Reconstitution of Functional 50S Ribosomes from in Vitro Transcripts of *Bacillus stearothermophilus* 23S rRNA†

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ABSTRACT: In vitro transcripts of *Bacillus stearothermophilus* 23S rRNA can be reconstituted into catalytically active 50S ribosomal subunits with an efficiency only 3−4-fold lower than that of natural 23S rRNA. Thus, post-transcriptional modifications in 23S rRNA are not essential for the assembly or function of the 50S subunit of the ribosome. This reconstitution sytem has been used to characterize the peptidyl transferase activity of site-directed mutations in 23S rRNA at positions G2252, U2506, U2584, and A2602 (*Escherichia coli* numbering), demonstrating its potential for the analysis of the role played by 23S rRNA in the function of the 50S subunit of the ribosome.

The 50S subunit of the ribosome is the site of its sole catalytic function, peptide bond formation. This macromolecular complex is composed of two large rRNAs, 5S [approximately 120 nucleotides (nt)] and 23S (approximately 2900 nt), and more than 30 different proteins. The in vivo assembly of this subunit, while not yet well-characterized, is clearly a complex, highly ordered event involving a series of noncovalent and covalent RNA and protein processing steps (*1*, *2*). Remarkably, the 50S subunit from several organisms, including eubacterial and archaeal species, can be reconstituted in vitro from the individual RNA and protein components (*3*−*7*). While the kinetics of in vitro assembly are slow and the efficiencies in different systems are variable (10−90%), it nonetheless seems likely that these processes share many fundamental features with those of in vivo assembly.

These reconstitution systems are a powerful tool for the analysis of the structure, function, and assembly of the 50S subunit (*8*−*13*). An in vitro reconstitution system dependent on in vitro-generated transcripts of 23S rRNA would provide an improved approach to in vitro genetics of 23S rRNA. Recently, we reported that in vitro transcripts of *Escherichia coli* 23S rRNA are severely compromised (5 orders of magnitude) in their ability to reconstitute into catalytically active 50S subunits in standard reconstitution reactions (*14*). Further analysis indicated that this is likely due to the absence of post-transcriptional rRNA modifications critical for either the in vitro assembly or function of this subunit of *E. coli*. Mapping studies identified an 80 nt region of 23S rRNA (containing six post-transcriptional modifications) responsible for this deficiency, allowing for the substitution of the remainder of the 2900 nt molecule with in vitro-transcribed rRNA in what was termed a "chimeric reconstitution". This system has been used to characterize several site-directed mutations in the peptidyl transferase center of 23S rRNA (*15*) and to identify a base-pairing interaction between G2252 in 23S rRNA and C74 of P-site-bound tRNA (*16*). However, the chimeric reconstitution approach is experimentally awkward, and the efficiency is relatively low. Furthermore, there is reason to suspect that the observed difficulties in reconstitution of *E. coli* 50S subunits may be species-specific. First, the 80 nt region of *E. coli* 23S rRNA that must be supplied as natural RNA for the in vitro reconstitution of catalytically active 50S subunits contains no universally conserved post-transcriptional modifications (*17*). Second, the magnesium and temperature requirements of the *E. coli* reconstitution system (*18*) are more elaborate than those of other 50S subunit reconstitution systems. Efficient reconstitution in the *E. coli* system depends on a two-step procedure (a first step at 4 mM Mg^{2+} and 44 °C and a second step at 20 mM Mg^{2+} and 50 °C) in which the first step exhibits an unusually sharp magnesium dependence profile.

We were interested in establishing whether the failure of in vitro transcripts of *E. coli* 23S rRNA to reconstitute into active 50S subunits is general or whether a system based on another organism might provide a more tractable in vitro reconstitution system. In this study, a DNA vector was constructed for runoff transcription of *Bacillus stearothermophilus* 23S rRNA by T7 RNA polymerase and for single-strand-based site-directed mutagenesis of this gene. Next it was shown that in vitro transcripts of *B. stearothermophilus* 23S rRNA can be reconstituted into catalytically active 50S subunits with efficiencies only 3−4-fold lower than that of natural rRNA. Different methods of preparation of *B. stearothermophilus* total r-proteins and rRNA were compared with respect to their ability to be reconstituted into catalytically active 50S subunits. Finally, transcripts containing site-directed mutations at functionally implicated positions of domain V of 23S rRNA were reconstituted, and their peptidyl transferase activity was tested. This system thus provides for convenient and sensitive mutational analysis of 23S rRNA using pure mutant rRNA populations.

