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Separation of mutant and wild-type ribosomes based on differences in their anti Shine–Dalgarno sequence

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ABSTRACT

We describe a system to isolate 30S ribosomal subunits which contain targeted mutations in their 16S rRNA. The mutations of interest should be present in so-called specialized 30S subunits which have an anti-Shine–Dalgarno sequence that is altered from 5′ ACCUCC to 5′ ACACAC. These plasmid-encoded specialized 30S subunits are separated from their chromosomally encoded wild-type counterparts by affinity chromatography that exploits the different Shine–Dalgarno complementarity. An oligonucleotide complementary to the 3′ end of wild-type 16S rRNA and attached to a solid phase matrix retains the wild-type 30S subunits. The flow-through of the column contains close to 100% mutant 30S subunits. Toeprinting assays demonstrate that affinity column treatment does not cause significant loss of activity of the specialized particles in initiation complex formation, whereas elongation capacity as determined by poly(Phe) synthesis is only slightly decreased. The method described offers an advantage over total reconstitution from *in vitro* transcribed mutant 16S rRNA since our 30S subunits contain the naturally occurring base modifications in their 16S rRNA.

INTRODUCTION

Although rRNA emerges as the key molecule for ribosome functioning (1), studies to assign functions to specific rRNA sequences have made only limited progress. Since most organisms have multiple rRNA gene copies, which are thought to be functionally identical, recessive mutations in one of these genes will be phenotypically suppressed by the wild-type rRNA expressed from the non-mutated genes. The use of plasmid-borne rRNA genes containing antibiotic resistance markers avoids this problem and enables *in vivo* selection (2) or detection (3–5) of rRNA mutants. *In vitro* studies on such 16S rRNA mutants are complicated, however, by the background activity of wild-type ribosomes (6, 7). Also, ribosomes with defective dominant mutations in their rRNA prove difficult to isolate because they impair growth or are lethal to the host cell (4, 6). One way to circumvent these difficulties is total reconstitution of ribosomal subunits using *in vitro* transcribed mutant 16S rRNA (8). A drawback of this approach is that the *in vitro* transcribed rRNA lacks the naturally occurring base modifications.

Here we present an alternative to *in vitro* reconstitution using the specialized ribosome system developed by Hui and de Boer (9) and recently further exploited by Brink *et al.* (10). In this system two 30S subunit populations coexist in the cell. One harbouring chromosomally encoded wild-type 16S rRNA and synthesizing the cell's regular proteins and the other containing plasmid-derived specialized 16S rRNA. The latter population contains an anti-Shine–Dalgarno (ASD) sequence at the 3′ terminal end altered from 5′ ACCUCC to 5′ ACACAC and these ribosomes translate only those messengers that have the corresponding SD complement. Taking advantage of the different ASD sequences on wild-type and specialized 16S rRNA we have developed an affinity chromatography procedure to isolate essentially pure specialized 30S subunits. Specialized ribosomes with targeted mutations in their 16S rRNA can be prepared using this method. Effects of these mutations on the partial reactions of protein synthesis can be studied *in vitro* which will presumably allow us to relate rRNA regions to specific functions. Due to their altered ASD sequence specialized ribosomes do not translate the majority of the endogenous mRNAs and they constitute a non-essential pool of ribosomes in which any mutation can be introduced without affecting the viability of the cell (10,11).

MATERIALS AND METHODS

Bacterial strains, plasmids and media

As a source of specialized ribosomes we used *E. coli*, strain K5637, described by Hui and de Boer (9), harbouring the plasmid pP$_L$ASDX-Spc^R-CATX, derived from pASDX-PSDRX-P$_L$ (9) and described by Brink *et al.* (10). pP$_L$ASDX-Spc^R-CATX contains the rrnB operon under the control of the thermo-inducible lambda P$_L$ promoter in which the 16S rRNA gene has an altered ASD sequence (5′ ACACAC as compared to 5′ ACCUCC for wild-type) and the spectinomycin resistance mutation, C$_{1192}$ to U$_{1192}$.

pGEMCAT-SDX, used for *in vitro* transcription of CAT mRNA, was constructed by cloning a HindIII–BamH1 fragment containing the *cat* gene with an altered SD sequence (5′ GUGUG,

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complementary to the specialized ASD sequence) from pP_LASDX-Spc^R-CATX into pGEM3Zf(−) (Promega) and was grown in JM101 (12). Strains were grown in LC medium with ampicillin added to a concentration of 100 mg/l. LC medium contains per litre 10 g bactotrypton, 5 g yeast extract, 1 ml 1 M Tris−chloride pH 7.9, 8 g NaCl, 20 mg thymine and 1 g $MgSO_4 \cdot 7H_2O$.

