Reconstitution of Functionally Active *Thermus aquaticus* Large Ribosomal Subunits with in Vitro-Transcribed rRNA†

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ABSTRACT: Functionally active large ribosomal subunits of thermophilic bacterium *Thermus aquaticus* have been assembled in vitro from ribosomal proteins and either natural or in vitro-transcribed 23S rRNA and 5S rRNA. Sedimentation properties of reconstituted subunits were similar to those of native ribosomal 50S subunits. Subunits reconstituted with in vitro-transcribed rRNAs exhibited high activity in the peptidyl transferase assay and in a poly(U)-dependent cell-free translation system (22 and 30%, respectively, compared to that of native 50S subunits). Catalytic activity of reconstituted subunits critically depended on the presence of 5S rRNA. rRNA mutations known to affect functions of the native ribosome produced similar effects in reconstituted *T. aquaticus* 50S subunits. Subunits assembled with in vitro-transcribed *T. aquaticus* 23S rRNA containing the G2267A mutation (G2252A in *Escherichia coli*), which interferes with binding of peptidyl-tRNA in the ribosomal P-site, showed drastically reduced peptidyl transferase activity, whereas clindamycin resistance mutation A2084G (A2058G in *E. coli*) rendered assembled subunits tolerant to clindamycin inhibition. Thus, reconstitution of functional subunits with in vitro-transcribed rRNA makes possible the use of in vitro genetics for mutational analysis of 23S rRNA functions in translation. In addition, the ability to assemble catalytically active 50S subunits from the rRNA transcript lacking any posttranscriptional modifications clearly demonstrates that modified nucleotides in 23S rRNA are dispensable for the principal activities of the ribosome.

rRNA plays a central role in most ribosomal functions, including catalysis of peptide bond formation (*1*, *2*). However, details of rRNA organization within the ribosome and molecular mechanisms of its functioning remain unclear due to the complexity of ribosome structure and limitations of methods that can be applied to rRNA studies. Thus, the use of a powerful technique of genetic analysis for rRNA research is hindered by the presence of multiple copies of rRNA genes in most organisms. This obstacle can be potentially overcome by introducing mutant rRNA genes on a multicopy plasmid or by using experimental organisms containing only one copy of rRNA genes (*3*, *4*). Unfortunately, both approaches have significant shortcomings. Introduction of mutated rRNA genes into a cell on a plasmid usually results in a mixed population of ribosomes where mutant ribosomes are "contaminated" to various extents by the wild type ribosomes containing chromosome-encoded rRNA. On the other hand, the use of unconventional single-rRNA operon organisms is limited because of the inadequate biochemical and/or genetic characterization of their translation apparatus. Furthermore, neither of the in vivo systems allows the study of dominant lethal mutations in rRNA.

An alternative promising way of studying rRNA functions is "in vitro genetics", where functional ribosomes or ribosomal subunits are assembled in a test tube from the ribosomal proteins and mutant rRNA and their functions are analyzed in the cell-free system. One of the most important breakthroughs that significantly advanced ribosomal research was the discovery that active ribosomal subunits could be reconstituted in vitro from their individual components: rRNA and ribosomal proteins (*5*). When ribosome complexity and the intricacy of the events and mechanisms involved in ribosome biosynthesis in vivo are considered, it is amazing that ribosomal components, rRNAs, and a number of different ribosomal proteins can combine in vitro in a precise, specific way to form functionally active ribosomal subunits. The conditions for reconstitution of the small and large ribosomal subunits from their natural components were found for both 30S and 50S subunits of *Escherichia coli* (*6*−*8*) and several other organisms (*9*−*12*). The use of in vitro-assembled ribosomal subunits was instrumental in understanding ribosome structure and functions (*13*−*17*).

An important advancement in studies of the 16S rRNA structure and functions in small ribosomal subunits was brought about by the finding that the *E. coli* natural 16S rRNA can be substituted by the in vitro-transcribed rRNA in the reconstitution reaction. The in vitro transcript successfully assembled into functional 30S subunits, though with somewhat reduced efficiency (*18*). This made possible studying effects of rRNA mutations on the functions of 30S subunits using in vitro mutational analysis (*19*−*22*). In

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contrast to the 30S subunit, replacement of the natural 23S rRNA with its in vitro transcript produced inactive large ribosomal subunits (*23*). In the in vitro complementation assay, Green and Noller (*24*) showed that the presence of an 80 nt segment of the natural *E. coli* 23S rRNA (extending from position 2445 to 2523) was absolutely essential for the activity of 50S subunits reconstituted from chimeric 23S rRNA composed of natural and synthetic rRNA segments. The failure to assemble synthetic 23S rRNA into functional 50S subunits significantly hampered the use of mutational analysis for studies of 23S rRNA functions (*17*).

A possible explanation for the failure to reconstitute functionally active 50S subunits from the in vitro-transcribed 23S rRNA was the lack of posttranscriptional modifications in the rRNA transcript. Both small and large ribosomal subunit rRNAs contain a significant number and variety of modified nucleotides. *E. coli* 23S rRNA has at least 23 posttranscriptional modifications (*25*−*27*). Though the functional importance of modified nucleotides in rRNA remains speculative, their clustering in the close vicinity of the peptidyl transferase center as well as the inability to substitute the segment of natural 23S rRNA, forming part of this center by synthetic transcript, implied their possible involvement in the activity of peptidyl transferase (*24*, *25*, *28*, *29*), and even a direct role of some residues in the peptidyl transfer catalysis was suggested (*30*).

On the other hand, there is no convincing evidence that modified nucleotides are indeed essential for catalysis of the peptidyl transferase reaction. Furthermore, the occurrence and location of posttranscriptional modifications differ between organisms, suggesting that they might play only an auxiliary function (*31*). These considerations raised a possibility that failure to assemble active subunits from the in vitro-transcribed *E. coli* 23S rRNA was due to improper folding of the transcript, suggesting in turn that it might be possible to reconstitute active large ribosomal subunits from synthetic rRNA using a model organism other than *E. coli*. A promising candidate for such a model was thermophilic bacterium *Thermus aquaticus*. *T. aquaticus* grows optimally at 70 °C. Ribosomes and ribosomal components isolated from the genus *Thermus* are commonly used for crystallographic studies of the ribosome (*32*−*35*) because they are significantly more robust than those from mesophilic organisms. This is confirmed by the finding that peptidyl transferase activity of *T. aquaticus* ribosomes withstands extensive deproteination procedures, reflecting a highly stable structure of the thermophilic large ribosomal subunit (*36*, *37*).