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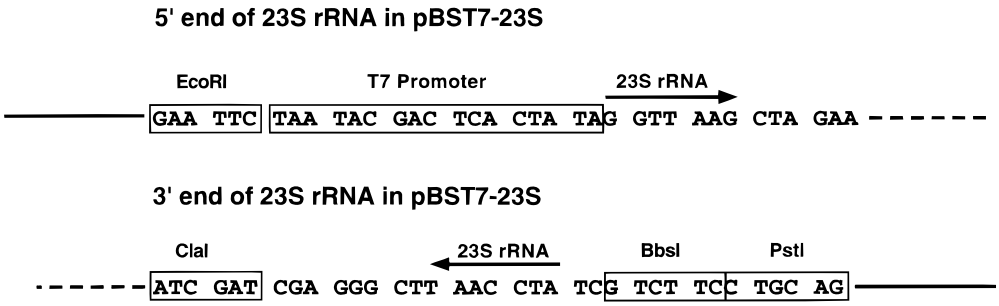

FIGURE 1: Nucleotide sequences found surrounding the 5′ and 3′ ends of the *B. stearothermophilus* 23S rRNA gene in pBST7-23S. Various restriction sites and the T7 RNA polymerase promoter are boxed, and the specific ends of the 23S rRNA transcriptional unit are indicated with arrows.

MATERIALS AND METHODS

Bacterial Growth and Ribosome Preparation. B. stearothermophilus strain 799 was grown and 70S ribosomes and 50S subunits were prepared essentially as described previously (*19*), except that a French press was used instead of alumina to lyse the bacterial cells.

Construction of Plasmid pBST7-23S. A 2.5 kilobase (kb) *Bam*HI−*Cla*I 23S rRNA fragment was isolated from *B. stearothermophilus* rRNA operon-containing plasmid pKW233 (*20*) grown in a methylation-deficient strain of *E. coli*, GM2163. This fragment was inserted, along with an oligonucleotide cassette composed of annealed oligonucleotides 26.1 (5′-GGA-AGA-CGA-TAG-GTT-AAG-CCC-TCG-AT-3′) and 32.1 (5′-CGA-TCG-AGG-GCT-TAA-CCT-ATC-GTC-TTC-CTG-CA-3′), into *Bam*HI−*Pst*I-restricted pUC118 in a three-piece ligation reaction to generate pBST7-3′. Next, the 5′ end (411 nucleotides) of the 23S rRNA gene was amplified by polymerase chain reaction (PCR) using the following oligonucleotide primers (53.1, 5′-GGT-AAC-ACG-CAT-ATG-TAA-TAC-GAC-TCA-CTA-TAG-GTT-AAG-CTA-GAA-AGG-GCG-CA-3′; and 21.13, 5′-TCA-GGA-TCC-GCT-CGG-GAG-GGA-3′). Reamplification of this product with primer 26.31 (5′-GCG-GAA-TTC-TAA-TAC-GAC-TCA-CTA-TA-3′) and 21.13 yielded a PCR DNA product that was digested with *Bam*HI and *Eco*RI and ligated into *Bam*HI−*Eco*RI-restricted pBST7-3′. The product of this ligation was pBST7-23S. Sequence data were obtained for the 5′ and 3′ ends of the 23S rRNA gene in the final clone covering the ligation junctions and the PCR-amplified region; the nucleotide sequences of these regions are shown in Figure 1.

Site-Directed Mutagenesis. Oligonucleotide-directed mutations were constructed in pBST7-23S as described previously (*16*) using the following mutagenic primers (*E. coli* numbering will be used throughout the paper): 2252 (U), 5′-GGA-GGC-GAC-CGC-(AT)CC-AGT-CAA-ACT-G-3′; 2506 (A, C, and G), 5′-TGC-GAT-GAG-CCG-(CGT)CA-TCG-AGG-TGC-C-3′; 2584 (A and C), 5′-ACG-ACG-TTC-TGA-(CGT)CC-CAG-CTC-GCG-T-3′; and 2602 (C), 5′-TAG-GGA-CCG-AAC-(ACG)GT-CTC-ACG-ACG-T-3′.

Preparation of Proteins and rRNA from Ribosomes. Total rRNA (urea/magnesium at pH 2.0) and TP70 (urea/magnesium at pH 2.0) were prepared from *B. stearothermophilus* 70S ribosomes (or 50S subunits) as described previously (*21*). The B-L3 protein fraction was prepared as described previously (*4*). Total rRNA (phenol) and TP70 (acetic acid) were alternatively prepared from *B. stearothermophilus* 70S ribosomes essentially as described for *E. coli* subunits (*22*), with the following exceptions. To completely extract the *B. stearothermophilus* proteins from the rRNAs using phenol, 50 mM ethylenediaminetetraacetic acid (pH 8.0) (EDTA) was added to the standard extraction buffer [20 mM Hepes/KOH (pH 7.6), 4 mM $MgCl_2$, 30 mM NH_4Cl, 2 mM spermidine, 0.2 mM spermine, 5 mM 2-mercaptoethanol, and 1% sodium dodecyl sulfate] and the resulting RNA pellet was resuspended in 30 mM Tris (pH 7.5), 20 mM $MgCl_2$, and 6 mM 2-mercaptoethanol. TP70 prepared with acetic acid extraction was dialyzed in the final step against 30 mM Tris (pH 7.5), 1 M KCl, 20 mM $MgCl_2$, and 6 mM 2-mercaptoethanol.