Buffers

Standard buffer contains 10 mM Tris−acetate pH 7.5, 10 mM Mg-acetate, 60 mM NH_4Cl, 0.1 mM EDTA, 2 mM dithiothreitol (Sigma). Buffers A, B and C are as standard buffer but in buffer A the Mg−acetate concentration is changed to 4 mM, in buffer B Mg−acetate is changed to 1 mM and buffer C contains 20 mM Mg−acetate and 100 mM NH_4Cl.

Preparation of 30S and 50S subunits

Bacterial strain K5637 harbouring plasmid pP_LASDX-Spc^R-CATX was grown for 1 h at 30°C to an OD_{650} of 0.1, followed by induction at 42°C for 2 h. After induction these cells harbour a mixture of wild-type 30S subunits containing chromosomally encoded 16S rRNA and specialized 30S subunits containing plasmid-transcribed 16S rRNA. The culture was cooled slowly on ice and cells harvested by centrifugation. An S30 cell extract was prepared by grinding with aluminum oxide as described by van Dieijen *et al.* (13) except that DNase was omitted. 70S tight couples were prepared by centrifuging the S30 extract through a 15−30% sucrose gradient in buffer A for 15 h at 18,000 rpm in a SW27 rotor. Fractions containing the 70S particles were collected and pelleted by centrifugation for 3.5 h at 50,000 rpm in a 50.2 Ti rotor. Pellets were dissolved overnight in buffer A and dialysed for 5 h against buffer B to dissociate the couples. 30S subunits were prepared by sucrose gradient centrifugation in buffer B. The Mg-acetate concentration in the 30S-containing fractions was raised to 10 mM and the subunits were pelleted by centrifugation for 5 h at 50,000 rpm in a 50.2 Ti rotor. Pellets were dissolved overnight in buffer C and 30S subunits were reactivated by incubation for 15 min at 40°C and stored at −80°C.

Wild-type 30S and 50S subunits were prepared from 70S tight couples isolated from frozen MRE600 cells following the procedure described above.

Preparation of the biotin-linked oligodeoxynucleotide

The oligodeoxynucleotide 5′ ATCATCATCTAAGGAGGT was prepared on a Gene Assembler (Pharmacia) using phosphoroamidite chemistry. An *N*-monomethoxytritylaminohexa-6-oxy-β-cyanoethyl *N,N*-diisopropylaminophosphoroamidite amine-linker (Biosearch) was attached to the 5′ end of the oligodeoxynucleotide as described by the supplier. The 5′ amine-activated oligodeoxynucleotide was coupled to the biotin *N*-hydroxysuccinimide (NHS) ester (Fluka) as described (14). The resulting 5′ biotin-linked oligodeoxynucleotide was passed through a Sephadex G25 (medium) column to remove the excess biotin NHS ester, precipitated with alcohol, dried and dissolved in H_2O to a concentration of 150 pmol/μl.

Preparation of the biotin−cellulose

Biotin−cellulose resin (Pierce) was mixed gently for 15 min in 5 volumes of standard buffer containing 500 μg/ml bovine serum albumin (Fluka) and 200 μg/ml herring sperm DNA (Fluka). The biotin−cellulose resin was spun down (3 min, 3000 rpm) and washed three times with 5 volumes of standard buffer by resuspending the pellet and mixing for 10 min. Biotin−cellulose pretreated in this way (binding capacity 1.4 mg streptavidin per ml) was mixed with 0.4 mg (= 7 nmol) streptavidin (Fluka) per ml biotin−cellulose resin in 2 volumes of standard buffer for 5 min at 30°C and for 20 min at 20°C. The resin was pelleted and washed three times with standard buffer as described above. 2 nmol of biotin-linked oligodeoxynucleotide were added per ml biotin−cellulose resin and mixed in 2 volumes of standard buffer for 10 min at 30°C and 20 min at 20°C. After washing three times with standard buffer the resin was ready for use in affinity chromatography.

