RNA (1999), *5*:832–843. Cambridge University Press. Printed in the USA.

Efficient reconstitution of functional *Escherichia coli* 30S ribosomal subunits from a complete set of recombinant small subunit ribosomal proteins

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ABSTRACT

Previous studies have shown that the 30S ribosomal subunit of *Escherichia coli* can be reconstituted in vitro from individually purified ribosomal proteins and 16S ribosomal RNA, which were isolated from natural 30S subunits. We have developed a 30S subunit reconstitution system that uses only recombinant ribosomal protein components. The genes encoding *E. coli* ribosomal proteins S2–S21 were cloned, and all twenty of the individual proteins were overexpressed and purified. Reconstitution, following standard procedures, using the complete set of recombinant proteins and purified 16S ribosomal RNA is highly inefficient. Efficient reconstitution of 30S subunits using these components requires sequential addition of proteins, following either the 30S subunit assembly map (Mizushima & Nomura, 1970, *Nature 226*:1214–1218; Held et al., 1974, *J Biol Chem 249*:3103–3111) or following the order of protein assembly predicted from in vitro assembly kinetics (Powers et al., 1993, *J Mol Biol 232*:362-374). In the first procedure, the proteins were divided into three groups, Group I (S4, S7, S8, S15, S17, and S20), Group II (S5, S6, S9, S11, S12, S13, S16, S18, and S19), and Group III (S2, S3, S10, S14, and S21), which were sequentially added to 16S rRNA with a 20 min incubation at 42 °C following the addition of each group. In the second procedure, the proteins were divided into Group I (S4, S6, S11, S15, S16, S17, S18, and S20), Group II (S7, S8, S9, S13, and S19), Group II′ (S5 and S12) and Group III (S2, S3, S10, S14, and S21). Similarly efficient reconstitution is observed whether the proteins are grouped according to the assembly map or according to the results of in vitro 30S subunit assembly kinetics.

Although reconstitution of 30S subunits using the recombinant proteins is slightly less efficient than reconstitution using a mixture of total proteins isolated from 30S subunits, it is much more efficient than reconstitution using proteins that were individually isolated from ribosomes. Particles reconstituted from the recombinant proteins sediment at 30S in sucrose gradients, bind tRNA in a template-dependent manner, and associate with 50S subunits to form 70S ribosomes that are active in poly(U)-directed polyphenylalanine synthesis. Both the protein composition and the dimethyl sulfate modification pattern of 16S ribosomal RNA are similar for 30S subunits reconstituted with either recombinant proteins or proteins isolated as a mixture from ribosomal subunits as well as for natural 30S subunits.

Keywords: 30S subunit assembly; *Escherichia coli* 30S subunit ribosomal proteins; in vitro reconstitution

INTRODUCTION

Studies on the structure, function, and assembly of the *Escherichia coli* 30S ribosomal subunit were revolutionized when it was discovered that a mixture of the 30S ribosomal proteins (TP30) could be reconstituted with 16S ribosomal RNA (rRNA) into functional 30S subunits (Traub & Nomura, 1968). Subsequently, the individual protein components (S1–S21) of the 30S subunit were identified, purified, and characterized (Hardy et al., 1969; Nomura et al., 1969; Traut et al., 1969; Kaltschmidt & Wittmann, 1970; Wittmann et al., 1971). Individually purified ribosomal proteins added as a mixture could also be reconstituted with 16S rRNA into functional 30S subunits (Mizushima & Nomura, 1970; Held et al., 1974). The reconstituted 30S subunits were shown to have the same sedimentation behavior and protein composition as natural 30S subunits. Additionally, the reconstituted 30S subunits were shown to function in tRNA binding and polyphenylalanine synthesis.

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Taken together, these experiments demonstrated that 30S subunits are capable of self-assembly, and that all of the information required for in vitro assembly is contained within these molecular components.

The purified proteins isolated from ribosomes were also used to study the in vitro assembly pathway of the 30S subunit by sequential and combinatorial addition of these proteins to 16S rRNA under reconstitution conditions (Mizushima & Nomura, 1970; Held & Nomura, 1973; Held et al., 1974). It was found that six proteins, S4, S7, S8, S15, S17, and S20, can interact directly with 16S rRNA in the absence of other proteins (Mizushima & Nomura, 1970; Schaup et al., 1971; Held et al., 1973, 1974). The remaining proteins were divided into two groups, depending on their requirements for stable incorporation into the ribonucleoprotein particle (RNP) under reconstitution conditions. Studies on the dynamics of in vitro 30S subunit assembly have shown that different regions of 16S rRNA undergo assembly at different rates (Powers et al., 1993), likely influenced by a combination of protein-dependent RNA conformational changes and rates of association of different proteins. The results of these in vitro assembly studies may reflect the pathway of 30S subunit assembly in vivo.

The ability to reconstitute the *E. coli* 30S subunit in vitro from purified components has allowed detailed investigation of the structure, function, and assembly of the 30S subunit. However, isolation of sufficient amounts of highly purified, functional small subunit ribosomal proteins from isolated subunits can be difficult, laborious, and costly. In particular, it is difficult to exclude cross-contamination between ribosomal proteins in large-scale purification. We sought to alleviate some of these problems by cloning and overexpressing a complete set of recombinant small subunit ribosomal proteins for use in studying the *E. coli* 30S ribosomal subunit. Initial attempts at reconstituting 30S subunits with recombinant proteins and isolated 16S rRNA, following standard procedures (Traub & Nomura, 1969), led to very inefficient production of 30S subunits. Therefore, we developed an ordered assembly protocol that allowed efficient reconstitution of 30S subunits using the purified recombinant proteins. Reconstitution of 30S subunits with the recombinant proteins is less efficient than those using a complete mixture of ribosomal proteins (TP30) but more efficient than 30S subunit reconstitution using proteins individually purified from ribosomal subunits. The molecular composition and sedimentation properties of the recombinant 30S subunits are similar to those of natural 30S subunits and those reconstituted with TP30. Also, the recombinant 30S subunits were active, as measured by in vitro assays. The large amounts of pure ribosomal proteins that can be obtained greatly facilitate studies that depend on reconstitution of 30S subunits from individual proteins, such as directed hydroxyl radical probing from single positions on individual proteins (Culver & Noller, 1998; Culver et al., 1999). Moreover, the potential for exhaustive mutational analysis of each small subunit ribosomal protein provides a new approach to investigation of their roles in structure, function, and assembly of ribosomes.