Preparation of in Vitro-Transcribed 23S rRNA. In vitro transcripts were transcribed by T7 RNA polymerase (*23*) from either PCR DNA templates obtained by amplifying wild-type *B. stearothermophilus* 23S rRNA (5′ primer for 23S rRNA, 5′-TAA-TAC-GAC-TCA-CTA-TAG-GTT-AAG-CTA-GAA-AGG-GCG-CA-3′; and 3′ primer for 23S rRNA, 5′-TAG-GTT-AAG-CCC-TCG-ATC-GAT-3′) from chromosomal DNA prepared from strain 799 or from *Bbs*I-restricted pBST7-23S and its mutant derivatives. GMP (10 mM) was included in the transcription reaction to generate transcripts initiated with a monophosphate, mimicking the natural processed 23S rRNA. Transcripts were separated from unincorporated nucleotides on a G50-Sephadex (Pharmacia) gel filtration column (1 cm × 20 cm) run in 30 mM Tris (pH 7.5) and 20 mM $MgCl_2$. Recovered RNA was precipitated with 0.5 M ammonium acetate (pH 6.0) and resuspended in 30 mM Tris (pH 7.5) and 20 mM $MgCl_2$ and stored at −80 °C.

In Vitro Reconstitution Reactions. In vitro reconstitution was performed with 23S rRNA at a concentration of $10A_{260}$ per milliliter and 5S rRNA at $0.3A_{260}$ per milliliter (*19*); other reagents, including buffers, salts, and proteins, were varied as described. The final optimized reconstitution for both natural and in vitro-transcribed 23S rRNA contained the following in a 30 μL total volume: $0.3A_{260}$ 23S rRNA, $0.01A_{260}$ 5S rRNA (*E. coli* from Boehringer Mannheim), 3 molar equiv of TP70, 3 molar equiv of B-L3 in 30 mM Tris (pH 7.5), 0.33 M KCl, 20 mM $MgCl_2$, 0.3 M NH_4Cl, and 6 mM 2-mercaptoethanol. Reactions were set up on ice by first adding the RNA components to the buffers and salts and then adding the protein components. The reaction mixtures were then incubated at 60 °C for 2 h, placed on ice for 10 min, and added directly to peptidyl transferase reaction mixtures.

Activity Assays. Poly(Phe) synthesis was measured as described previously using *E. coli* 30S subunits (*19*). Peptidyl

transferase activity was measured essentially as described previously (*15*) using wild-type and mutant oligonucleotide substrates. Reaction products were analyzed either by using high-voltage paper electrophoresis and phosphorimager quantitation or by directly quantitating the radioactivity in the ethyl acetate-extracted fraction by scintillation counting.

Sucrose Gradient Analysis. Reconstitution reaction mixtures were layered onto 10 mL 10 to 40% sucrose gradients in 30 mM Tris (pH 7.5), 0.1 M KCl, and 5 mM $MgCl_2$ (as indicated). Gradients were spun in an SW41 rotor at 22 000 rpm for 18 h (*14*).

RESULTS

Reconstitution of 50S Subunits from Natural 23S and 5S rRNA and Total Proteins Extracted from B. stearothermophilus Ribosomes. Catalytically active *B. stearothermophilus* 50S subunits can be reconstituted from their individual rRNA (5S and 23S) and r-protein components in a single-step 60 °C incubation in buffer containing 30 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.33 M KCl, and 6 mM 2-mercaptoethanol (*3*, *4*, *21*). The two-step protocol found to be essential for the reconstitution of active *E. coli* 50S subunits (including a low-temperature, low-magnesium incubation followed by a high-temperature, high-magnesium incubation) is not required for the reconstitution of *B. stearothermophilus* 50S subunits. Overall, the efficiency of the in vitro reconstitution of *B. stearothermophilus* 50S subunits is comparable to that of the analogous *E. coli* reconstitution (using the fragment reaction, ca. 30% reconstitution of peptidyl transferase activity compared to that of native 50S subunits) (*3*−*5*, *21*, *22*).

Natural ribosomal RNAs and total protein mixtures from *B. stearothermophilus* were prepared from 50S subunits and 70S ribosomes with several different procedures. In one procedure, essentially that of Cohlberg and Nomura (*21*), total proteins (TP50) were prepared from *B. stearothermophilus* 50S subunits by removing them from 23S and 5S rRNA by treatment with 4.5 M urea/0.5 M $MgCl_2$ (pH 2.0). In a modification of this procedure, the proteins were isolated in two separate fractions (*4*). In the first fraction, the less tightly bound proteins were removed from the 50S subunits using 4 M urea/2 M LiCl (TP50-L3). The more tightly bound proteins (including predominantly *B. stearothermophilus* L3, the homologue to *E. coli* L2) were removed from the remaining core using 4.5 M urea/0.5 M $MgCl_2$ (pH 2.0) (the L3 fraction). In each step, the rRNAs were removed from the protein fraction by centrifugation and the protein-containing supernatant was dialyzed into reconstitution buffer with 30 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 M KCl, and 6 mM 2-mercaptoethanol. Total natural rRNA (including 5S, 16S, and 23S rRNA) was prepared reciprocally by removing all proteins from 70S ribosomes with 4.5 M urea/0.5 M $MgCl_2$ (pH 2.0), recovering the rRNAs by centrifugation, and then resuspending in H_2O followed by dialysis against low-salt reconstitution buffer [30 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 6 mM 2-mercaptoethanol].