Determination of 30S subunit identity by primer extension on 16S rRNA

The ratio of specialized to wild-type 30S in any preparation was determined using primer extension on 16S rRNA (15). Due to the C_{1192} to U_{1192} base substitution in specialized 16S rRNA, extension of a [^{32}P]-end-labelled oligodeoxynucleotide complementary to region 1194−1210 with the use of ddGTP gives extension products of 19 nt and 39 nt on wild-type and specialized 16S rRNA, respectively. Isolation of 16S rRNA from 30S subunits and primer extension were performed as described by Triman *et al.* (2). AMV reverse transcriptase was obtained from Promega and ddGTP from Pharmacia. Extension products were analyzed on 12.5% polyacrylamide/8 M urea gels. The percentage of specialized 30S subunits present before and after affinity-column treatment was determined by measuring the radioactivity present in the 19-mer or 39-mer extension products using the Betascope 603 Blot Analyzer (Betagen).

Toeprint analysis

Primer extension inhibition (or toeprinting) was performed essentially as described by Hartz *et al.* (16). CAT mRNA containing the specialized Shine−Dalgarno (SD) sequence 5′ GUGUG was transcribed *in vitro* from pGEMCAT-SDX with SP6 RNA polymerase (Pharmacia). pGEMCAT-SDX was linearized by cutting the BamHI restriction site situated about 750 nt downstream of the *cat* gene start and *in vitro* transcription was performed as described by Krieg and Mellon (17) but omitting the DNase treatment. CAT mRNA was purified by phenol extraction and column chromatography through Sephadex G50, precipitated with alcohol and dissolved in H_2O to a concentration of 0.5 μg/μl. The primer, 5′ GCAACTGACTGA-AATGCCTC, complementary to residues 64−83 from the CAT start codon on the CAT mRNA, was end-labelled with ^{32}P (16). Toeprinting reactions were performed in 10 mand 1 μM $tRNA_f$ met (Boehringer-Mannheim). Primer extension reactions include 0.5 U/μl RNase inhibitor (RNAguard, Pharmacia). Relative toeprints were determined by measuring the radioactivity of the inhibited and uninhibited extension products on the gels using a Betascope 603 Blot Analyzer (Betagen) and were defined as the percentage extension inhibition compared to total primer extension (18).

Poly(U) translation

Translation of poly(U) was performed as described by van Dieijen *et al.* (19). The reactions were carried out in a total volume of 50 μl containing 0.1 μM 30S subunits and 50S subunits. Samples of 5 μl were taken 0, 5, 15 and 30 min after starting the reaction and incorporation of [^{3}H]Phe into poly(Phe) was determined by TCA precipitation.

RESULTS

Fractionation of a mixed ribosome population using the Shine–Dalgarno interaction

To isolate 30S subunits containing specialized 16S rRNA, a nucleic acid affinity chromatography method was developed to trap wild-type ribosomes on a column via their anti-SD sequence. An oligodeoxynucleotide (5′ ATCATCATCTAAGGAGGT) was prepared in which the last nine nucleotides provide a ribosome binding site, whereas the first nine residues function as spacer to better expose the SD sequence. The spacer sequence is chosen in such a way that secondary structure formation is minimized and S1-mediated ribosome binding via polypyrimidine stretches (20) is avoided. The oligodeoxynucleotide is attached to a biotin–cellulose matrix as described in Materials and methods. Wild-type 30S subunits are expected to bind to the immobilised oligodeoxynucleotide *via* their complementary 16S rRNA 3′ end. Specialized 30S subunits have an altered ASD sequence which restricts the base pairing with the ribosome binding site on the oligodeoxynucleotide. These mutant ribosomes are expected to pass through the column.

1.5 ml of oligonucleotide-containing biotin–cellulose suspension was poured into a 2 ml sterilized syringe (Becton Dickinson) on sterilized and siliconized glass wool. 450 pmol of 30S subunits containing both specialized and wild-type 30S (prepared from induced K5637 cells containing the plasmid pP_LASDX-Spc^R-CATX and called 'mixed 30S subunits' throughout this paper) were diluted in standard buffer to a concentration of 5 pmol/μl and loaded on the column. Elution with standard buffer (4°C) under mild pressure allowed a steady flow of 100 μl/min. Fractions containing 30S subunits were identified by their absorbance at 260 nm. About 280 pmol 30S subunits were recovered (0.4 pmol/μl).