In this paper, we report successful reconstitution of functionally active 50S subunits from *T. aquaticus* using large ribosomal subunit proteins and the in vitro-transcribed 23S and 5S rRNAs. Assembled subunits exhibited high activity in the peptidyl transferase assay and in a poly(U)-dependent cell-free translation system. These results demonstrate that posttranscriptionally modified nucleotides in 23S rRNA are dispensable for peptidyl transferase activity and general catalysis of polypeptide synthesis and open the possibility of applying in vitro mutational analysis to studies of large ribosomal subunit rRNA.

MATERIALS AND METHODS

Cell Growth and Isolation of Ribosomes and the S100 Enzyme Fraction. *T. aquaticus* cell cultures were grown in a modified Castenholz TYE medium (*38*) containing 1× Castenholz salts, 1× Nitsch's trace elements, 4 g/L yeast extract, 8 g/L bactopeptone, and 2 g/L NaCl (*36*). Cells were harvested in the late logarithmic phase ($A_{600} = 1.0$) and stored as a frozen pellet at −75 °C. For isolation of ribosomes, 15 g of frozen *T. aquaticus* cells was resuspended in 40 mL of buffer A [20 mM Tris-HCl (pH 7.5)/25 mM $MgCl_2$/100 mM NH_4Cl/6 mM β-mercaptoethanol] and passed twice through a French press (18 000 psi). The lysate was cleared by centrifugation in a Beckman JA-20 rotor at 20 000 rpm for 20 min at 4 °C and layered over a 1.1 M sucrose cushion prepared in buffer B [20 mM Tris-HCl (pH 7.5)/25 mM $MgCl_2$/500 mM NH_4Cl/6 mM β-mercaptoethanol]. Ribosomes were pelleted by centrifugation in a Beckman Ti-70 rotor at 22 000 rpm for 17 h at 4 °C. The pellet was washed three times with 3 mL of dissociation buffer [DB; 50 mM Tris-HCl (pH 7.8)/2 mM $MgCl_2$/100 mM NH_4Cl/6 mM β-mercaptoethanol], resuspended in 500 μL of DB, and dialyzed for 5 h at 4 °C against 500 mL of the same buffer. The dissociated subunits were layered over a 10 to 40% sucrose gradient prepared with DB buffer and centrifuged in a Beckman SW-28 rotor at 26 000 rpm for 15 h at 4 °C. Peaks corresponding to 30S and 50S subunits were collected; the $MgCl_2$ concentration was adjusted to 25 mM, and subunits were pelleted by centrifugation in a Beckman Ti-70 rotor at 45 000 rpm for 20 h at 4 °C. Ribosomal subunits were resuspended in buffer A, aliquoted, and stored at −75 °C.

For preparation of the S100 extract, 10 g of *T. aquaticus* cell mass was resuspended in 30 mL of buffer A. Cells were lysed by passing them through the French press (12 000 psi), and the cell lysate was clarified by centrifugation in a Beckman JA-20 rotor at 16 000 rpm for 40 min at 4 °C. The S100 extract was prepared by centrifugation in a Beckman Ti-70 rotor at 33 000 rpm for 12 h at 4 °C. After buffer composition was adjusted to 20 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/22 mM NH_4Cl/6 mM β-mercaptoethanol, the extract was loaded onto a 40 mL DEAE-cellulose column and washed with 400 mL of the same buffer, and the enzyme fraction was eluted with buffer containing 20 mM Tris-HCl (pH 7.6)/10 mM $MgCl_2$/0.3 M KCl/6 mM β-mercaptoethanol. The yellowish fractions were combined and dialyzed against two changes (1 L each) of buffer L [10 mM Tris-HCl (pH 8.0)/12 mM $MgCl_2$/60 mM KCl/60 mM NH_4Cl/β-mercaptoethanol] and then against 400 mL of buffer L containing 50% glycerol. The S100 extract was aliquoted and stored at −75 °C.

Preparation of Ribosomal Proteins and Natural rRNA. Total proteins from the 50S subunit (TP50)[1] were isolated by acetic acid extraction according to ref *39*. 23S and 5S rRNA were isolated from *T. aquaticus* 50S ribosomal subunits by phenol extraction. Fifty A_{260} units of 50S subunits in 500 μL of buffer solution containing 300 mM NaAc (pH 5.5), 5 mM EDTA, and 0.5% SDS was mixed with 500 μL of water-saturated phenol, and the mixture was vortexed 10 min at room temperature and spun for 5 min in a microcentrifuge. The aqueous phase was transferred to a fresh tube, and phenol extraction was repeated two more times followed by extraction with water-saturated phenol/chloroform (1:1

[1] Abbreviations: TP50, total proteins of 50S ribosomal subunits; TCA, trichloroacetic acid.

v/v) and chloroform. RNA was precipitated from the aqueous phase with 3 volumes of ethanol and resuspended in 200 μL of buffer RB [10 mM Tris-HCl (pH 7.4)/4 mM $MgCl_2$]. 23S and 5S rRNA were separated by sucrose gradient centrifugation as described in ref *40*. *E. coli* 5S rRNA was isolated in a similar way from *E. coli* 50S ribosomal subunits.

Cloning and in Vitro Transcription of 23S and 5S rRNA. The *T. aquaticus* 23S rRNA gene was cloned into the pEMBL9 vector as a 7.2 kb *Hin*dIII fragment of *T. aquaticus* genomic DNA. The 23S rRNA gene was PCR amplified from this plasmid using a pair of primers, PT5 (GGAATTCTAATACGACTCACTATAGGTCAAGATGCTAAGGGCCCACG) and PT3 (GGAATTCGAAGGGTCAAGACCTCGGACGATTGG), corresponding to the 5′ and 3′ termini of the 23S rRNA gene, respectively, and cloned into the *Eco*RI site of the pUC18 plasmid to produce the plasmid pT7TA. Primer PT5 had the sequence of the T7 RNA polymerase promoter immediately followed by the 5′ terminal sequence of the 23S rRNA gene. Primer PT3 contained the *Bsp*119I site used for "opening" the final construct for runoff in vitro transcription. The amplified PCR product was cut with *Eco*RI and cloned into the *Eco*RI site of the pUC18 plasmid. The entire *T. aquaticus* 23S rRNA gene was sequenced in several randomly picked recombinant clones, and a clone was found that did not contain mutations resulting from PCR errors or cloning artifacts. This clone was used for the in vitro transcription. Due to introduction of the *Bsp*119I site, the runoff transcript of the 23S rRNA gene contained an additional uridine at the 3′ end compared to the natural rRNA.