The RNA and protein fractions were combined in a standard *B. stearothermophilus* 50S subunit reconstitution at 60 °C for 120 min, and the efficiency of the reconstitution was tested using the "fragment" reaction assay for peptidyl transferase and poly(U)-templated poly(Phe) synthesis. In the fragment reaction, the minimal P- and A-site substrates, CACCA-*N*-Ac-[^{35}S]Met and puromycin, are combined with the various reconstitution mixtures in the presence of high salt and magnesium and 33% methanol, and the production of *N*-Ac-[^{35}S]Met-puromycin is followed (*14*, *24*). In both assays, the 50S reconstitution mixtures (using both protein preparations, TP50 and TP50-L3/L3) have approximately 5−10-fold less activity than untreated *B. stearothermophilus* 50S subunits (data not shown). Although these results are generally consistent with previous observations (*21*), we found certain aspects of the experimental procedure to be undesirable. While treatment of the ribosomes with urea/$MgCl_2$ at low pH yielded RNAs that were intact when analyzed on urea denaturing polyacrylamide gels (data not shown), it also resulted in an rRNA pellet that was extremely difficult to resolubilize, even following incubation for extended periods of time in H_2O at room temperature. For *E. coli* 50S subunit reconstitution, the preparation of rRNAs that reconstitute with high efficiency has been shown to be highly dependent on careful treatment at all stages of the purification, including the continuous presence of polyamines and divalent cations (*25*).

Therefore, we next prepared total protein mixtures and rRNAs from *B. stearothermophilus* 70S ribosomes by modification of gentler procedures adapted for *E. coli* 50S reconstitution (*5*, *22*). Total proteins were extracted from the ribosomes by treatment with acetic acid and magnesium acetate, precipitated with acetone, and then dialyzed against protein reconstitution buffer (as above) (*26*). Total rRNA was prepared by successive phenol extraction in the presence of 0.5% SDS and 50 mM EDTA in a buffer containing 20 mM Hepes/KOH (pH 7.6), 4 mM $MgCl_2$, 30 mM NH_4Cl, 2 mM spermidine, 0.2 mM spermine, and 5 mM 2-mercaptoethanol (*26*); while the presence of 50 mM EDTA obviates the presence of the stabilizing Mg^{2+} ions, the polyamines may continue to stabilize the rRNAs. Following five phenol extractions, the rRNAs were precipitated with ethanol and resuspended in RNA reconstitution buffer (as above). No proteins (including the extremely tightly bound B-L3) were found to remain associated with these extracted rRNAs when analyzed by SDS−PAGE (data not shown); notably, when EDTA is not included in the phenol extraction procedure, the tightly bound *B. stearothermophilus* proteins remain associated with the rRNAs. Using these differently prepared rRNAs and r-proteins in standard *B. stearothermophilus* reconstitution reactions resulted in efficiencies that were slightly higher than those obtained with reactions using the urea, $MgCl_2$, low-pH extraction procedures, as assessed using the fragment reaction (see Table 1, experiment 1-A vs 3−5-A). Given the favorable comparison in efficiency, the phenol extraction procedure for rRNAs offers a considerable advantage over the urea, LiCl, low-pH procedure in terms of ease and yield.

Cloning of B. stearothermophilus 23S rRNA for in Vitro Transcription by T7 RNA Polymerase. A pUC118 plasmid derivative carrying the *B. stearothermophilus* 23S rRNA gene under transcriptional control of a T7 RNA polymerase promoter, with a *Bbs*I restriction site (nonpalindromic) at the 3′ end of the gene for runoff transcription, was constructed as follows. The rDNA plasmid pKW233 (*20*) was used as a source of restriction fragments and as a PCR template for all manipulations. In an initial three-piece

Table 1: Peptidyl Transferase Activity of Reconstituted 50S Subunits Derived from either Natural 23S rRNA or Transcripts Generated by T7 RNA Polymerase Expressed as a Fraction of the Activity of Untreated *B. stearothermophilus* 50S Subunits[a]

	23S rRNA	5S rRNA	proteins	relative activity
experiment 1				
A	natural	natural	TP50 (urea/$MgCl_2$)	0.24
B	T7	*E. coli*	TP50 (urea/$MgCl_2$)	0.033
C	T7	*B. stearo*	TP50 (urea/$MgCl_2$)	0.025
experiment 2				
A	T7	*E. coli*	TP50 (urea/$MgCl_2$)	0.044
B	T7	*E. coli*	TP70 (AcOH)	0.066
experiments 3−5 (averaged)				
A	natural	natural	TP70 (AcOH)	0.34 ± 0.11
B	T7	*E. coli*	TP70 (AcOH)	0.093 ± 0.026