Performance of the affinity column

The spectinomycin resistance mutation C_{1192} to U_{1192}, introduced into specialized 16S rRNA by Hui and de Boer (9), was used to discriminate between wild-type and specialized 30S subunits by primer extension (15). Primer extension using a [^{32}P]-end-labelled primer complementary to a sequence downstream of position 1192, yields a 39-mer and a 19-mer as extension products on specialized and wild-type 16S rRNA, respectively, if dGTP is replaced by ddGTP. The results of the primer extension experiments on the various ribosomal preparations are shown in Fig.1. As can be seen in lane 2, 'mixed 30S subunits' already contain far more specialized than wild-type ribosomes before purification. After affinity chromatography, fractions contain hardly detectable amounts of wild-type subunits (lanes 3 and 4). The results are presented quantitatively in Table 1. The percentage of specialized 30S in the samples increases from about 80% to more than 95% by affinity chromatography. Clearly the column is able to selectively bind virtually all the wild-type subunits. Attempts to raise the purity of the subunits even further by a second column cycle were unsuccessful (Fig.1, lane 4 and Table 1). This probably means that the residual amount of wild-type material in the preparation consists of inactive 30S subunits that have their ASD region not exposed and thus do not bind to the affinity-column (21,22).

Table 1. Calculated percentage of wild-type and specialized 30S subunits before and after affinity chromatography

Ribosome preparation	specialized 16S rRNA	wild-type 16S rRNA
1 Wild-type 30S subunits (MRE600)	0%	100%
2 Mixed 30S subunits (K5637)	82 ± 4%	18 ± 4%
3 As 2 but after affinity chromatography	97 ± 1%	3 ± 1%
4 As 3 but after a second run through an affinity column	97 ± 1%	3 ± 1%

Primer extension was carried out as described for Fig.1. Amounts of wild-type and specialized 30S subunits were determined by direct measurement of the radioactivity present in the 19-mer extension products representing wild-type 16S rRNA or 39-mer extension products representing specialized 16S rRNA.

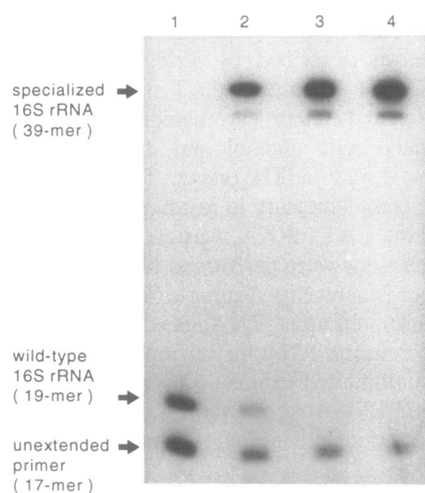

Figure 1. Primer extension analysis on 16S rRNA prepared from various 30S samples. Lane 1, wild-type 30S (MRE600). Lane 2, mixed 30S subunits (K5637). Lane 3, as lane 2 but after one cycle of affinity chromatography. Lane 4, as lane 3 but after a second run through a (fresh) affinity column. Secondary bands, visible in lanes 2–4 (moving slightly faster than the specialized 16S rRNA extension product) are prematurely terminated 38-mer extension products on specialized 16S rRNA. Radioactivity present in these bands is counted as primer extension on specialized 16S rRNA as quantitated in Table 1.