RESULTS

Cloning, expression, and purification of ribosomal proteins S2–S21

Genomic DNA from *E. coli* MRE600 was used as a template for polymerase chain reaction (PCR) amplification of the genes encoding ribosomal proteins S2–S4, S6–S16, and S18–S21. The genes encoding S1 (Sorensen et al., 1998), S5 (Heilek & Noller, 1996b), and S17 (G.M. Heilek & H.F. Noller, unpubl. results) have previously been cloned. Primers for the PCR reactions were designed to facilitate cloning (by the inclusion of restriction enzyme sites) and expression (by optimizing the distance between the start codon and the ribosome binding site within the vector). After amplification and restriction enzyme digestion, the PCR products were cloned directly into the pET24b vector (Novagen), which contains an inducible promoter and an f1 origin for production of single-stranded DNA. The integrity of each individual clone was confirmed by restriction enzyme digestion and DNA sequence analysis (data not shown). Induction of protein expression in the *E. coli* strain BL21(DE3) results in production of differing amounts of the various proteins of which some are soluble and some insoluble (Fig. 1A; Table 1); the perceived differences in induction levels may in part reflect varying staining efficiencies of the different proteins (see Fig. 1). For some constructs, it appears that multiple species are overexpressed upon induction (Fig. 1A); this may be an artifact of the expression system, since similarly sized contaminants are observed with more than one construct (see Fig. 1A; compare, for example, S18 and S21). Nevertheless, these contaminants do not appear to interfere with purification (Figs. 1B and 2). The level of overexpression and the highly charged nature of most of the proteins enables single-column FPLC purification (Figs. 1B and 2; Table 1). Representative chromatograms of FPLC cation-exchange purification of two overexpressed proteins are shown in Figure 2. S2 has a relatively low isoelectric point (Kaltschmidt, 1971), is insoluble, and is purified at lower pH (Fig. 2A; Table 1), compared to S4, which is quite basic, soluble, and purified at the higher of the two pHs used during chromatography (Fig. 2B; Table 1; Kaltschmidt, 1971). Each of the overexpressed proteins was similarly purified to near homogeneity by FPLC cation-exchange chromatography, except for S6, which was purified by FPLC anion-exchange chromatography (Fig. 1B; Table 1). Some proteins (for example, see S11) are quite insoluble, and

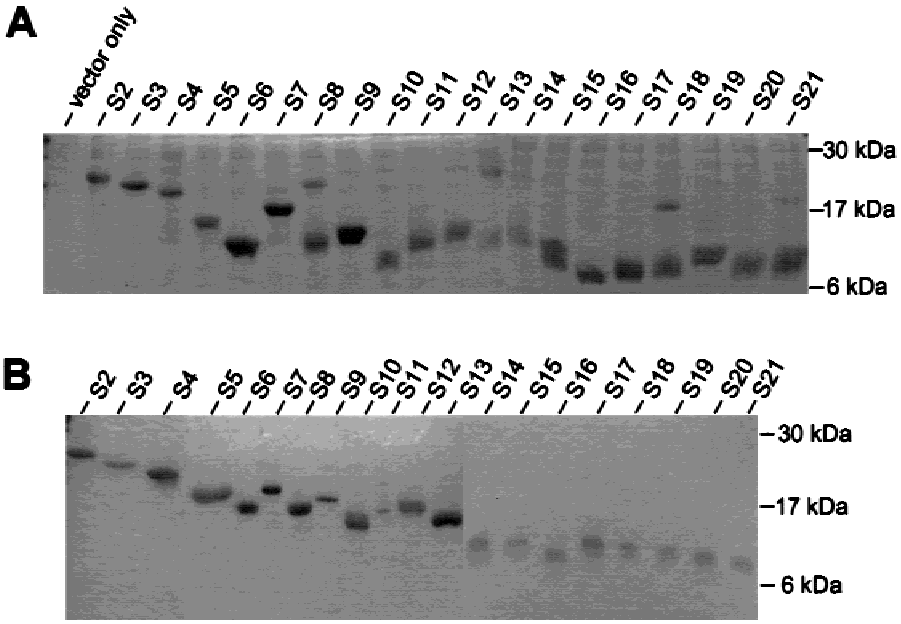

FIGURE 1. Overexpressed and purified recombinant small subunit ribosomal proteins S2–S21. **A**: Induction of the ribosomal proteins S2–S21 expression. Vector only: cells harboring pET24b; S2–S21: cells harboring the ribosomal protein genes encoding S2–S21 cloned into pET24b. **B**: 7.5 μg of individually purified recombinant ribosomal proteins S2–S21 post-FPLC purification and dialysis.

TABLE 1. Properties of *E. coli* ribosomal proteins S2–S21 relevant to purification of the recombinant proteins.

Protein	Solubility[a]	Buffer[b]	Elution [KCl]	pI[c]	Mw[d]	Yield[e]
S2	I	C	140	6.7	26,613	70
S3	I	B	210	12	25,852	6.0
S4	S	B	200	10.4	23,137	47
S5	I	B	160	9.9	17,515	50
S6	I	C	140	4.9	15,704	430
S7	I	B	200	12.2	19,732	50
S8	I	B	110	9.1	13,996	70
S9	I	B	240	12	14,725	5.0
S10	S	C	170	7.9	11,736	10
S11	I	B	230	12	13,728	2.5
S12	S	B	230	12	13,606	40
S13	S	B	230	12	12,968	50
S14	S	B	270	>11	11,191	60
S15	S	B	210	>12	10,137	75
S16	S	B	190	11.6	9,191	65
S17	I	C	190	9.7	9,573	25
S18	I	B	250	>12	8,896	30
S19	S	B	260	>12	10,229	90
S20	S	B	230	>12	9,553	40
S21	I	B	230	>12	8,369	40

[a]Solubility of the majority of overexpressed protein in extract; I: insoluble; S: soluble.

[b]Buffer used for dialysis and FPLC column chromatography; B: 20 mM Tris-HCl (pH 7.0), 20 mM KCl, 6 M Urea, and 6 mM BME; C: 20 mM NaOAc (pH 5.6), 20 mM KCl, 6 M Urea, and 6 mM BME.

[c]Isoelectric point taken from Kaltschmidt (1971).

[d]Molecular weight, taken from Giri et al. (1984).

[e]Milligrams of purified protein obtained from a 500-mL starting culture.

thus the level of overexpressed protein appears to be significantly greater than what is obtained in purified form (compare Fig. 1A and Table 1).

Reconstitution of 30S subunits using a complete set of recombinant proteins

Once purified, the recombinant proteins were assayed for their ability to support 30S subunit reconstitution with 16S rRNA by sucrose gradient sedimentation analysis. Natural 30S subunits and particles reconstituted using TP30 were used as controls for sedimentation and reconstitution (Fig. 3). Following procedures of Nomura and coworkers (Traub & Nomura, 1969; Mizushima & Nomura, 1970), initial attempts at reconstitution using up to an eightfold excess of an equimolar mixture of the recombinant proteins over 16S rRNA resulted in very inefficient reconstitution (Fig. 3A).