[a] Reconstitution conditions included 23S rRNA at $10A_{260}$ per milliliter and 5S rRNA at $0.3A_{260}$ per milliliter in 30 mM Tris (pH 7.5), 0.33 M KCl, 0.3 M NH_4Cl, 20 mM $MgCl_2$, and 6 mM 2-mercaptoethanol. TP50 (urea/$MgCl_2$) or TP70 (AcOH) and B-L3 were included at a level of 3 molar equiv relative to the RNA input in each reaction; these protein fractions were prepared as described in the text. Natural RNA consisted of a mixture of 5S, 16S, and 23S rRNA, prepared directly from 70S ribosomes by phenol extraction. *E. coli* 5S rRNA was purchased from Boehringer Mannheim, and *B. stearothermophilus* 5S rRNA was prepared as described previously (*26*) from total rRNA.

ligation, a 2.5 kilobase (kb) *Bam*HI−*Cla*I 23S rRNA fragment isolated from plasmid pKW233 (grown in a methylation-deficient strain of *E. coli*, GM2163) was inserted with an oligonucleotide cassette (see Materials and Methods) into *Bam*HI−*Pst*I-restricted pUC118. At this stage, nucleotides extending from position 411 in the 23S rRNA gene through its 3′ end were in place, including a *Bbs*I restriction site at the 3′ end of the gene, in a vector designated pBST7-3′. In a subsequent cloning step, the 5′ end (approximately 400 nt) of the 23S rRNA gene was amplified by polymerase chain reaction (PCR) (see Materials and Methods). The resulting PCR fragment was digested with *Bam*HI−*Eco*RI and inserted into *Bam*HI−*Eco*RI-digested pBST7-3′ to generate the final plasmid, pBST7-23S. The final clone, pBST7-23S, was sequenced at both the 5′ and 3′ ends of the 23S rRNA gene to confirm that no mutations were incorporated during the PCR amplification or ligation steps. Restriction of plasmid pBST7-23S with *Bbs*I and subsequent transcription with T7 RNA polymerase result in the generation of *B. stearothermophilus* 23S rRNA with a sequence identical to that of the in vivo-processed transcript; the exact sequences of pBST7-23S at the 5′ and 3′ ends of the 23S rRNA gene are described in Figure 1. This vector also carries an F1 origin, allowing for the preparation of single-stranded DNA for site-directed mutagenesis approaches.

50S Reconstitution Reactions Using in Vitro-Transcribed 23S rRNA. Previous results indicated that in vitro transcripts of *E. coli* 23S rRNA are unable to reconstitute efficiently into catalytically active 50S ribosomal subunits (*14*). To determine whether this property was general for 50S subunit reconstitution or specific to *E. coli*, we prepared in vitro transcripts of *B. stearothermophilus* 23S rRNA using T7 RNA polymerase and either a PCR-derived DNA template or a *Bbs*I-digested version of plasmid pBST7-23S. Transcripts were incubated with 5S rRNA (from *E. coli*) and TP50 in a standard *B. stearothermophilus* 50S reconstitution reaction, and the peptidyl transferase activity of the resulting mixture was assayed. Reconstituted peptidyl transferase activity was readily detected from reaction mixtures containing the in vitro transcripts, although the signal was approximately 10-fold lower relative to those from reconstitution reaction mixtures containing natural 23S rRNA transcripts. Notably, the reconstituted peptidyl transferase activity was the same independent of the DNA template (PCR or plasmid) used for generating transcript (data not shown). *E. coli* 5S rRNA is known to effectively substitute for *B. stearothermophilus* 5S rRNA in *B. stearothermophilus* 50S subunits (*27*, *28*); we confirmed these results initially using *B. stearothermophilus* 5S rRNA purified on 10 to 30% sucrose gradients from the total rRNA mixture (Table 1, experiment 1) (*26*). Omission of 5S rRNA from the reconstitution reaction mixtures resulted in a 1.5−2-fold decrease in peptidyl transferase activity (data not shown).

We subsequently varied the reconstitution conditions in search of parameters that would increase the level of peptidyl transferase activity associated with the particles containing both natural and in vitro transcripts of *B. stearothermophilus* 23S rRNA. While not exhaustive, the search included alteration of incubation temperature and length of incubation (including two-step procedures similar to those used for *E. coli*), high-temperature preincubation of the rRNAs (prior to addition of proteins), variation of mono- and divalent cations and their concentrations, addition of methanol, and supplemental 5S rRNA or B-L3 protein fraction. Several alterations resulted in an increase in reconstitution efficiency of about 3-fold for the natural rRNA and about 9-fold for the in vitro transcript. The optimized conditions include the addition of 3 equiv of TP70 (rather than 1.5 equiv), extra B-L3 protein (3 equiv), and the addition of 0.3 M NH_4Cl to the standard 0.33 M KCl (Figure 2). Complete substitution of the monovalent ion with either NH_4Cl or KCl did not improve the efficiency of the reconstitution reaction. Multiple cation requirements have also been documented for the in vitro reconstitution of 50S subunits in the archaebacterium *Haloferax mediterranei* (*7*). Thus, the reconstituted particles derived from natural 23S rRNA are 30% as active, and the particles derived from in vitro transcripts of *B. stearothermophilus* 23S rRNA are 10% as active, as natural *B. stearothermophilus* 50S subunits (Table 1). The final activity associated with a reconstituted *B. stearothermophilus* 50S subunit containing an in vitro transcript of 23S rRNA (ca. 10%) is enhanced 10000-fold compared to that observed with similar *E. coli* in vitro reconstitutions (*14*).