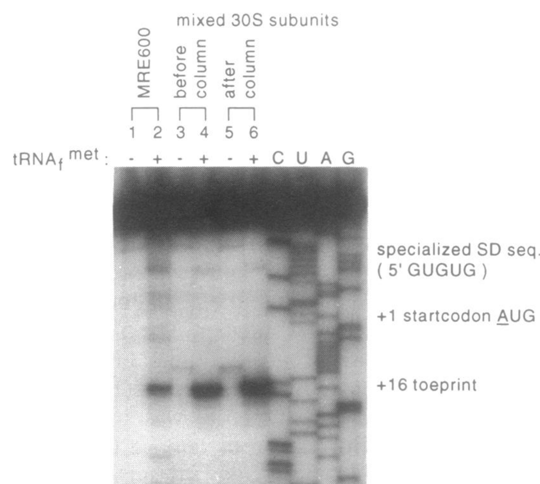

Figure 2. Toeprint analysis of initiation complex formation on specialized CAT mRNA using 30S subunit preparations before and after affinity chromatography. Lanes 1 and 2, wild-type 30S subunits (MRE600); lanes 3 and 4, mixed 30S subunits (K5637); lanes 5 and 6, mixed 30S subunits after affinity chromatography; lanes 7–10, sequencing reactions on CAT mRNA, reaction mixtures contain ddGTP, ddATP, ddTTP and ddCTP respectively.

Table 2. Efficiency of initiation complex formation on specialized CAT mRNA of various 30S subunit preparations before and after affinity chromatography

Ribosome preparation	Relative toeprint	Relative toeprint corrected for the presence of chromosome encoded 30S subunits
1 Wild-type 30S (MRE600)	8%	–
2 Mixed 30S subunits (K5637)	17%	19%
3 As 2 but after affinity chromatography	20%	20%

Relative toeprints were calculated as described by Hartz *et al.* (18). Relative toeprints of pure specialized 30S subunits were calculated by subtracting the activity by chromosomally encoded 30S and extrapolating the amount of specialized 30S to 100%. It is thereby assumed that 30S subunits prepared from MRE600 have the same activity as those chromosomally encoded in K5637.

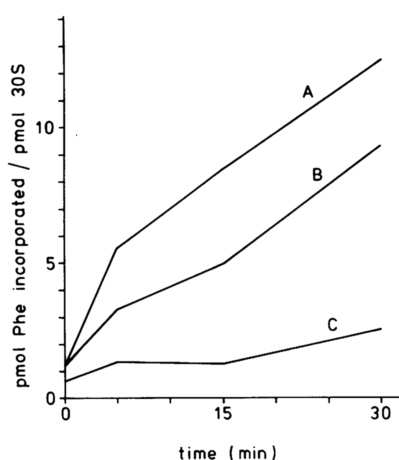

Figure 3. Translation of poly(U) by mixed 30S subunits before and after affinity chromatography. **A**: poly(Phe) incorporation before affinity chromatography. **B**: after affinity chromatography. **C**: poly(Phe) incorporation in the absence of mixed 30S subunits. This curve serves as a control for the presence of 30S subunits in our 50S preparation.

Activity of the isolated specialized 30S subunits

To examine whether affinity chromatography has any adverse effects on translational activity of the purified 30S subunits two representative functional tests were performed. First, the ability to form a ternary initiation complex was checked by toeprinting on CAT mRNA containing the specialized SD sequence, GUGUG. Accordingly, the toeprinting activity of mixed 30S subunits before and after fractionation over the affinity column was measured.

Fig. 2 shows that toeprints are $tRNA_f^{met}$ dependent and occur, as expected, 15 nucleotides downstream of the start codon of the CAT mRNA indicating that 30S subunits exclusively use the authentic initiation codon for ternary complex formation under toeprinting conditions. As can be seen in Fig. 2 (lanes 4 and 6), mixed 30S subunits are about equally capable of forming an initiation complex before and after affinity chromatography. Relative toeprints were calculated and are shown in Table 2. Relative toeprinting activity of the mixed 30S subunits increases after affinity chromatography from 17% to 20% as would be expected from the increased fraction of specialized particles. Contrary to our expectations, wild-type 30S subunits, isolated from MRE600, also form an initiation complex at the initiation codon of the CAT mRNA, albeit with an efficiency of about one third of that of specialized 30S ribosomes as shown in Fig. 2 (lanes 2 and 6) and Table 2 (see Discussion). Correction for the presence of wild-type 30S in the mixed 30S starting material yields virtually equal relative toeprints of about 20% before and after affinity chromatography showing that the column has no negative effect on this activity (Table 2).

