrophoresis, proteins were precipitated with 10% trichloroacetic acid, and the pellets washed in succession with 1 ml of acetone and 1 ml of ether, lyophilized, and redissolved in the appropriate sample buffer. Sodium dodecyl sulfate-urea and pH 4.5 urea slab gels were prepared and run

TABLE 1. Reconstitution of peptidyl transferase activity from lithium chloride core particles

Components present in reconstitution mixture	Peptidyl transferase activity of reconstituted particles	
	Acetyl-[³ H]leucyl-puromycin formed (cpm)	% Activity
50S subunits	938	(100)
LiCl cores	20	2
LiCl SP	0	0
LiCl cores + LiCl SP	625	67

Mixtures were subjected to reconstitution conditions, as described in *Materials and Methods*, using 7.5 A_{260} units of LiCl cores or 50S subunits, and, where appropriate, 160 μ g of LiCl SP. Aliquots (1.5 A_{260} units, or the equivalent amount of LiCl SP) were assayed for peptidyl transferase activity by the fragment reaction (see *Materials and Methods*).

as described by G. Thomas, R. Sweeney, C. Chang, and H. F. Noller (manuscript in preparation). Two-dimensional gel electrophoresis was done by a modification of the method of Kaltschmidt and Wittmann (13). Urea was omitted from the running buffer, and its pH was adjusted to 8.6 with NaOH. Samples were polymerized in a 1 cm band in the middle of the cylindrical pH 8.6 first-dimension gel (overall dimensions, 2.5 × 130 mm). After electrophoresis at 200 V (2.5 mA per tube) for 4.5 hr at room temperature, the first-dimension gel was dialyzed against second-dimension buffer and polymerized into a two-dimensional slab (0.15 × 13 × 16 cm). The second dimension of electrophoresis was performed in the device described by Reid and Bieleski (14) at 80 V (10 mA) at room temperature for 14 hr. One-dimensional urea gels and two-dimensional gels were stained with 0.3% Coomassie blue in methanol/water/acetic acid (5:5:1) at room temperature for 1 hr, and destained electrophoretically at 12 V (1–2 amp). Sodium dodecyl sulfate-urea gels were stained and destained as described (15).

RESULTS

Reconstitution of Active Particles from Inactive LiCl Cores and LiCl Split Proteins. Treatment of 50S subunits with 2 M LiCl removes a set of ribosomal proteins (LiCl SP) and gives rise to a particle (LiCl core) that lacks peptidyl transferase activity. Active particles can be reconstituted from inactive LiCl cores and LiCl SP by incubating them together in a high salt buffer for 90 min at 50° (Table 1). From 50 to 80% of the original activity is restored by this procedure. The heating procedure alone does not restore activity to LiCl cores, indicating that one or more proteins from the LiCl SP fraction are required for reconstitution of peptidyl transferase.

The protein composition of the LiCl core and LiCl SP fractions was determined by two-dimensional gel electrophoresis

TABLE 2. Peptidyl transferase activity of particles reconstituted from LiCl cores and purified LiCl split proteins

Expt.	Column peak	Protein components	cpm	%	
			Ac-[³ H]Leu formed	Activity	
1	—	—	32	4	
	Flow-through	I	L7, L8/9, L10, L12	22	3
		II	L1, L5, L6, L11, L25	176	22
		III	L6, L11	135	17
		IV	L18	98	12
		V	L15, L27, L32, L33	111	14
		VI	L16, L26	674	84
LiCl SP	L2, L28	89	11		
2	—	—	798	(100)	
	V-1	L16	63	9	
	V-2	L26*	692	99	
	LiCl SP	—	168	24	
			698	(100)	

Reconstitutions and assays were performed in duplicate, as described in *Materials and Methods*. Column peak designations refer to Fig. 2. Proteins were concentrated and dialyzed into SP buffer prior to reconstitution, as described in *Materials and Methods*. Aliquots containing 1.5 A_{260} units of reconstituted particles were used for each assay.

* The L26 sample used in this experiment was contaminated with minor amounts of L16.