The structural compactness of the *B. stearothermophilus* 50S subunits reconstituted from natural and in vitro-transcribed 23S rRNA was compared by sucrose gradient centrifugation analysis. As seen in Figure 3, both natural and in vitro-transcribed 23S rRNA are incorporated into 50S-

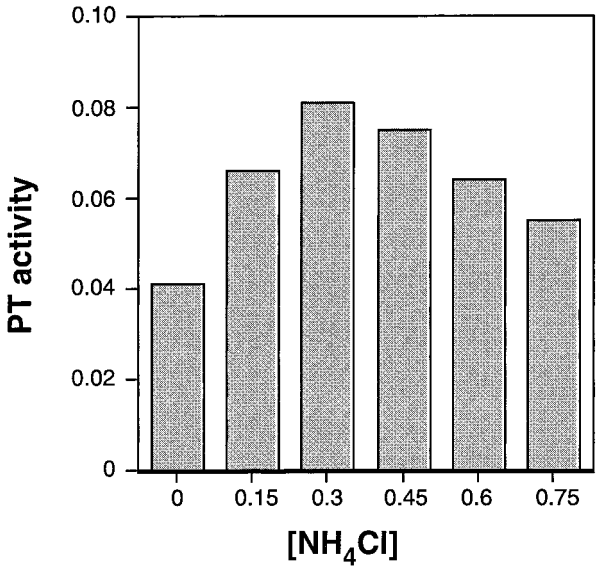

FIGURE 2: Dependence of the peptidyl transferase activity (expressed as a fraction of the activity of untreated natural *B. stearothermophilus* 50S subunits) of *B. stearothermophilus* reconstitution reaction mixtures on the concentration (in molarity) of NH_4Cl added as a supplement to the standard reconstitution reaction mixture at 0.33 M KCl.

like peaks on sucrose gradients with comparable efficiencies. Further, the 50S-like particles derived from natural and in vitro-transcribed 23S rRNA are observed with magnesium concentrations varying from 5 to 20 mM (data not shown), unlike the 50S-like particles observed with in vitro-transcribed *E. coli* 23S rRNA which dissociate in the presence of lowered Mg^{2+} concentrations (*14*). These data are consistent with the significant levels of peptidyl transferase activity associated with these in vitro reconstitution reactions, although there is no direct structural correlation to the 3-fold higher activity associated with natural RNA.

Effect of Mutations at G2252, U2506, U2584, and A2602 (E. coli Numbering) of 23S rRNA on Peptidyl Transferase Activity. To test the utility of such an in vitro reconstitution system, site-directed mutations were incorporated into *B. stearothermophilus* 23S rRNA at positions G2252 (U), U2506 (A, C, and G), U2584 (A and C), and A2602 (C), nucleotides previously implicated as interacting, directly or indirectly, with the 3′ terminus of P-site-bound tRNA (*16*, *29*, *30*) (the *B. stearothermophilus* numbering for these nucleotides is G2280, U2534, U2612, and A2630, respectively). In the *E. coli* chimeric reconstitution system, G2252 was shown using in vitro genetics to interact directly with C74 of P-site-bound tRNA in a Watson−Crick pairing interaction (*16*). In 70S ribosomes, nucleotides U2506, U2584, and U2585 are protected from chemical modification by carbodiimide in the presence of P-site-bound tRNA; protection is lost, however, when the terminal adenosine of tRNA is selectively removed (*29*). In addition, these nucleotides are efficiently cross-linked by benzophenone-derivatized A- and P-site-bound tRNA (*30*). These data suggest the possibility of direct pairing interactions between these positions and the terminal adenosine of the P-site tRNA. Previously, using the *E. coli* chimeric reconstitution, we characterized the effects of mutations at position U2585, where 3−10-fold decreases in peptidyl transferase activity were observed; no suppression of these defects was observed