In an attempt to facilitate reconstitution, the results of earlier studies that mapped the pathway and order of in vitro protein assembly into 30S subunits were used as a guide to subdivide the proteins into three groups (Table 2), based on their requirements for assembling on the growing RNP (Mizushima & Nomura, 1970; Held et al., 1974). Sequential addition of the recombinant proteins, using these groupings, and incubation with 16S rRNA results in efficient reconstitution (Fig. 3B). Optimal reconstitution was observed using a fourfold molar excess of protein to 16S rRNA (Fig. 3B). Approx-

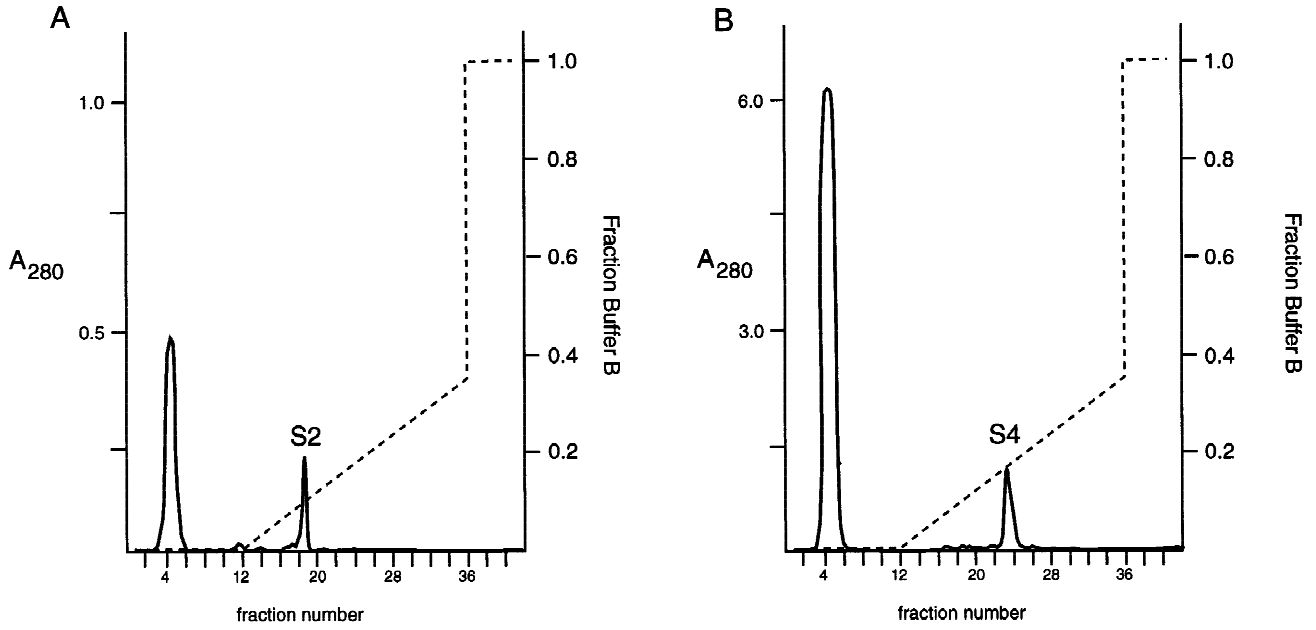

FIGURE 2. Representative chromatograms of FPLC purification of overexpressed (**A**) ribosomal protein S2 and (**B**) ribosomal protein S4 on a cation exchange column (Resource S). Samples were loaded in Buffer A and a linear 125 mL gradient of Buffer B was introduced, starting at fraction 12. Peaks corresponding to S2 and S4 are indicated.

TABLE 2. Subsets of proteins used for ordered reconstitution.

Group	Assembly map[a]	Assembly kinetics[b]
I	S4	S4
	S7	S6
	S8	S11
	S15	S15
	S17	S16
	S20	S17
		S18
		S20
II	S5	S7
	S6	S8
	S9	S9
	S11	S13
	S12	S19
	S13	
	S16	
	S18	
	S19	
II′		S5
		S12
III	S2	S2
	S3	S3
	S10	S10
	S14	S14
	S21	S21

[a]Based on Held et al. (1974) and Mizushima & Nomura (1970).
[b]Based on Powers et al. (1993).

imately 45% of the input 16S rRNA was incorporated into 30S subunits following this procedure (Table 3; Fig. 3); in contrast, when all the recombinant proteins were added in a single step only 18% of the input 16S rRNA was incorporated into 30S subunits (Fig. 3A,C). There is a small amount of rapidly sedimenting material that is observed in the 30S subunits reconstituted with recombinant proteins (Fig. 3B), which may be composed of 30S subunit dimers.

An alternative ordered assembly protocol was tested, in which the proteins were divided into four groups, based on their order of assembly inferred from the kinetics of in vitro assembly monitored by chemical probing of 16S rRNA (Powers et al., 1993; Table 2). Sequential addition of the recombinant proteins, following these groupings, also results in efficient reconstitution (Fig. 3C, IV), with a similar protein:RNA optimum of 4:1 (data not shown). These data suggest that sequential addition of the recombinant proteins overcomes a kinetic barrier to reconstitution. Since sequential addition of proteins following either grouping (Table 2) results in efficient reconstitution (Fig. 3C), all subsequent experiments were done using the groupings based on the in vitro assembly map (Mizushima & Nomura, 1970; Held et al., 1974).

A potential use of the recombinant protein reconstitution system is construction of 30S subunits containing a single Fe(II)-derivatized protein for directed hydroxyl radical probing of 16S rRNA. In previous stud-