To test the elongation potential of our purified ribosomes, poly(U) translation was performed. The kinetics of poly(Phe) synthesis of 30S ribosomes isolated from K5637 before and after column purification were measured. As shown in Fig. 3, the rate of poly(Phe) synthesis for purified specialized ribosomes is about 75% (curve B) of the unfractionated sample (curve A). Affinity chromatography seems to cause some decrease in elongating capacity of the purified 30S subunits. As a control, poly(Phe) synthesis without added 30S subunits is shown by curve C.

Poly(U) translation by wild-type 30S subunits isolated from MRE600 is comparable to that of the mixed 30S subunits, showing that the activity in poly(Phe) synthesis which we measure for the purified specialized ribosomes is associated with the bulk of the material and not with a minor fraction of the chromosomally encoded ribosomes. A secondary conclusion from this result is that the never tested assumption that poly(U) translation is insensitive to SD–ASD interaction, appears correct.

DISCUSSION

We describe a method for isolating 30S subunits containing plasmid-derived specialized 16S rRNA assembled *in vivo*. The base pairing capacity of the ASD sequence in the 16S rRNA to a matrix-bound oligonucleotide was exploited to separate 30S subunits containing specialized rRNA from those harbouring wild-type rRNA. Following affinity chromatography, 30S ribosomes prepared from K5637, harbouring the pP_LASDX-Spc^R-CATX plasmid containing the specialized 16S rRNA gene, were almost free of wild-type 30S subunits. Apparently, the formation of an rRNA–DNA hybrid of, at most, nine base pairs with the wild-type 16S rRNA is sufficient to retain wild-type 30S on the column. Three mismatches are sufficient to prevent the SD interaction with specialized 16S rRNA and allows for the isolation of specialized ribosomes as the flow-through of the column.

Induction of the P_L controlled rrnB operon on pP_LASDX-Spc^R-CATX yielded already about 80% specialized ribosomes when isolated from 70S tight couples. This is more than obtained in other studies using P_L controlled rRNA operons (5, 10, 23). The only apparent difference in experimental approach is that we use LC instead of LB medium for growing the cells.

Isolated specialized 30S subunits were tested for their activity in initiation complex formation by toeprinting. Purifying the specialized ribosomes on the biotin–cellulose affinity column did not seem to affect this activity as witnessed by their equal activity before and after column treatment. Wild-type 30S subunits formed initiation complexes on specialized CAT mRNA with an unexpected high efficiency. *In vivo*, translation of the specialized CAT mRNA by wild-type ribosomes is less than 5% of the specialized translational activity (10). It may be that competition with other mRNAs containing 'better' (wild-type) ribosome binding sites, in combination with the *in vivo* conditions, makes binding of wild-type ribosomes to specialized CAT mRNA less favourable *in vivo*.

Specialized 30S subunits were also examined for their elongating activity. Poly(Phe) synthesis seems to be slightly lowered by the column treatment. This decrease may be due to rRNA degradation or partial loss of ribosomal proteins during passage over the column although it is not clear why such damage would not show up in the toeprint assay.

Our method for isolating mutant 30S subunits can be of help in assigning functions to defined regions in 16S rRNA. For example, mutations in the central pseudoknot at the 5′ end of 16S rRNA have been shown to impair translation of specialized CAT mRNA *in vivo* (10). Specialized 30S subunits containing these mutations have now been isolated by us and the nature of their defect is being studied *in vitro*.

Using *in vitro* transcribed mutant 16S rRNA, Ofengand and his group were able to study the effect of defined 16S rRNA mutations on ribosome function (24, 25). Isolating mutant 30S subunits from growing cells can serve as an alternative to this *in vitro* reconstitution. 30S subunits assembled *in vivo* have the advantage of containing the naturally occurring base modifications in their 16S rRNA. Although the function of the modified bases in rRNA is still unknown, the observation that almost all of them are located in regions related to some well defined activity indicates that they are important for optimal ribosomal functioning (26–28). The fact that ribosomes harbouring *in vitro* transcribed 16S rRNA are less active in translational assays than ribosomes containing natural 16S rRNA (24) may be due to the absence of RNA modification.

The thermo-inducibility of the specialized 16S rRNA gene combined with the fact that specialized 30S subunits hardly recognize endogenous mRNAs, allows the isolation of 30S subunits with dominant lethal 16S rRNA mutations (11, 29). The method described here may also have applications in studying the consequences of 16S rRNA mutations for the processing of 16S rRNA and the assembly of the 30S subunit.

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