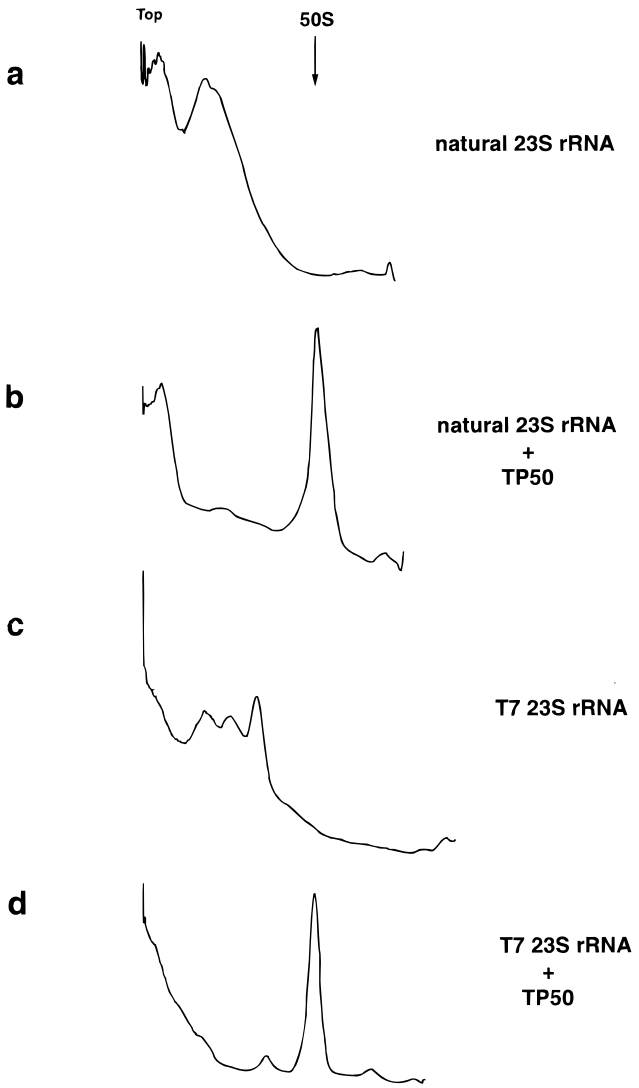

FIGURE 3: Sucrose gradient analysis of in vitro reconstitution reactions using natural (a and b) and in vitro-transcribed (T7) (c and d) 23S rRNA. Panels a and c contain data for only 23S rRNA (natural or T7), and panels b and d represent data for complete reconstitutions including 23S rRNA (natural or T7), 5S rRNA, and TP50. The magnesium concentration included in these gradients was 5 mM; an arrow indicates the mobility of a 50S subunit, and Top indicates the top of the gradient.

in the presence of A76 mutant tRNA oligonucleotide substrates (*15*). Finally, nucleotide A2602 displays an interesting modification pattern in the presence of tRNA substrates. In the presence of P-site-bound tRNA, the chemical reactivity of A2602 is enhanced, whereas in the presence of A-site-bound tRNA, its reactivity is decreased (i.e., it becomes protected); both the enhancement and protection are dependent on the presence of the acyl moiety of the bound tRNA (*29*). Intriguingly, in the presence of hybrid (A/P)-bound aminoacyl tRNA (non-puromycin-reactive), A2602 is protected from chemical modification, consistent with this nucleotide behaving as an indicator of the "reactive" state of the P-site-bound tRNA (*31*). It has been speculated that A2602 is intimately involved in the catalysis of peptide bond formation or in the mechanism of tRNA movement.

The mutant 23S rRNA transcripts described above were reconstituted with 5S rRNA and TP70, and peptidyl transferase activity was assayed (Figure 4). G2252U mutant

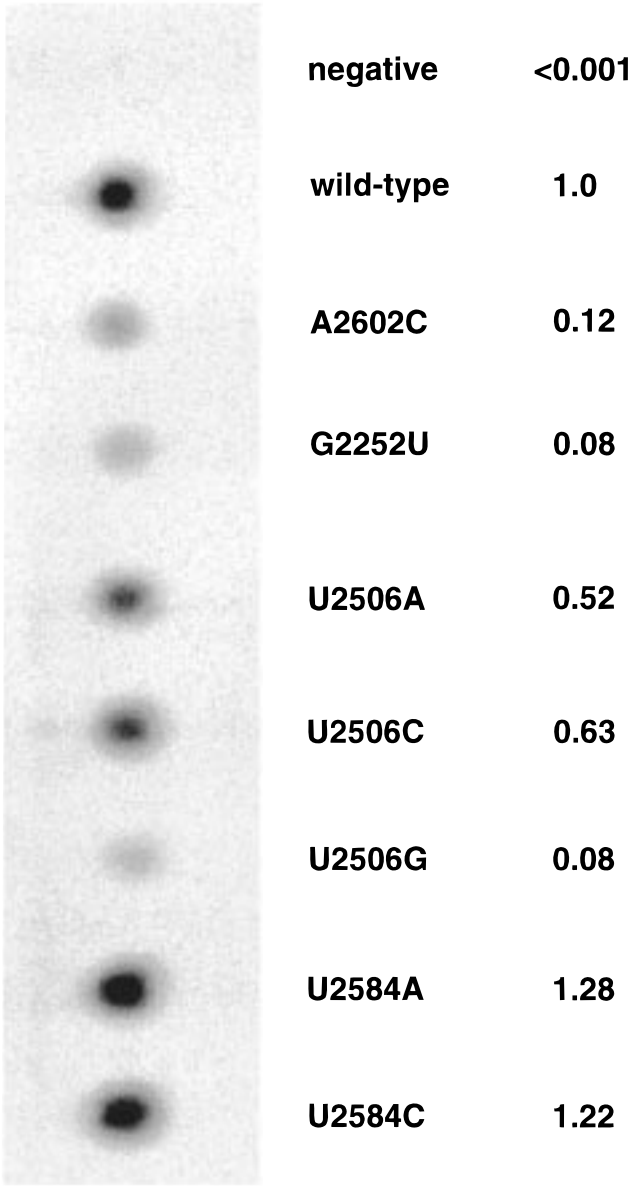

FIGURE 4: Peptidyl transferase assay. Phosphorimager exposure of paper electrophoresis analysis of the peptidyl transferase reaction catalyzed by the 50S subunits reconstituted from wild-type and mutant in vitro transcripts of *B. stearothermophilus* 23S rRNA. Spots represent the product *N*-Ac-[^{35}S]Met-puromycin; the fractional activity relative to subunits reconstituted from wild-type transcripts of *B. stearothermophilus* 23S rRNA is indicated.