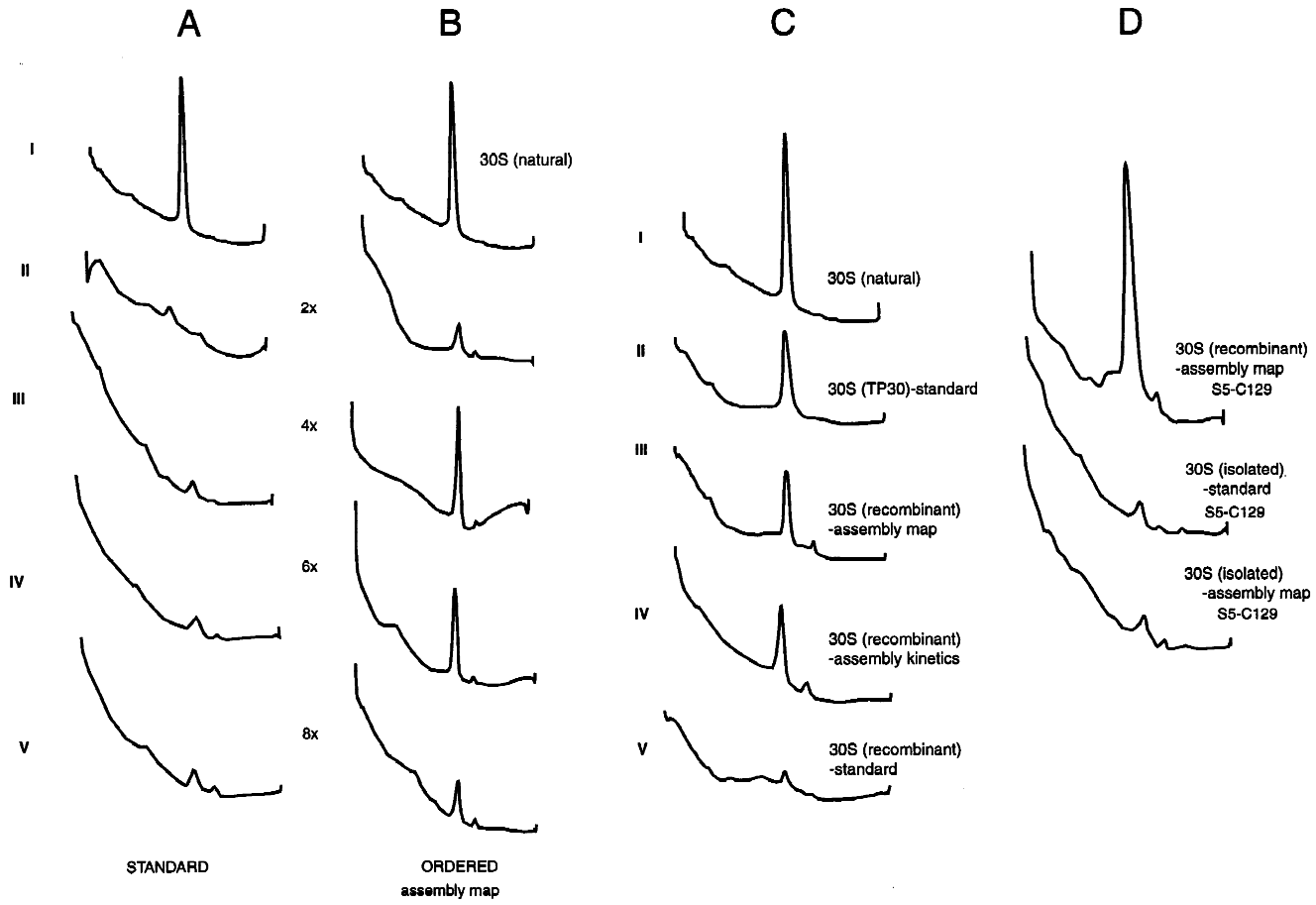

FIGURE 3. Sedimentation analysis of in vitro reconstitution of 30S subunits using a complete set of recombinant small subunit proteins. Reconstitution of 30S subunits using a complete set of small subunit recombinant proteins and (**A**) standard reconstitution conditions (Traub & Nomura, 1969) or (**B**) ordered assembly following the assembly map (Table 2; Mizushima & Nomura, 1970; Held et al., 1974). (I) natural 30S subunits; 30S subunits reconstituted with 16S rRNA (30 pmol) and a complete set of recombinant small subunit proteins; (II) 2 molar equivalents protein; (III) 4 molar equivalents protein; (IV) 6 molar equivalents protein; (V) 8 molar equivalents protein. **C**: Comparison of 30S subunit reconstitution systems and procedures. (I) natural 30S subunits; 30S subunits reconstituted with 16S rRNA (30 pmol) and (II) TP30, a mixture of total proteins isolated from 30S subunits, following the standard protocol (Traub & Nomura, 1969); a complete set of recombinant small subunit proteins following (III) the assembly map groupings (Table 2); (IV) the assembly kinetics groupings (Table 2); (V) the standard protocol (Traub & Nomura, 1969). **D**: Comparison of 30S subunit reconstitution with Fe(II)-derivatized S5. 30S subunits reconstituted with 16S rRNA (60 pmol), Fe(II)-C129-S5 and (I) the remaining recombinant small subunit proteins following the assembly map groupings; the remaining small subunit proteins individually isolated from 30S subunits following (II) the standard protocol (Traub & Nomura, 1969); (III) the assembly map groupings. Sedimentation is from left to right, and absorbance was monitored at 254 nm.

TABLE 3. Recovery, tRNA binding, and polyphenylalanine synthesis of reconstituted 30S subunits.

30S subunits	Recovery[a] (%)	$tRNA^{Phe}$ binding[b] poly(U)-dependent (% activity)	$tRNA^{Phe}$ binding[b] poly(U)-independent (% activity)	Polyphe synthesis[c] (% activity)
Natural[d]		100 ± 3	20 ± 4	100 ± 2
TP30[e]	70	72 ± 5	16 ± 3	79 ± 3
Recombinant[f]	45	48 ± 4	12 ± 3	34 ± 2

[a]Recovery is based on comparison of amount of input 16S rRNA to the amount of 30S subunits isolated and purified from sucrose gradients.
[b]For tRNA binding, 100% binding is equal to 0.7 pmol $tRNA^{Phe}$ bound/pmol of 30S subunits.
[c]For polyphenylalanine synthesis, 100% activity is equal to 9.0 pmol of polyphenylalanine synthesized/pmol 30S subunits.
[d]Natural 30S subunits.
[e]30S subunits reconstituted with 16S rRNA and proteins isolated from 30S subunits.
[f]30S subunits reconstituted with 16S rRNA and a complete set of recombinant proteins.

ies, 30S subunits containing a single Fe(II)-derivatized protein were reconstituted from proteins that had been individually isolated from ribosomes (Heilek et al., 1995; Heilek & Noller, 1996a, 1996b); therefore, we wished to compare the efficiencies of these two reconstitution systems for preparing such constructs. Ribosomal protein S5 derivatized with Fe(II) at a unique site (Fe-C129-S5; Heilek & Noller, 1996b; Culver et al., 1999), was reconstituted with 16S rRNA and a full complement of the remaining recombinant proteins or proteins that had been individually purified from ribosomes (Fig. 3D). Reconstitution of 30S subunits was dramatically more efficient using ordered assembly with the recombinant proteins (Fig. 3D, I) compared to reconstitution using proteins individually isolated from ribosomes, either under standard conditions (Fig. 3D, II) or using the ordered assembly protocol (Fig. 3D, III). Clearly, the recombinant protein system is advantageous for obtaining efficient reconstitution of 30S subunits containing a single derivatized protein.

Structural characterization of the reconstituted 30S subunits

The structural integrity of the recombinant protein reconstituted 30S subunits was assessed by base-specific chemical probing of 16S rRNA and by analysis of the protein composition of the reconstituted particles. Base-specific chemical modification of 16S rRNA is a sensitive probe of both folding of 16S rRNA and proper assembly of proteins in the reconstituted subunits. Dimethyl sulfate (DMS) was used to probe the conformation of naked 16S rRNA, 16S rRNA in natural 30S subunits, and 30S subunits reconstituted with either TP30 or recombinant proteins (Fig. 4). The modification patterns of 16S rRNA in all isolated 30S subunits are very similar and distinct from that of naked 16S rRNA (Fig. 4). Many nucleotides that are available for modification on naked 16S rRNA are protected from modification in all 30S subunits probed (Fig. 4). Additionally, there are nucleotides that have enhanced reactivity to DMS in 30S subunits compared to naked 16S rRNA. The similar DMS modification pattern observed for 30S subunits reconstituted with recombinant proteins, natural 30S subunits, and 30S subunits reconstituted with TP30 suggests similar folding of 16S rRNA and protein protections in these three sets of 30S subunits. Additionally, the enhanced reactivity of a few nucleotides suggests that the local RNA environment is similar in all 30S subunits probed, yet distinct from naked 16S rRNA.