The 5S rRNA gene was PCR amplified from *T. aquaticus* genomic DNA using primers 5S-T7 (TAATACGACTCACTATAGGAATCCCCCGTGCCCTTAG) and 5S-R (TTGATATCCCCCGCACCGACCTAC) and cloned into the *Sma*I site of the pUC19 vector. The 5S-T7 primer contained the sequence of the T7 RNA polymerase promoter. Two additional guanines were introduced between the T7 promoter and the 5S rRNA gene sequence to ensure efficient initiation of transcription in vitro. The 5S-R primer contained an *Eco*RV site that could be used for opening the plasmid for the in vitro runoff transcription.

The 23S and 5S rRNAs were transcribed from corresponding recombinant plasmids linearized with *Bsp*119I (23S rRNA) or *Eco*RV (5S rRNA) using an in vitro RNA transcription MegaScript kit (Ambion). The transcribed rRNAs were purified by gel filtration on a 2 mL Sephadex G-50 column using 300 mM NaAc (pH 5.5) as the elution buffer. Fractions containing the rRNA transcript were combined, extracted with water-saturated phenol, phenol/chloroform, and chloroform, and precipitated with 3 volumes of ethanol. RNA pellets were resuspended in RB buffer and stored at −75 °C.

Introducing Mutations in 23S rRNA. To facilitate subsequent cloning, the *Pst*I site was removed from the polylinker of the pT7TA plasmid by cutting the plasmid with *Bam*HI and *Hin*dIII, filling up protruding DNA ends with Klenow DNA polymerase in the presence of dNTPs, and religating the plasmid. Two mutations, A2084G (position 2058 in *E. coli*) and G2267A (position 2252 in *E. coli*), were introduced into 23S rRNA by mutagenic primer-mediated PCR (*41*). For introduction of the A2084G transition, the *Pau*I−*Pst*I segment (positions 1688−2109) of the wild type gene was replaced with a corresponding mutant fragment prepared by PCR using a combination of primers CCTAAGGTGAGGCCGAAAG/CGGGGTCTTCTCGTCCTGCC and TGGTATTTCACCGGCGC/GGCAGGACGAGAAGACCCCG (mutant positions are underlined). For introduction of the G2267A mutation, the mutant *Pst*I−*Cla*I fragment prepared by PCR from a combination of primers GTGAAGATGCGGCCTACCCG/AGGCGACCGCTCCAGTCAA and GTTTGACTGGAGCGGTCGCCT/CAACCCTTGGGACCTTCTT was used to replace the corresponding wild type segment in the pT7TA plasmid. The presence of introduced mutations was confirmed by sequencing the 23S rRNA gene around the site of mutation. In vitro transcripts of mutant 23S rRNAs were prepared using the same procedure that was used for transcription of the wild type 23S rRNA.

Reconstitution of 50S Subunits. For reconstitution of 50S subunits from natural rRNA, 1 A_{260} unit of an unfractionated mixture of 23S and 5S rRNA was combined with 1.2 equivalent units of TP50 (1 equivalent unit is the amount of protein corresponding to 1 A_{260} unit of 50S subunits) in 100 μL of reconstitution buffer containing 20 mM Tris-HCl (pH 7.4)/400 mM NH_4Cl/4 mM $MgCl_2$/6 mM spermidine/5 mM β-mercaptoethanol. The mixture was incubated for 90 min at 44 °C. The concentration of $MgCl_2$ was adjusted to 20 mM, and the reaction mixture was incubated for 60 min at 60 °C.

For reconstitution of 50S subunits from in vitro-transcribed 23S and 5S rRNAs, 1 A_{260} unit of 23S rRNA transcript was mixed with 0.05 A_{260} unit of 5S rRNA transcript and 1.2 equivalent units of TP50 in 100 μL of reconstitution buffer. The reaction mixture was placed in a 0.5 mL tube in the PCR cycler (Techne), quickly heated to 70 °C, and incubated for 1 min at this temperature. The reaction mixture was cooled to 44 °C over a period of 2 min and then incubated at 44 °C for 60 min. The concentration of $MgCl_2$ was increased to 20 mM, and a second incubation step was performed at 60 °C for 30 min.

The reconstituted subunits were directly tested in the peptidyl transferase assay or poly(U)-dependent cell-free translation.

Peptidyl Transferase Assay. Peptidyl transferase activity was assayed using a modified peptidyl transferase "fragment" reaction, with f-Met-$tRNA^{Met}$ as a donor substrate and puromycin as the acceptor (*42*). Formyl-[^{35}S]Met-tRNA was prepared as described previously (*43*) and HPLC purified. A typical peptidyl transferase assay was performed in 60 μL of reaction buffer [20 mM Tris-HCl (pH 8.0)/400 mM KCl/20 mM $MgCl_2$/1 mM puromycin] containing 15 pmol of native or reconstituted 50S subunits and 30 pmol (300 000 cpm) of formyl-[^{35}S]Met-tRNA. The reaction was initiated by addition of 30 μL of cold methanol and the mixture incubated for 20 min on ice. To terminate the reaction, 15 μL of 10 M KOH was added and the mixture incubated for 20 min at 37 °C. After addition of 300 μL of 1 M KH_2PO_4, reaction mixtures were extracted with 1 mL of ethyl acetate. Five hundred microliters of the ethyl acetate phase was dried down in an Eppendorf tube in the SpeedVac concentrator. The dry pellet was resuspended in 10 μL of ethyl acetate, and the solution was spotted on a strip of Whatman 3MM (20 cm × 80 cm) paper. High-voltage electrophoresis was carried out in 500 mM formic acid (pH 2.0) at 1500 V for 75 min (*24*); paper was dried, and radioactivity was visual-

ized using a β-scanner (Ambis). Under these conditions, the relationship between the amount of *N*-formyl-methionyl-puromycin formed and ribosome concentration was linear.

In experiments with clindamycin, the antibiotic was added to a final concentration of 12 μM prior to addition of methanol.