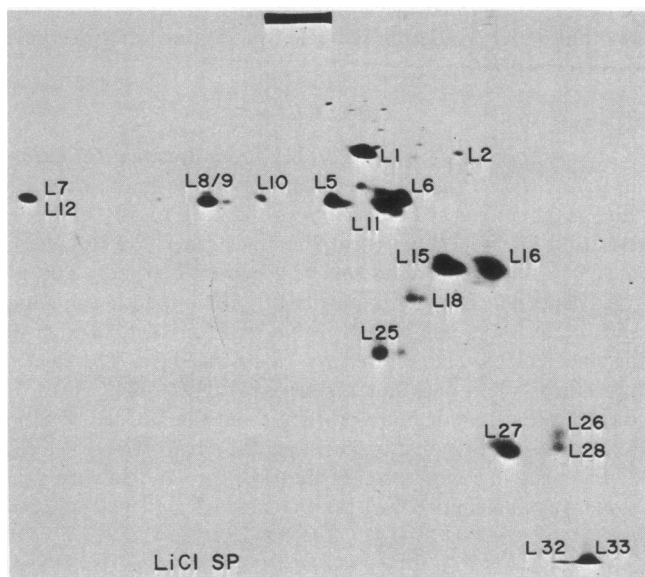
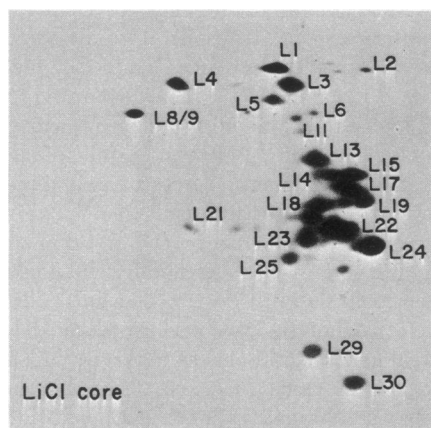


Fig. 1. Protein composition of LiCl core and split fractions. Protein samples (about 3 μ g per protein) were prepared and subjected to two-dimensional gel electrophoresis, as described in *Materials and Methods*. Individual proteins are identified according to the nomenclature of Kaltschmidt and Wittmann (29).

(Fig. 1). Although more than half of the 33 50S proteins are found to some extent in the LiCl SP fraction, some of these (L1, L2, L5, L8/9, L15, L18, and L25) are found in significant amounts in the LiCl cores. Proteins L6 and L11 are found almost exclusively in the LiCl SP fraction, and L7, L10, L12, L16, L26, L27, L28, L32, and L33 are detectable only in the LiCl SP fraction (Fig. 1). No significant amount of 5S or 23S RNA is found in the LiCl SP fraction.

Isolation and Identification of the Active Protein Component from LiCl SP. Attempts to recover active protein fractions after CM-cellulose chromatography of the LiCl SP proteins under standard conditions (16) were unsuccessful. It was found that activity could be maintained if the chromatography is performed above pH 7. Fractionation of the LiCl SP proteins under such conditions is shown in Fig. 2. The activity of each protein peak was estimated by the ability of particles reconstituted from LiCl cores and individual protein fractions to catalyze the "fragment reaction" (see *Materials and Methods*). Peptidyl transferase activity was clearly localized in