Although the change in DMS modification pattern between naked 16S rRNA and 16S rRNA in 30S subunits likely reflects protein-dependent changes in 16S rRNA conformation and protection of nucleotides by assembled proteins, we also analyzed the protein composition of the reconstituted particles directly, by two-dimensional gel electrophoresis (Fig. 5). There is very little qualitative difference between the proteins isolated from natural 30S subunits (Fig. 5A) and the two sets of reconstituted particles (Fig. 5B,C). No proteins appear to be either missing from or greatly overrepresented in 30S subunits reconstituted with recombinant proteins. Thus, these reconstituted 30S subunits have a protein composition similar to that of natural 30S and TP30 reconstituted 30S subunits.

Functional characterization of the recombinant reconstituted particles

The functional state of the recombinant reconstituted 30S particles was assessed by their ability to associate with 50S subunits, to bind tRNA and to participate in poly(U)-directed polyphenylalanine synthesis. Reconstituted, unpurified 30S subunits, as well as control natural 30S subunits, were incubated with natural 50S subunits, and the formation of 70S ribosomes was monitored by sucrose gradient sedimentation (Fig. 6). It is clear from the absence of material sedimenting at 30S that all of the 30S subunits were competent for association with 50S subunits (Fig. 6). The lower yield of 70S ribosomes using the recombinant reconstitution system (Fig. 6D) parallels the lower yield of 30S subunits in reconstitution (Fig. 3C). Isolated and purified 30S subunits (natural, recombinant protein, and TP30) were assayed for their ability to bind tRNA using poly(U) as a template. Recombinant-protein reconstituted 30S subunits were approximately 50% as active as natural 30S subunits in tRNA binding (Table 3). For comparison, 30S subunits reconstituted with TP30 showed intermediate activity, approximately 70% of that of natural 30S subunits (Table 3). The activity observed for 30S subunits reconstituted with recombinant proteins is comparable to that previously reported for 30S subunits reconstituted from proteins individually isolated from subunits (Table 3; Nomura et al., 1969). The residual poly(U)-independent binding is in the range of that expected under the $MgCl_2$ concentrations used in the reconstitutions and subsequent assays (Lill et al., 1986). Lastly, the reconstituted 30S subunits were assayed for their ability to function in poly(U)-directed polyphenylalanine synthesis. In the presence of natural 50S subunits and cofactors, purified recombinant-protein reconstituted 30S subunits support polyphenylalanine synthesis, with an activity, relative to natural 30S subunits, that is similar to that observed for tRNA binding (Table 3), and very similar to that previously reported for 30S subunits reconstituted from individually purified natural proteins (Nomura et al., 1969).

DISCUSSION

These experiments demonstrate efficient reconstitution of functional 30S subunits from a complete set of

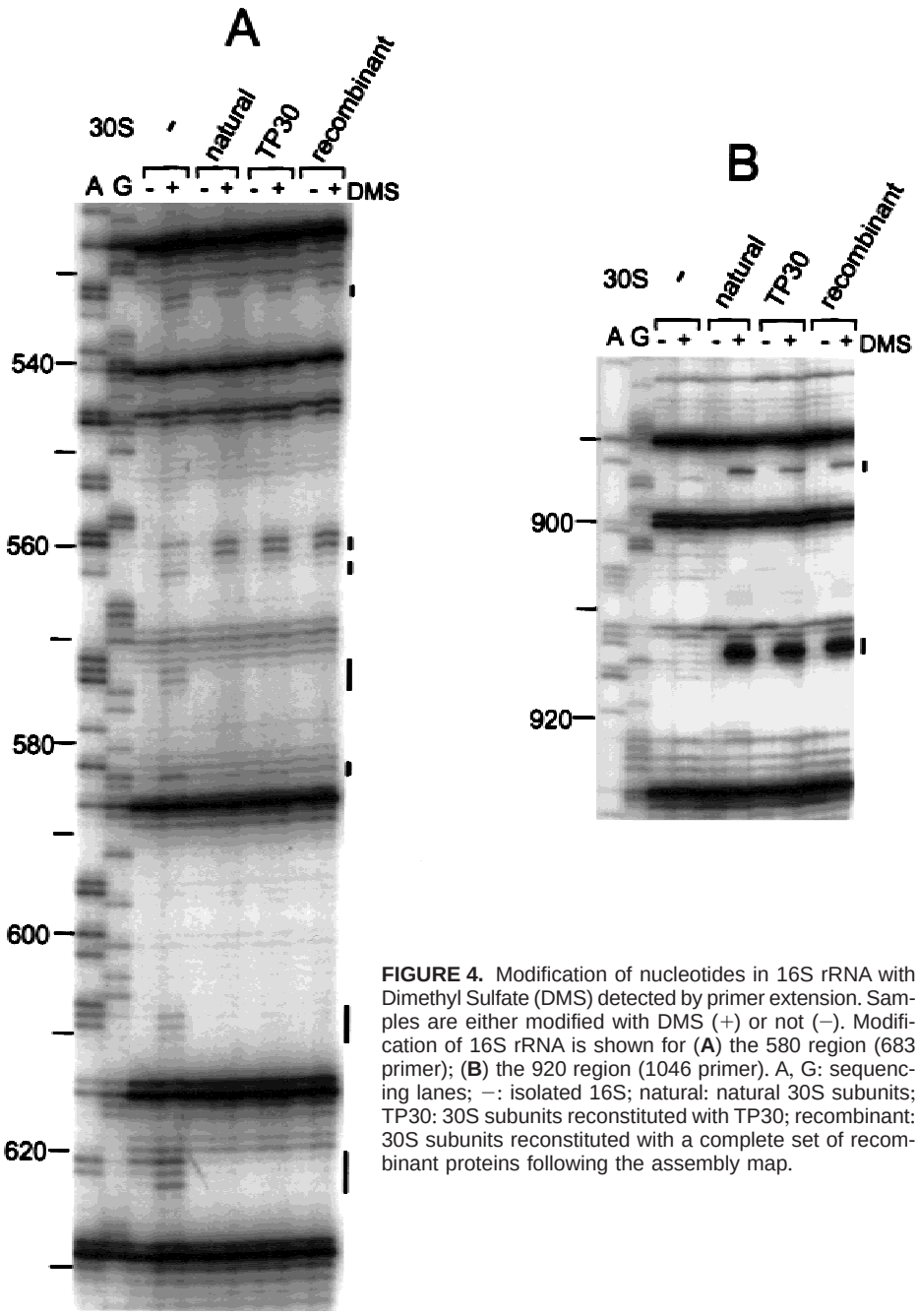

FIGURE 4. Modification of nucleotides in 16S rRNA with Dimethyl Sulfate (DMS) detected by primer extension. Samples are either modified with DMS (+) or not (−). Modification of 16S rRNA is shown for (**A**) the 580 region (683 primer); (**B**) the 920 region (1046 primer). A, G: sequencing lanes; −: isolated 16S; natural: natural 30S subunits; TP30: 30S subunits reconstituted with TP30; recombinant: 30S subunits reconstituted with a complete set of recombinant proteins following the assembly map.