Poly(U)-Dependent Cell-Free Translation. Fifteen picomoles of reconstituted or native 50S subunits in 50 μL of reconstitution buffer was combined with 15 pmol of 30S subunits, 10 μL of S100 enzyme fraction, 2 μg of poly(U), 2 μCi (140 pmol) of [^{3}H]phenylalanine, 2 μg of *E. coli* tRNA^{Phe} (Sigma), 100 nmol of GTP, and 300 nmol of ATP in a total volume of 100 μL. The final buffer concentrations were adjusted to 50 mM Tris-HCl (pH 7.8), 60 mM $MgCl_2$, 100 mM NH_4Cl, 2 mM spermidine, and 6 mM β-mercaptoethanol. The reaction mixture was incubated for 30 min at 65 °C. The reaction was stopped by addition of 250 μL of 1 M NaOH. After incubation for 15 min at 37 °C, 1 mL of solution containing 25% trichloroacetic acid (TCA) and 2% casamino acids was added and the mixture was incubated on ice for 30 min. The solution was filtered through Fisher G4 glass fiber filters. Filters were washed with ice-cold 5% TCA, dried, and counted.

Sucrose Gradient Analysis. Approximately 3 A_{260} units of reconstructed or native 50S subunits was loaded onto a 10 to 40% linear sucrose gradient prepared in buffer G [20 mM Tris-HCl (pH 7.4)/400 mM NH_4Cl/20 mM $MgCl_2$/5 mM β-mercaptoethanol] in a centrifuge tube for the SW41 rotor (Beckman). The gradients were centrifuged at 4 °C for 15 h at 26 000 rpm. Gradients were fractionated through the ISCO flow spectrophotometer. Peak fractions were combined and precipitated with 3 volumes of ethanol. Pellets were resuspended in 50 μL of RB20 buffer and immediately used for peptidyl transferase activity assay.

Protein Analysis. Reconstituted or native 50S subunits were purified by sucrose gradient centrifugation, and proteins were extracted by acetic acid as described in ref *39*. Proteins were separated by two-dimensional gel electrophoresis (*44*). Gels were stained with GelCode Blue Stain Reagent (Pierce), and images were captured on the Hewlett-Packard 6100C ScanJet scanner.

RESULTS

Total Reconstitution of T. aquaticus 50S Subunits from Natural rRNA and Ribosomal Proteins. Functionally active *E. coli* 50S subunits can be assembled from total proteins of the 50S subunit (TP50) and natural 23S and 5S rRNA in a two-step protocol developed by Nierhaus and co-workers (*39*, *45*). After optimizing the reconstitution conditions with respect to ion requirements, polyamine concentration, incubation temperatures, and the duration of incubation steps, we found that *T. aquaticus* 50S subunits can be reconstituted from the natural 23S rRNA, 5S rRNA, and TP50 proteins in a similar two-step reaction by incubating a mixture of rRNA and proteins for 90 min at 44 °C in a buffer containing 4 mM $MgCl_2$ and then, after adjusting the $MgCl_2$ concentration to 20 mM, for 30 min at 60 °C (details of the reconstitution procedure are described in Materials and Methods). Sucrose gradient analysis demonstrated that reconstituted subunits sedimented as a sharp symmetric peak with a sedimentation rate similar to that of the intact 50S

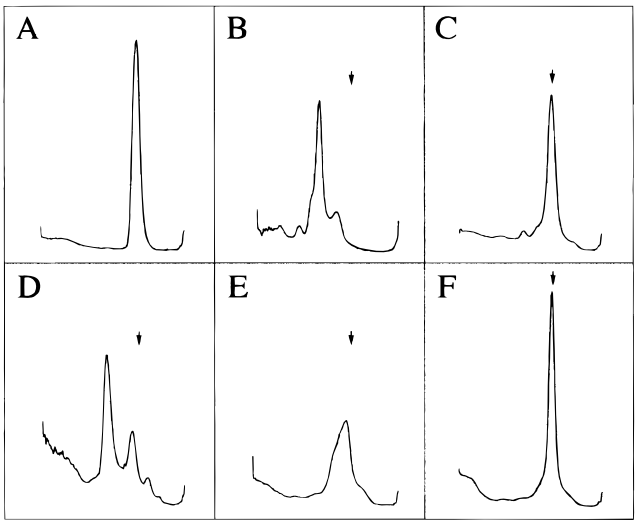

FIGURE 1: Sucrose gradient analysis of *T. aquaticus* 50S subunits reconstituted from ribosomal proteins and either natural or in vitro-transcribed rRNA: (A) native 50S subunits, (B) natural deproteinated 23S rRNA, (C) 50S subunits assembled from TP50 and natural 23S rRNA and 5S rRNA, (D) in vitro-transcribed 23S rRNA, and (E and F) 50S subunits assembled from TP50 and in vitro-transcribed 23S rRNA and 5S rRNA in a two-step (E) or three-step (F) reconstitution reaction. Arrows in panels B−F indicate the position of the peak of native 50S subunits. The direction of sedimentation is from left to right. Shown profiles represent absorbance at 254 nm.

(Figure 1C). The functional activity of reconstituted subunits was tested in both the peptidyl transferase reaction and poly-(U)-dependent cell-free translation system. The peptidyl transferase assay was performed under "fragment reaction" conditions where the reaction between a donor substrate (formyl-[^{35}S]methionyl-tRNA) and an acceptor substrate (puromycin) is catalyzed by isolated large ribosomal subunits in the presence of 33% methanol (*42*, *46*). Reconstituted *T. aquaticus* 50S subunits exhibited ca. 25% activity in the peptidyl transferase reaction compared to that of the native 50S subunits, which was similar to the 30−40% peptidyl transferase activity reported previously for reconstituted *E. coli* 50S subunits (*24*, *45*). Activity of the assembled *T. aquaticus* large ribosomal subunits in the cell-free translation system was even higher, more than 50% of the activity of the native 50S subunits (Table 1).

Reconstitution of T. aquaticus 50S Subunits with in Vitro-Transcribed rRNA. Replacement of the natural *T. aquaticus* 23S rRNA with an in vitro-transcribed 23S rRNA in a two-step reconstitution procedure reduced the peptidyl transferase activity of the assembled particles to approximately 1% compared to that of the untreated natural 50S subunits (data not shown). The particles reconstituted with the 23S rRNA transcript formed a broad heterogeneous peak on the sucrose gradient (Figure 1E). When fractions of the peak were assayed for peptidyl transferase activity, only the more compact (faster sedimenting) material exhibited peptidyl transferase activity, while slower sedimenting fractions were inactive (data not shown).