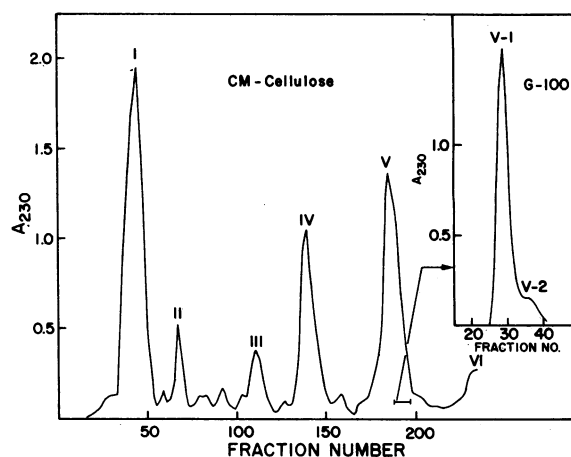


Fig. 2. Fractionation of LiCl SP proteins on carboxymethyl-cellulose. A sample (80 mg) of LiCl SP in 10 mM KCl, 10 mM Tris·HCl (pH 7.5), 6 M urea, was applied to a column (1 \times 10 cm) of carboxymethyl-cellulose (Whatman CM-52), and eluted at 6° with a 1200-ml linear gradient of KCl (0.01–0.2 M) in 10 mM Tris·HCl (pH 7.5), 6 M urea. Fractions (5 ml) were collected and their absorbance at 230 nm was determined. The insert shows repurification of peak V by gel filtration on a column (1 \times 87 cm) of Sephadex G-100, carried out in 6 M urea, 0.1 M KCl, and 10 mM Tris (pH 7.5), at 6°. Twenty-drop fractions were collected. Proteins were identified by two-dimensional gel electrophoresis and are indicated in Table 2.

peak V, although some stimulation was obtained from the other protein fractions (Table 2, Exp. 1). Preliminary experiments, in which arbitrary pools of all column fractions were tested, indicated that fractions located between the major peaks did not have significant activity.

One- and two-dimensional gel electrophoresis showed that peak V contained two major protein components, L16 and L26. These two proteins were resolved by gel filtration on Sephadex G-100 (Fig. 2, insert). Although L26 obtained by this method contained minor amounts of L16, L16 was obtained in high purity, and migrated as a single polypeptide chain in both urea and sodium dodecyl sulfate gels (Fig. 3a and b). Peptidyl transferase activity equivalent to that of total LiCl SP was found for purified protein L16 (Table 2, Exp. 2). Identification of protein L16 by two-dimensional gel electrophoresis is shown in Fig. 3c.

DISCUSSION

Previous attempts to identify molecular components of peptidyl transferase have involved partial reconstitution (6, 17), affinity labeling (18–25), and enzymatic modification (26, 27). Affinity labels designed to react covalently with ribosomal sites in the neighborhood of the 3' end of bound tRNA react with proteins L2 (18) and L27 (18, 19, 22), as well as with ribosomal RNA (23, 24). Reactive analogs of antibiotics known to interact with peptidyl transferase have also been used as affinity labels. Monoiodoamphenicol, a chloramphenicol analog, reacts with protein L16 (20), whereas *N*-iodoacetyl-puromycin reacts mainly with protein L6 (21). Another puromycin analog, 5'-*O*-(*N*-bromacetyl-*p*-aminophosphoryl)-3'-*N*-*L*-phenylalanyl puromycin aminonucleoside, reacts with ribosomal RNA (25). Mild enzymatic digestion with either proteases (27) or nucleases (26) lead to loss of peptidyl transferase activity, consistent with the inter-

pretation that protein as well as RNA components are essential for this function.

Staehelein *et al.* used the partial reconstitution approach to attempt to identify essential protein components of peptidyl transferase (6). These authors showed that removal of a group of proteins from the 50S subunit by centrifugation through cesium chloride gradients resulted in loss of peptidyl transferase activity. Activity could be substantially restored by incubation of the split proteins with the protein-depleted cores under conditions of elevated temperature and high ionic strength.

More recently, Nierhaus and Montejó (17) have reported reconstitution experiments based on lithium chloride extraction of 50S subunits, similar to the system described here. Use of this system affords a simple and rapid means for obtaining the large amounts of split proteins required for subsequent fractionation procedures. By combined use of gel filtration chromatography and gel electrophoretic analysis, the latter authors identified protein L11 as the protein in the split fraction which restores activity to LiCl cores. In contrast, we show here that purified protein L16 is as active as total split protein in restoring peptidyl transferase activity to LiCl cores. Almost no L11 is present in the cores, yet reconstitution of cores with protein L11 gives little stimulation of activity. It could be argued that the small amount of L11 remaining in the core particles is responsible for the observed activity and that addition of L16 is required for stimulation of L11. Another possible explanation for this discrepancy is that the gel filtration method used by Nierhaus and Montejó affords inadequate resolution of the LiCl split proteins, necessitating indirect identification of proteins from the activity obtained by reconstitution with mixtures of proteins. For example, the elution position of protein L16 strongly overlaps that of L11, making difficult the unambiguous assignment of activity to a single protein. In fact, the latter authors report that purified L11 did not restore activity to LiCl cores, in agreement with the results reported here. However, this finding could be attributed to inactivation of the protein during purification. In fact, we have found that purification procedures carried out at low pH lead to loss of activity of protein L16, a difficulty that has prevented the use of standard ion-exchange chromatographic procedures in this system.