recombinant small subunit ribosomal proteins and 16S rRNA. Initial attempts at reconstitution using the recombinant proteins, following standard procedures (Traub & Nomura, 1969), resulted in very inefficient reconstitution (Fig. 3A). We hypothesized that reconstitution might be facilitated by addition of the ribosomal proteins in an ordered manner reflecting 30S subunit assembly. Therefore, the proteins were divided into discrete sets based on either the in vitro 30S subunit assembly map (Mizushima & Nomura, 1970; Held et al., 1974; Table 2) or the results of in vitro 30S subunit assembly kinetics (Powers et al., 1993; Table 2). Sequential addition of the recombinant proteins using either grouping resulted in efficient reconstitution (Table 2; Fig. 3). The 16S rRNA in 30S subunits reconstituted with recombinant proteins had a DMS modification pattern indistinguishable from that of natural 30S subunits or subunits reconstituted from TP30 (Fig. 4). The protein composition of the recombinant 30S subunits is similar to that of natural 30S subunits and 30S subunits reconstituted from TP30 (Fig. 5). In addition, 30S subunits reconstituted with recombinant proteins function in subunit association (Fig. 6), tRNA binding (Table 3), and polyphenylalanine synthesis (Table 3), although they are

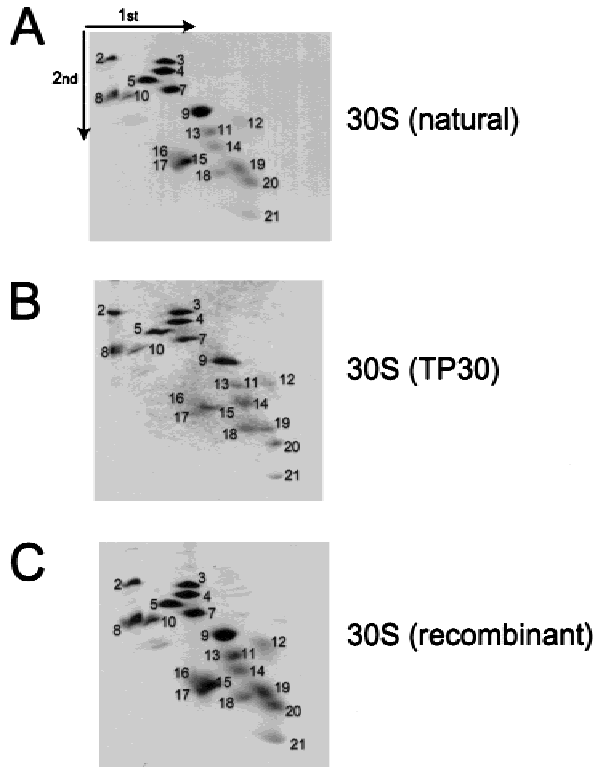

FIGURE 5. Analysis of protein composition of 30S subunits by two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis analysis (Geyl et al., 1981) of proteins extracted from (**A**) natural 30S subunits; 30S subunits reconstituted with 16S rRNA and (**B**) TP30, total protein isolated from 30S subunits following the standard protocol (Traub & Nomura, 1969); (**C**) recombinant, a complete set of recombinant small subunit proteins following the assembly map groupings.

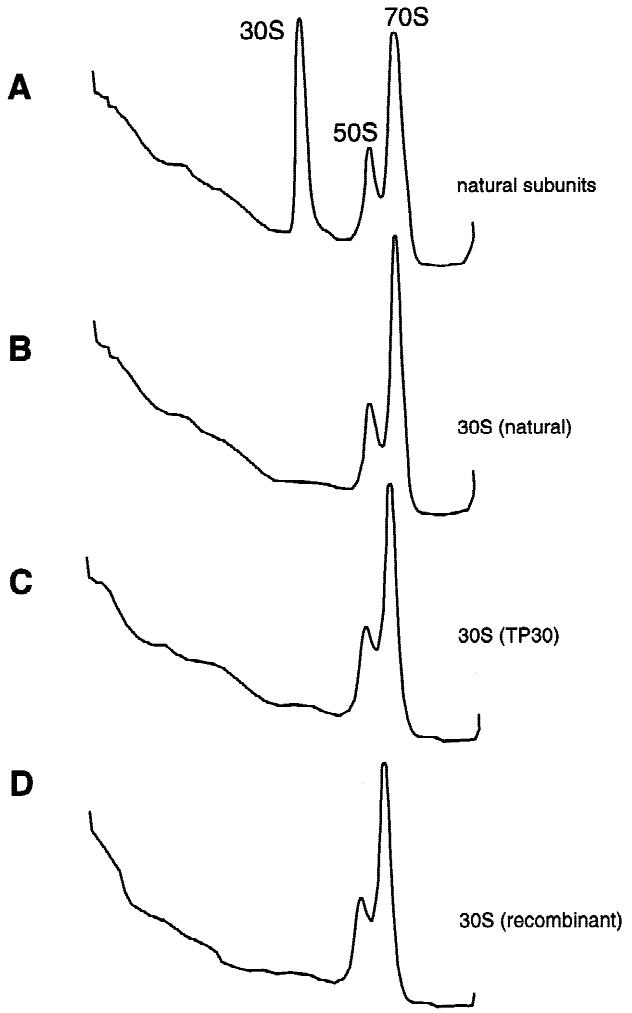

FIGURE 6. Sedimentation analysis of ribosomal subunit association using 30S subunits reconstituted in vitro with a complete set of recombinant small subunit proteins. **A**: Natural 30S (50 pmol) and 50S (40 pmol) subunits incubated under conditions to yield a mixture of free subunits and 70S ribosomes. **B–D**: Full association of 50S subunits with (**B**) natural 30S subunits (natural); (**C**) 30S subunits reconstituted with TP30 (total protein isolated from 30S subunits) and 16S rRNA (40 pmol) (TP30); (**D**) a complete set of recombinant small subunit proteins and 16S rRNA (60 pmol) (recombinant). Sedimentation is from left to right, and absorbance was monitored at 254 nm.

somewhat less active than either natural 30S subunits or 30S subunits reconstituted with TP30. This is in agreement with previous work that showed that 30S subunits reconstituted with proteins individually isolated from subunits also had lower activity than 30S subunits reconstituted with TP30 (Mizushima & Nomura, 1970). These data provide evidence that the recombinant reconstituted 30S subunits are structurally and functionally very similar to subunits reconstituted with proteins isolated as a mixture from ribosomes and sufficiently similar to natural 30S subunits to provide a useful approach to the study of their structure and function.

Reconstitution of 30S ribosomal subunits from 16S rRNA and ribosomal proteins that were individually purified from subunits was achieved nearly 25 years ago (Held et al., 1973). One major difference between these earlier findings and the work presented here is the necessity of ordered addition of the recombinant proteins. The basis of this difference is still unknown. It is possible that addition of the proteins in discrete sets based on their order of incorporation onto the assembling RNP increases the efficiency of reconstitution by avoiding kinetically trapping incorrectly assembled complexes. In this study and in previous work, a decrease in reconstitution efficiency was observed as the ratio of protein to 16S rRNA was increased above the optimum, suggesting that the presence of additional protein could result in nonproductive interactions, such as dead-end assembly complexes (Nomura et al., 1969; see Fig. 3B). Because reconstitution is optimal with a fourfold molar excess of recombinant proteins to 16S rRNA for both groupings (Fig. 3; data not shown), as compared to a 1.8- to 2-fold molar excess of TP30 (Nomura et al., 1969), it is possible that, while one or more of the recombinant proteins are required in a larger amount, increased levels of the other recombinant proteins could contribute to nonproductive interactions when

added en masse. Sequential addition of the proteins may reduce the interference of the excess protein with productive interactions.