Several factors could contribute to the difference in efficiency of reconstitution between 50S subunits from natural rRNA and the in vitro transcript (*24*). (i) One or several ribosomal proteins may remain associated with the natural rRNA during RNA purification. (ii) The conformation

Table 1: Activity of Reconstituted *T. aquaticus* 50S Subunits in the Peptidyl Transferase Reaction and Poly(U)-Dependent Cell-Free Translation System

large ribosomal subunit component	PT[a] (% activity[c])	poly(U)[b] (% activity[d])
native 50S	100 ± 2	100 ± 4
native 50S, no poly(U)	–	4 ± 2
50S reconstituted with natural 23S rRNA and 5S rRNA[e]	25 ± 3	53 ± 4
native 23S rRNA and 5S rRNA (no TP50)[f]	0	0
50S reconstituted with in vitro-transcribed 23S rRNA and 5S rRNA[g]	22 ± 2	30 ± 3
in vitro-transcribed 23S rRNA and 5S rRNA (no TP50)[h]	0	0
TP50 (no rRNA)[f]	0	2 ± 2
natural 23S rRNA and TP50 (no 5S rRNA)[e]	0	–
in vitro-transcribed 23S rRNA and TP50 (no 5S rRNA)[g]	0	–

[a] Peptidyl transferase activity was measured under "fragment reaction" conditions as described in Materials and Methods. Reaction mixtures contained 2.5 pmol of native or reconstituted 50S subunits or an equivalent amount of 50S subunit components. Reconstituted subunits were purified by sucrose gradient centrifugation prior to their addition into the reaction mixture. [b] The poly(U)-dependent cell-free translation reaction mixtures contained 15 pmol of *T. aquaticus* ribosomal 30S subunits in addition to 15 pmol of native or assembled 50S subunits (or equivalent amounts of rRNA or TP50 proteins). Assembled 50S subunits were added to the reaction mixture without sucrose gradient purification. Other components of the poly(U)-dependent cell-free translation system are specified in Materials and Methods. [c] Activity values are an average of two independent experiments. Background radioactivity of the control sample containing no ribosomal components (2% of the radioactivity of the native 50S subunit sample) was subtracted from all activity values. [d] Activity values are an average of two independent experiments; the TCA-precipitable radioactivity of the sample containing no ribosomes (17% of the radioactivity of the native 50S subunit sample) was subtracted from all radioactivity values. [e] Natural rRNA was assembled with TP50 proteins in a two-step reconstitution reaction. [f] Natural rRNA (without TP50) or TP50 (without rRNA) was incubated under two-step reconstitution conditions. The integrity of rRNA after incubation was tested by gel electrophoresis. [g] In vitro-transcribed rRNA was assembled with TP50 proteins in a three-step reconstitution reaction. [h] In vitro-transcribed rRNA (without TP50) was incubated under three-step reconstitution conditions. The integrity of rRNA after incubation was tested by gel electrophoresis.

of the in vitro transcript may be different from that of natural 23S rRNA. (iii) Posttranscriptional modifications, present in the natural rRNA and missing in the in vitro transcript, may be required for optimal activity of the assembled particles. Since no protein bands were detected on the Coomassie-stained SDS gel overloaded with 23S rRNA isolated from *T. aquaticus* 50S subunits, association of proteins with natural rRNA was unlikely to be the reason for the difference in activity of subunits reconstituted with natural or with synthetic rRNA (data not shown). To test the importance of rRNA conformation for subunit assembly, the natural and synthetic 23S rRNA was denatured by heating it in water at 100 °C for 2 min and fast cooling on ice prior to combining it with 5S rRNA and TP50 proteins in the reconstitution reaction. Denaturation of natural rRNA resulted in a significant drop (ca. 25-fold) in activity of the reconstituted material, while activity of subunits reconstituted from the heat-denatured in vitro 23S rRNA transcript was slightly increased (Figure 2A). Hence, levels of activity of 50S subunits assembled from the denatured natural 23S rRNA or in vitro-transcribed 23S rRNA were practically the same. This result indicated that low efficiency of reconstitution of functional 50S subunits from the rRNA in vitro transcript could be due to unfavorable transcript conformation, rendering it incompetent for reconstitution. Therefore, to increase the yield of active subunits reconstituted with synthetic rRNA, we introduced an additional "refolding" step in the assembly protocol during which the reconstitution reaction mixture containing in vitro-transcribed 23S and 5S rRNAs and TP50 proteins was first heated to 70 °C in the presence of 4 mM $MgCl_2$ and then, after incubation for 1 min, rather rapidly cooled to 44 °C. Addition of this step dramatically changed the sedimentation pattern of reconstituted material which now formed a sharp symmetric peak with a sedimentation rate close to that of 50S subunits (Figure 1F). The "improved" sedimentation pattern correlated with increased activity of the assembled subunits; subunits assembled in a three-step procedure from TP50 and synthetic rRNA and

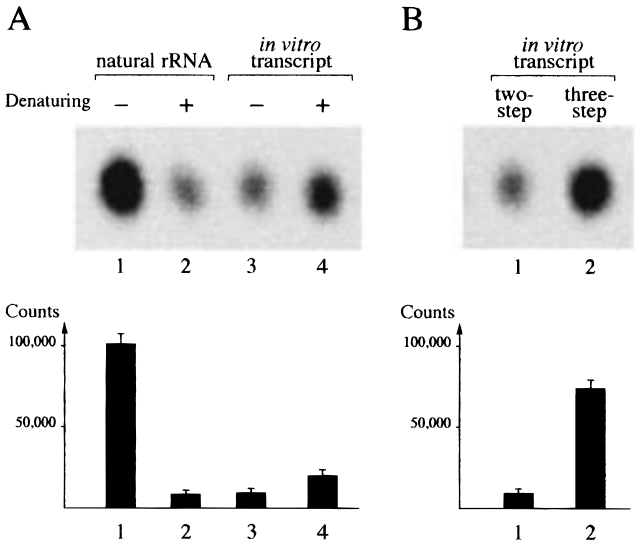

FIGURE 2: Peptidyl transferase activity of *T. aquaticus* large ribosomal subunits assembled with natural or synthetic 23S rRNA. Peptidyl transferase activity was measured by accumulation of formyl-[^{35}S]methionyl-puromycin (see Materials and Methods for experimental details). The upper panel shows the fMet-puromycin spot resolved by paper electrophoresis and visualized with a β-scanner. The histogram in the bottom panel shows the radioactivity of fMet-puromycin spots (counts accumulated after exposure for 12 h). Reconstituted subunits were added directly into the assay reaction mixture without prior sucrose gradient purification. (A) Effect of heat denaturing of natural or in vitro-transcribed 23S rRNA (2 min at 100 °C, in water) on the catalytic activity of 50S subunits assembled in a two-step reconstitution reaction; heat-denatured samples are marked by +. (B) Peptidyl transferase activity of 50S subunits reconstituted from TP50 and in vitro-transcribed rRNA in two-step (left) and three-step (right) reactions.