In addition to L11, protein L6 is also found in greatly reduced amounts in LiCl core particles, and is thus probably not essential for peptidyl transferase activity. Proteins L7, L10, L12, L26, L27, L28, L32, and L33 appear to be completely dispensable, and thus cannot play an essential role in catalysis of peptide bond formation. The lack of requirement for L27 is of interest because of its apparent proximity to the 3' terminus of peptidyl tRNA (18, 19, 22). Protein L2, another 50S component strongly implicated in the peptidyl transferase function, is found in large amounts in LiCl core particles, and thus no conclusion can be drawn from its lack of significant stimulation in the present study.

The requirement of L16 for peptidyl transferase activity is consistent with a number of related observations. Affinity labeling of L16 by monoiodoamphenicol (20) and demonstration that L16 is required for binding of chloramphenicol to 50S subunits (28) show that L16 interacts with chloramphenicol, an antibiotic whose inhibitory effect on protein synthesis is closely associated with the acceptor moiety in the peptidyl transferase reaction.

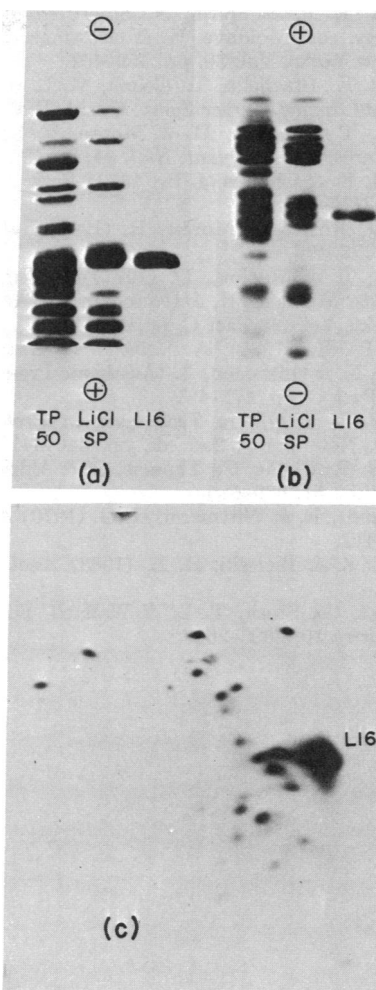


FIG. 3. Identification and purity of protein L16. Gel electrophoresis of total 50S proteins (TP 50), 50S LiCl split proteins (LiCl SP), and purified L16. Proteins were run (a) in 15% acrylamide/sodium dodecyl sulfate/urea gels, or (b) in 7.5% acrylamide (pH 4.5)/urea gels, in slabs 1.5 mm thick and 13 cm high. (c) Proteins were identified by two-dimensional gel electrophoresis, as described in *Materials and Methods*, with 10 μ g of pure L16 mixed with a 1 μ g per protein background of total 50S proteins.

The studies reported here do not shed light on the precise functional role of L16 in peptidyl transferase, apart from the suggestion from previous workers that it may be involved with the acceptor, rather than the donor, moiety. We have been unable to detect peptidyl transferase activity with L16 in the absence of LiCl cores, nor have we detected esterase activity with the purified protein. Whether or not L16 possesses catalytic activity *in situ*, or whether this is a function of other protein or RNA components of the 50S ribosomal subunit, remains to be shown.

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