The requirement for ordered addition of the recombinant proteins for efficient reconstitution could be due to a number of possible factors. Some of the small subunit ribosomal proteins are subject to posttranslational modification (Leibowitz & Soffer, 1971; Cumberlidge & Isono, 1979; Reeh & Pedersen, 1979; Isono & Isono, 1980; Kowalak & Walsh, 1996). Therefore, one or more of the overproduced, recombinant proteins might be substoichiometrically modified. Although all of the recombinant proteins were overexpressed in *E. coli* and thus available to their natural modification enzymes, the levels of expression may exceed the capacity of the endogenous enzymes. Ordered assembly could obviate the need for specific modifications; binding a subset of proteins to 16S rRNA could promote or increase the lifetime of transiently formed intermediates, thus allowing an inadequately modified protein, perhaps with a weakened binding affinity, the opportunity to interact productively with its target, prior to the addition of other proteins and subsequent conformational changes. Proteins that were isolated from ribosomes may be in a more functional conformation if prior ribosome assembly involves rearrangement of protein structure; therefore, it is possible that one or more of the recombinant proteins is incompletely folded as isolated, and that other proteins and/or RNA could stimulate their folding into a more functional conformation. Thus, ordered assembly could help this folding problem if incubating the incompletely folded protein with a subset of small subunit proteins or 16S rRNA potentiates folding. This could reflect the process that occurs in vivo, where a subset of proteins might initiate assembly cotranscriptionally. Either of the above possibilities could result in overestimation of the concentration of functional protein. The same would be true if a subpopulation of protein(s) were inactivated during purification, as was hypothesized for proteins isolated from ribosomes (Nomura et al., 1969).

A complete set of recombinant small subunit ribosomal proteins was produced with the hope of generating an easily renewable source of large quantities of highly purified individual proteins, obviating the difficult and laborious purification of individual proteins from ribosomal subunits. This set of proteins will be of great use, not only in studying the proteins themselves but, also as tools for studying the structure, function, and assembly of 30S subunits and 70S ribosomes. Along with the ability to purify tens of milligrams of ribosomal proteins with relative ease, the high levels of overexpression of these proteins can provide proteins that are essentially free of contamination with other ribosomal and cellular proteins. Finally, systematic mutational analysis of the small subunit ribosomal proteins is facilitated by the availability of useful clones encoding all of their genes.

MATERIALS AND METHODS

Reagents

Preparation of 16S rRNA, 30S, 50S, and 70S ribosomes was as described (Moazed et al., 1986). Preparation of TP30 was as previously described (Nierhaus, 1990). $tRNA^{Phe}$ was transcribed from p67CF10 (Sampson et al., 1989) as previously described (Milligan et al., 1987). Purification of individual ribosomal proteins from subunits was as previously described (Stern et al., 1988). Buffer A consisted of 80 mM K^+-HEPES (pH 7.6), 20 mM $MgCl_2$, 330 mM KCl, and 0.01% Nikkol. Buffer B consisted of 20 mM Tris-HCl (pH 7.0), 20 mM KCl, 6 M Urea, and 6 mM β-mercaptoethanol (BME). Buffer C consisted of 20 mM NaOAc (pH 5.6), 20 mM KCl, 6 M Urea, and 6 mM BME. Buffer D consisted of 80 mM K^+-HEPES (pH 7.6), 20 mM $MgCl_2$, 1 M KCl, and 6 mM BME. Buffer E consisted of 20 mM K^+-HEPES (7.6), 20 mM KCl, and 6 mM BME.

Cloning the genes encoding ribosomal proteins S2–S21

The genes encoding S5 and S17 had previously been cloned (Heilek & Noller, 1996b; G.M. Heilek & H.F. Noller, unpubl. results). The genes encoding ribosomal proteins S2–S4, S6–S16, and S18–S21 were amplified by polymerase chain reaction (PCR) of *E. coli* MRE600 genomic DNA. An *Nde*I restriction enzyme site was included at the 5′ end of every clone. Either a *Bam*HI (S2–S4, S6, S8–S16, S18, S19, and S21) or a *Hin*dIII (S7 and S20) restriction enzyme site was included at the 3′ end of the genes. The PCR products were cleaved with the appropriate enzymes (see above) and ligated into pET24b that had been cleaved with the same enzymes and purified. Wild-type clones were identified by sequence analysis and transformed into BL21(DE3), where the proteins were overexpressed from an inducible T7 promoter on the plasmid (Studier et al., 1990; Novagen). For overexpression, strains harboring the plasmids were grown to an approximate OD_{600} of 0.4, IPTG was added to a final concentration of 1 mM, and the cultures were grown for an additional 4 h prior to harvesting. Cells were washed once with Buffer E and stored at −20 °C. For analysis of overexpression, equal volumes of induced cell culture and SDS-PAGE loading dye containing 6 M Urea were mixed, and 30 μL of this mixture was analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970; 4% stacking gel; 12% resolving gel, both containing 6 M Urea).

Purification of recombinant ribosomal proteins S2–S21

Cells containing overexpressed protein were disrupted by sonication at 4 °C in Buffer E. Postsonication centrifugation at 4 °C either cleared the protein-containing lysate or pelleted the protein-containing inclusion bodies. Inclusion body pellets containing overexpressed protein (see Table 1) were resuspended in Buffer B (S3, S5, S8, S9, S11, S18, and S21) or Buffer C (S2, S6, and S17) and dialyzed overnight against two changes of the same buffer at 4 °C. Soluble proteins (see Table 1) were dialyzed overnight at 4 °C against

three changes of Buffer B (S4, S7, S12–S16, S19, and S20) or Buffer C (S10). Proteins (S2–S5, S7–S21) were purified at 4 °C by FPLC cation exchange chromatography using a Resource S column (Pharmacia) with 125 mL linear gradients (20–350 mM KCl) starting in dialysis buffer (B or C, as appropriate). Ribosomal protein S6 was purified at 4 °C by FPLC chromatography on a Resource Q anion exchange column (Pharmacia) developed in Buffer C with a 125 mL linear gradient from 20–350 mM KCl. The concentration of KCl at which each protein eluted from the column is given in Table 1. Protein-containing fractions (5 mL) were identified by SDS-polyacrylamide electrophoresis (Laemmli, 1970; see above) and dialyzed against Buffer D, except for S10 and S18, which were dialyzed against Buffer D plus 4 M Urea. Protein concentration was determined by Bradford assay (Bio-Rad). Aliquots of the proteins were frozen at −80 °C.