purified with a sucrose gradient showed peptidyl transferase activity approaching that of subunits reconstituted with natural 23S rRNA (22 and 25% of the activity of native 50S subunits, respectively) (Figure 2B and Table 1). A longer incubation of the assembly mixture at high temperatures or increasing the temperature during the refolding step reduced

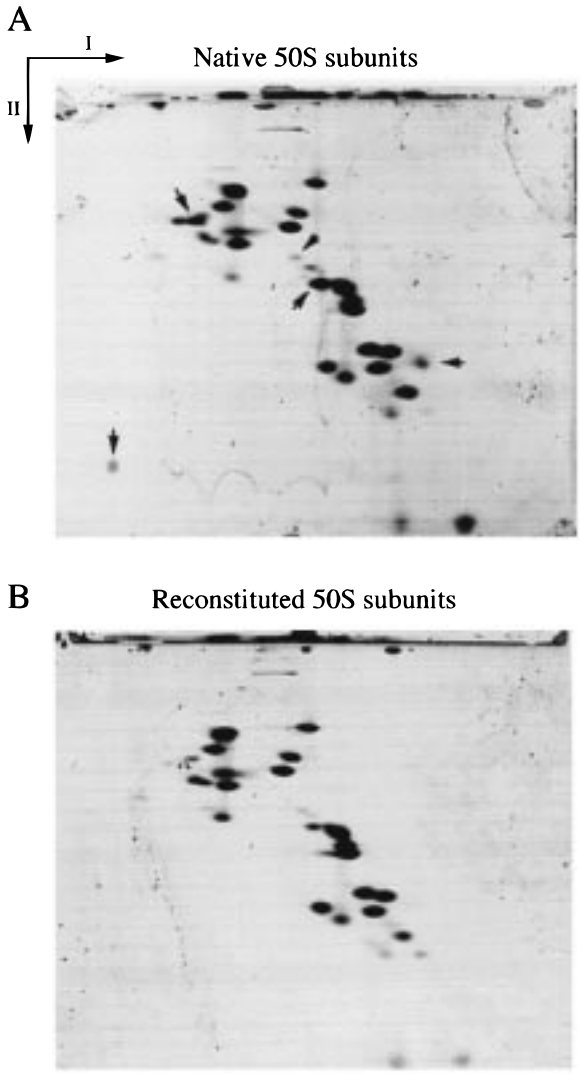

FIGURE 3: Two-dimensional gel electrophoresis analysis of the protein composition of (A) native 50S subunits and (B) subunits reconstituted from TP50 and in vitro-transcribed rRNA. Arrows indicate protein spots whose intensity was significantly reduced in reconstructed particles compared to those of native 50S subunits.

the activity of reconstituted particles. The refolding step did not affect the activity of subunits reconstituted with natural rRNA (data not shown).

50S subunits reconstituted from TP50 and synthetic rRNA showed considerable activity not only in the peptidyl transferase assay but also in poly(U)-dependent polypeptide synthesis. Though their activity was lower than that of the subunits assembled from the natural rRNA, it still reached approximately 25% of the activity of the native 50S subunits (Table 1).

Protein Composition of Reconstituted Subunits. To determine the protein composition of subunits reconstituted from the in vitro-transcribed rRNA, assembled subunits were purified by sucrose gradient centrifugation and proteins were analyzed by two-dimensional gel electrophoresis. Comparison of the protein pattern of reconstituted subunits with that of the native 50S subunits showed that some protein spots were missing in reconstituted material or were present in a reduced amount (Figure 3). Since only some ribosomal proteins of *T. aquaticus* have been characterized, it was impossible to assign proteins corresponding to the missing

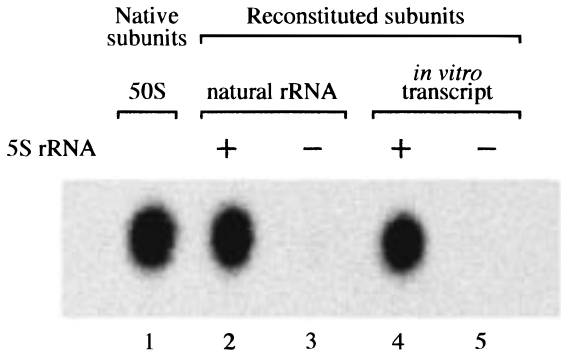

FIGURE 4: Effect of the presence of 5S rRNA on the peptidyl transferase activity of reconstituted 50S subunits. Subunits were reconstituted with TP50 proteins and either natural (samples 2 and 3) or in vitro-transcribed (samples 4 and 5) 23S rRNA in the absence (−) or in the presence (+) of 5S rRNA. Sample 1 was native 50S subunits. Reconstituted subunits were added directly to the peptidyl transferase assay reaction mixture without sucrose gradient purification. fMet-Puromycin formed in the reaction was resolved by paper electrophoresis and visualized with a β-scanner (see Materials and Methods for details).

spots. Incomplete incorporation of certain ribosomal proteins in the reconstructed subunits could partly explain the reduced activity of reconstituted subunits in the peptidyl transferase reaction and poly(U) translation system. Interestingly, the reconstituted particles maintained sedimentation properties of native 50S subunits despite the missing proteins (Figure 1F). This result was in accordance with our previous observation that peptidyl transferase-active particles, prepared by treatment of *T. aquaticus* large ribosomal subunits with proteinase, SDS, and phenol and containing only a limited number of ribosomal proteins, maintained their compactness and sedimented as 50S subunits (*37*).

Importance of 5S rRNA for Efficient Reconstitution of Catalytically Active Subunits. The presence of 5S rRNA was absolutely essential for the activity of *T. aquaticus*-reconstituted subunits; the activity of subunits assembled without 5S rRNA was below the detection threshold (at least 3 orders of magnitude lower compared to the activity of subunits reconstituted under standard conditions) (Figure 4). However, the nature of 5S rRNA was less critical; natural *T. aquaticus* 5S rRNA could be substituted with the in vitro-transcribed *T. aquaticus* 5S rRNA or with *E. coli* 5S rRNA without any decrease of peptidyl transferase activity of assembled subunits (data not shown).

Reconstitution of 50S Subunits with the Mutant 23S rRNA Transcript. To test whether 50S subunits reconstituted with synthetic 23S rRNA can be used for in vitro mutational analysis and to prove unequivocally that the activity of assembled 50S subunits depended on the in vitro-transcribed rRNA, we introduced mutations into the in vitro-transcribed 23S rRNA and tested the activity of reconstituted subunits.

The Watson−Crick interaction between G2252 of *E. coli* 23S rRNA and C74 of peptidyl-tRNA is essential for the binding of peptidyl tRNA in the ribosomal P-site (*17*). A mutation of G2252 to A compromises the binding of peptidyl-tRNA and renders ribosomes significantly less active in the peptidyl transferase reaction (*17*). The corresponding mutation (position 2267 in *T. aquaticus*) was engineered in the *T. aquaticus* 23S rRNA gene by site-directed mutagenesis. Subunits reconstructed with the in vitro transcript carrying the G2267A mutation displayed a sedimentation

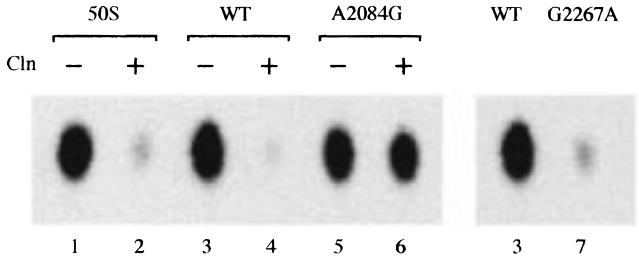

FIGURE 5: Peptidyl transferase activity of *T. aquaticus* large ribosomal subunits assembled with in vitro-transcribed wild type or mutant 23S rRNA. Radioactive spots represent fMet-puromycin formed as a result of the peptidyl transferase reaction catalyzed by native or assembled 50S subunits (see the legends of Figures 2 and 4): effect of clindamycin (Cln) on the activity of native 50S subunits (lanes 1 and 2), subunits assembled with in vitro-transcribed wild type 23S rRNA (lanes 3 and 4), or subunits assembled with in vitro-transcribed 23S rRNA containing the A2084G mutation (A2058G in *E. coli*) (lanes 5 and 6) and peptidyl transferase activity of subunits assembled with in vitro-transcribed 23S rRNA containing the G2267A mutation (G2252A in *E. coli*) (lane 7).

pattern similar to that of subunits assembled with the wild type 23S rRNA transcript (not shown); however, they exhibited a significantly reduced level of peptidyl transferase activity (Figure 5).

The mutation A2058G was shown to render *E. coli* ribosomes resistant to a peptidyl transferase inhibitor clindamycin (*47*). When *T. aquaticus* 23S rRNA transcripts carrying the corresponding mutation, A2084G, were used in reconstitution, the peptidyl transferase activity of the reconstituted subunits showed markedly increased resistance to clindamycin compared to subunits reconstituted with the wild type 23S rRNA transcript (Figure 5). Thus, in vitro genetics experiments clearly demonstrated that the peptidyl transferase activity of reconstituted subunits was due to incorporation of the in vitro-transcribed 23S rRNA and that the developed reconstitution system can be used for in vitro mutational analysis of 23S rRNA functions.

DISCUSSION

In this paper, we demonstrate that functionally active 50S ribosomal subunits of *T. aquaticus* can be reconstituted from total large subunit proteins and in vitro-transcribed rRNA. Assembled subunits efficiently catalyze the peptidyl transferase reaction and exhibit high activity in poly(U)-dependent cell-free translation which opens a possibility for using the *T. aquaticus* reconstitution system for in vitro mutational analysis of large ribosomal subunit rRNA. In addition, this result demonstrates that posttranscriptionally modified nucleotides in 23S rRNA are dispensable for translation.

Previous attempts to reconstitute functional ribosomal subunits from in vitro-transcribed 23S rRNA were unsuccessful. Active *E. coli* 50S subunits could be efficiently assembled from ribosomal proteins and natural rRNA (*7*), whereas the activity of particles reconstituted from the in vitro-transcribed rRNA was 6 orders of magnitude lower than that of native 50S subunits (*23*, *24*). Our success in reconstitution of functional *T. aquaticus* 50S subunits with synthetic 23S rRNA suggests that the reason for the lack of activity in the *E. coli* system was incorrect folding of the rRNA transcript rather than the lack of posttranscriptional modifications. In vivo, rRNA folding and ribosome assembly occur cotranscriptionally; ribosomal proteins and chaperone factors bind to the growing nascent rRNA and guide its folding into the functional conformation (*48*−*51*). The aberrant conformation of rRNA may hinder ribosome assembly. Mutations in rRNA interfering with its natural folding cause assembly defects (*52*, *53*). Furthermore, *E. coli* 23S rRNA transcribed in vivo by the bacteriophage T7 RNA polymerase fails to assemble into functional 50S subunits. The transcription rate of T7 RNA polymerase is much faster than that of cellular RNA polymerase which probably results in misfolding of the rRNA transcript (*54*). It is conceivable that *E. coli* 23S rRNA transcribed in vitro by T7 RNA polymerase may also be trapped in a kinetically stable conformation incompatible with rRNA assembly into active subunits.

Even in our first experiments with thermophilic ribosomes from *T. aquaticus*, the activity of 50S subunits reconstituted from the 23S rRNA in vitro transcript and TP50, though still low, was at least 4 orders of magnitude higher than that reported previously for the *E. coli* system (*24*), demonstrating a high potency of *T. aquaticus* 23S rRNA transcript to fold into a conformation competent for the assembly of functional subunits. It was possible to "activate" further the in vitro transcript by partially denaturing it at 70 °C (the natural temperature for assembly of *T. aquaticus* ribosomes in vivo) and then allowing it to refold in the presence of TP50 proteins. Binding sites for at least some ribosomal proteins might be formed only in the native rRNA conformation; therefore, binding of ribosomal proteins might lock the partially denatured rRNA transcript in its functional conformation during the refolding step. The activity of 50S subunits reconstituted by a three-step procedure, which included a refolding step, was improved more than 1 order of magnitude compared to the two-step assembly protocol optimized for the natural *T. aquaticus* 23S rRNA.