Fe(II)-derivatization of S5 proteins

Synthesis of 1-(*p*-bromoacetamidobenzyl)-EDTA (BABE), and preparation of Fe(II)-BABE complex was done as previously described (DeRiemer et al., 1981; Heilek et al., 1995). Conjugation of Fe(II)-BABE to cysteine-containing mutant of S5–C129 and purification of derivatized protein from unreacted reagent was done essentially as described (Heilek & Noller, 1996b). Briefly, 3 nmol of S5–C129 were incubated with 150 nmol of Fe(II)-BABE at 37 °C for 20 min in 100 μL buffer containing 20 mM Tris-HCl, pH 7.5, 1 M KCl, and 0.01% Nikkol. Fe(II)-C129-S5 was purified from excess BABE by centrifugation at 6,500 rpm in Microcon 3 microconcentrators for 30 min. After four 400 μL washes with modification buffer, the concentration of recovered protein was determined by Bradford assay.

Reconstitution and purification of 30S subunits

Prior to reconstitution, mixtures of pure recombinant proteins were prepared following either the 30S subunit assembly map (am; see Table 2): Group I (am), containing equimolar amounts of S4, S7, S8, S15, S17, and S20; Group II (am), containing equimolar amounts of S5, S6, S9, S11, S12, S13, S16, S18, and S19; Group III, containing equimolar amounts of S2, S3, S10, S14, and S21; or following assembly kinetics (ak; see Table 2): Group I (ak), S4, S6, S11, S15, S16, S17, S18, and S20; Group II (ak), S7, S8, S9, S13, and S19; Group II′ (ak), S5 and S12; Group III, same as above. These protein mixtures were concentrated on Microcon 3 microconcentrators at 4 °C, and protein concentration determined by Bradford assay. Protein mixtures were aliquoted and stored at −80 °C. For experiments using the proteins that were individually isolated from subunits, mixtures were prepared as described above following the assembly map. The salt concentration of the protein mixtures was maintained at 1 M KCl (Buffer D) to ensure that the proteins remained in solution. Since reconstitution proceeds at 330 mM KCl (Buffer A), the KCl concentration must be readjusted after addition of each mixture of proteins during reconstitution. Reconstitution of 30S particles was done using a fourfold molar excess of each purified recombinant protein over 16S rRNA, following an ordered assembly protocol. In a standard reaction, 40 pmol of 16S rRNA were incubated in 5 μL of Buffer A minus KCl at 42 °C for 15 min. Group I proteins were added to 16S rRNA, the buffer conditions were adjusted to those of Buffer A, and the reaction was incubated at 42 °C for 20 min. Group II proteins were then added to the reconstitution reaction, buffer conditions were again adjusted to those of Buffer A, and the resulting reaction was again incubated at 42 °C for 20 min. Lastly, Group III proteins were added to the reaction, the KCl concentration adjusted to that of Buffer A in a final volume of 100 μL, and the reaction incubated at 42 °C for 20 min. For reconstitution using the assembly kinetics groupings, the same procedure was followed except for an additional incubation with Group II′ proteins, after the addition of Group II and prior to the addition of Group III. Reconstitution using TP30 was performed as previously described (Powers et al., 1993). Reconstitution of 30S subunits was analyzed by sucrose gradient sedimentation using 10–40% sucrose gradients in 20 mM K^+-HEPES (pH 7.6), 20 mM $MgCl_2$, and 330 mM KCl centrifuged in a SW41 rotor (32,000 rpm) for 15.5 h at 4 °C. Peaks sedimenting at 30S were isolated, and sucrose was removed from 30S subunits by centrifugation at 4 °C for 60 min at 2,400 rpm in Centricon 100 ultraconcentrators in a JA-20 rotor with three to four sequential 2 mL washes with buffer A.

Subunit association of reconstituted 30S subunits and native 50S subunits

In a standard reaction, unpurified reconstituted 30S subunits were incubated with 30 pmol natural 50S subunits in 80 mM K^+-HEPES (pH 7.6), 20 mM $MgCl_2$, 100 mM KCl, and 0.003% Nikkol at 37 °C for 30 min. Sucrose gradient sedimentation using 10–40% sucrose gradients in 20 mM K^+-HEPES (pH 7.6), 20 mM $MgCl_2$, and 100 mM KCl centrifuged in a SW41 rotor (32,000 rpm) for 15.5 h at 4 °C was used to analyze subunit association.

Dimethyl sulfate probing of 16S rRNA in 30S subunits

Chemical probing using dimethyl sulfate (DMS) was performed as described by Stern et al. (1988), with slight modification. Briefly, 5 pmol of isolated 16S rRNA or 30S subunits were incubated in 50 μL Buffer A on ice for 60 min with 12 mM DMS (final concentration). Samples were precipitated with 3 vol ethanol and 0.1 vol 3 M NaOAc (pH 5.2). RNA was isolated and analyzed as previously described (Stern et al., 1988).

Polyphenylalanine synthesis

Polyphenylalanine synthesis was assayed essentially as described by Nomura and co-workers (Traub et al., 1981). Briefly, 10 pmol isolated 30S subunits and 10 pmol natural 50S subunits were incubated at 37 °C for 20 min. To the ribosomes, 100 pmol $tRNA^{Phe}$, 1.2 μL 0.5 mg/mL pyruvate kinase, 2.4 μL ^{14}C-phenylalanine, 1 mM phenylalanine, 1 μL S100 extract, 9 μL polyphenylalanine buffer were added, the final volume adjusted to 30 μL with water, and incubated at 30 °C for 10 min. Poly(U) (8 μg) was added to initiate the reaction and the sample was incubated at 30 °C. Reactions were stopped

by spotting on Whatman filter paper at 0, 5, 10, and 20 min and filters were submerged in ice-cold 10% TCA after spotting. Filters were boiled twice in 5% TCA, washed with 95% ethanol, dried, and counted.

Transfer RNA binding

Transfer RNA binding (Nirenberg & Leder, 1964) was performed as previously described with slight modification (Moazed & Noller, 1986). Ribosomal particles (5 pmol) were incubated with 10 pmol [^{32}P]tRNA^{Phe} (3′-end labeled with [^{32}P]-pCp) and 7.5 mg poly(U) in 50 μL 20 mM $MgCl_2$, 100 mM KCl, 80 mM K^+-HEPES (pH 7.6) for 15 min at 37 °C followed by 10 min on ice. Reactions were spotted on nitrocellulose filters, washed, and counted.

Two-dimensional protein gel electrophoresis

Proteins were extracted from 100 pmol of isolated 30S subunits as previously described (Siegmann & Thomas, 1987). Two-dimensional protein gel electrophoresis of the recovered proteins was performed as previously described (Geyl et al., 1981).

ACKNOWLEDGMENTS

We thank Gabriella Heilek for the clones of S5 and S17 and Lisa Newcomb for preparation of TP30. We thank Lovisa Holmberg, Kate Lieberman, and Lisa Newcomb for critical comments on the manuscript and J. Moran, D.P. Greiner, and S. Joseph for 1-(*p*-bromoacetamidobenzyl)-EDTA (BABE), which was synthesized in the laboratory of C. Meares. This work was supported by National Institutes of Health (NIH) grant No. GM-17129 (to H.F.N.), NIH postdoctoral fellowship No. 1F32GM18065-01 (to G.M.C.), and a grant to the Center for Molecular Biology of RNA from the Lucille P. Markey Charitable Trust.

Manuscript accepted without revision April 5, 1999

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