Though the origin of 23S rRNA appeared to be important for subunit reconstitution, the nature of the second rRNA molecule of the 50S subunit, 5S rRNA, was less essential; equally active subunits could be reconstituted using natural 5S rRNA, in vitro-transcribed 5S rRNA, or even 5S rRNA from *E. coli*. Though 5S rRNA was previously shown to play an important role during the last steps of assembly of *E. coli* 50S subunits (*13*), subunits assembled in the absence of 5S rRNA exhibited significant peptidyl transferase activity (ca. 10% of that of native 50S subunits) (*55*). In contrast, despite the relaxed requirements regarding the 5S rRNA nature, the actual presence of 5S rRNA was absolutely essential for the assembly of *T. aquaticus* functional subunits, irrespective of whether natural or in vitro-transcribed 23S rRNA was used in reconstitution (Figure 4). The function of 5S rRNA in the ribosome remains unclear (*56*). 5S rRNA can be cross-linked to segments of 23S rRNA located in domains II and V, both of which might be involved in formation of the peptidyl transferase catalytic center (*57*). Therefore, even not being directly involved in peptidyl transfer catalysis, 5S rRNA might be important for aligning domains II and V of 23S rRNA during 50S subunit assembly. This notion is supported by the fact that activity of *T. aquaticus* 50S subunits assembled in the absence of 5S rRNA can be partly restored if reconstitution is performed in the presence of antibiotics known to interact simultaneously with

domains II and V of 23S rRNA (P. Khaitovich and A. S. Mankin, in preparation).

The possibility of reconstructing functionally active large ribosomal subunits from ribosomal proteins and in vitro-transcribed rRNA resolved a long-standing question of the importance of posttranscriptionally modified nucleotides in rRNA for the activity of the 50S subunit. The central role of 23S rRNA in the catalysis of peptide bond formation has been unequivocally established in a variety of experiments (see refs *2* and *58*−*60* for reviews). The majority of modified nucleotides in 23S rRNA are located in the vicinity of the peptidyl transferase center: in domain V, which is known to be an integral part of peptidyl transferase, and in domains IV and II, which are located in the immediate proximity of the catalytic center in the tertiary structure of the ribosome and may constitute essential parts of peptidyl transferase. Such clustering of the modified nucleotides around the peptidyl transferase center may reflect their involvement, direct or indirect, in some aspects of ribosome functioning (*25*, *29*, *30*). Though many of the posttranscriptionally modified nucleotides found in *E. coli* 23S rRNA are also present in 23S rRNA of *T. aquaticus* (P. F. Crain, J. A. McCloskey, P. Khaitovich, and A. S. Mankin, unpublished results), ribosomal subunits assembled with the *T. aquaticus* 23S rRNA transcript containing no modifications not only are active in the peptidyl transferase assay but also exhibit high activity in a cell-free translation system. Therefore, it is clear that modified nucleotides in 23S rRNA are dispensable for the principal activities of the ribosome. This conclusion is consistent with the fact that location of modified nucleotides in rRNA is not well conserved (*31*, *61*, *62*) and supplements the previous finding that functional small ribosomal subunits can be reconstituted using the in vitro transcript of 16S rRNA containing no modified nucleotides (*19*, *22*). It should be emphasized, however, that our results do not exclude the functional significance of posttranscriptional modifications. Indeed, in the poly(U)-dependent cell-free translation system 50S subunits assembled with natural rRNA showed higher activity than subunits reconstituted with the 23S rRNA in vitro transcript, suggesting that posttranscriptional modification might contribute to subunit association, fine-tuning of peptidyl transferase activity, or communicating signals from small to large ribosomal subunits. Furthermore, the presence of modified nucleotides might facilitate rRNA folding during ribosome biogenesis in vivo (*29*, *63*, *64*), and in the case of *E. coli* 50S subunits, the lack of some modification may preclude 23S rRNA from in vitro assembly into functional subunits (*24*).

Large ribosomal subunits assembled from ribosomal proteins and in vitro-transcribed rRNA maintain functional characteristics of native 50S subunits. A mutation, known to interfere with the catalytic activity of the ribosome as well as a mutation which conferred antibiotic resistance in vivo, produced similar effects on the function of large ribosomal subunits assembled from the in vitro transcript of rRNA (Figure 5). These observations open the possibility to apply site-directed or random mutagenesis for elucidation of 23S rRNA involvement in ribosomal functions, in particular, catalysis of peptide bond formation. Combining rRNA mutagenesis and reconstitution of large ribosomal subunits with the recently developed methods of selection of functional 50S subunits (*65*, *66*) makes it possible to approach directly the involvement of individual 23S rRNA nucleotides in ribosomal catalytic functions and interactions with ribosomal ligands.

There have been several exciting reports of functional activity of deproteinated *E. coli* 23S rRNA or even its in vitro transcript (*67*, *68*). Under our reaction conditions, neither natural *T. aquaticus* 23S rRNA nor its in vitro transcript exhibited any activity in the puromycin reaction or cell-free translation system (Table 1). Similar negative results were obtained in other laboratories with isolated *E. coli* 23S rRNA (*24*) (R. Green, personal communication).

The only other example of reconstituting active large ribosomal subunits from the in vitro 23S rRNA transcript is presented in the preceding paper by Green and Noller (*69*). It is probably not a coincidence that in this case, similar to our work, the ribosomes were from a thermophilic source, *Bacillus stearothermophilus*. Thus, it appears that the thermophilic nature of ribosomal constituents, rRNA and/or ribosomal proteins, facilitates assembly of functional subunits with the rRNA in vitro transcript. It is possible that the high G-C content of the thermophilic rRNA (63% in *T. aquaticus* and 58% in *B. stearothermophilus* vs 53% in *E. coli*) may be beneficial for the correct folding of the in vitro-transcribed 23S rRNA during 50S subunit reconstitution. On the other hand, proteins from thermophilic ribosomes may be more robust and prone for association with the 23S rRNA transcript than *E. coli* ribosomal proteins (*37*).

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