ribosomes had 8% of the activity of wild-type ribosomes; this level of activity is consistent with the 7% activity previously reported for this mutant in the chimeric *E. coli* reconstitution system (*16*) and confirms the authenticity of this in vitro reconstitution system. Of the three mutations at U2506, U2506G had the most severe peptidyl transferase defect (8% of the wild-type activity) while U2506A and U2506C had activities comparable to that of the wild type (52 and 63%, respectively). Neither of the mutations at U2584 (U2584A and U2584C) had any detectable effect on peptidyl transferase activity (128 and 122%, respectively). Peptidyl transferase assays were performed with the U2506 and U2584 mutant reconstituted ribosomes and A76 mutant tRNA oligonucleotide fragments [CACCX-*N*-Ac-[^{35}S]Met where X is A, C, G, and U (*15*)]; no evidence for altered substrate specificity using these substrates was observed (data not shown). Recent site-directed mutagenesis of these same positions and an analysis of peptidyl transferase function using in vivo-generated *E. coli* ribosomes yielded similar results (*32*). Finally, mutation of A2602 to C resulted in a 10-fold decrease in peptidyl transferase activity, consistent with this nucleotide playing an important, though not essential, role in the catalysis of peptide bond formation. These data indicate the potential of this reconstitution system, dependent on in vitro-transcribed 23S rRNA, to rapidly screen mutant species for peptidyl transferase phenotypes worthy of further characterization.

DISCUSSION

The inability to reconstitute 50S ribosomal subunits using in vitro-transcribed 23S rRNA has been a barrier to the use of in vitro genetic approaches for the study of ribosomes. In most organisms, ribosomes carrying mutagenized rRNAs can only be produced in a background of wild-type ribosomes because of the multiple rRNA genes located in the chromosome. And, while the incorporation of antibiotic resistance mutations in plasmid-borne rRNA genes has allowed for some analysis of the effects of expression of mutant ribosome populations in vivo (*33*), the isolated study of specific translational processes is not possible. Analysis of ribosomes using the rRNA operon-based transformation system in *H. halobium*, where there is a single-copy rRNA gene, is limited by the requirement that only mutations with nonlethal, or conditionally lethal, phenotypes can be maintained (*34*).

Several clever approaches that take advantage of the differential sensitivities of mutant 23S rRNA populations to peptidyl transferase and translocation specific antibiotics have been developed for studying mixed populations of ribosomes in vitro (*35*, *36*). Yet, while these systems offer important advantages (in particular, the high yield and quality of in vivo-generated ribosomes), they are generally limited by the fact that they only reduce the background signal from the wild-type ribosome population by 20-fold (ca. 5%). In situations where a mutant 23S rRNA species of interest has diminished, but significant, activity, it may be impossible to detect such a signal over the 5% background level of the wild type.

Previous work demonstrated that in vitro transcripts of *E. coli* 23S rRNA are compromised severely (5 orders of magnitude) in their ability to be reconstituted in vitro into catalytically competent 50S ribosomal subunits (*14*). The cause of this deficiency was localized to an 80 nt region of the *E. coli* 23S rRNA, containing six post-transcriptional modifications, one or more of which is likely to be involved in in vitro assembly or subunit function. However, the low degree of phylogenetic conservation of these six post-transcriptional modifications (*17*) and the elaborate reconstitution procedure required for the *E. coli* 50S subunit (relative to, for example, that of *B. stearothermophilus*) suggested that the difficulties might be peculiar to the *E. coli* system (*3*, *5*).

We therefore extended the reconstitution approach to 50S subunits from *B. stearothermophilus*, a thermophilic eubacterial species that is significantly distant from *E. coli* phylogenetically, and whose ribosomes and their reconstitution have been extensively characterized (*3*, *4*). In initial studies, urea/LiCl precipitation was used to remove a

majority of bound proteins from the ribosomal RNAs (*3*), and the remaining tightly bound proteins [in particular, *B. stearothermophilus* L3 (B-L3), the homologue of *E. coli* L2] were removed with a subsequent urea/magnesium treatment at pH 2.0 (*4*). RNA and proteins recovered from these treatments were dialyzed against appropriate buffers (containing potassium as the monovalent cation) and used directly in a single-step (2 h at 60 °C) reconstitution. The efficiency of the reconstitution is high; using poly(Phe) synthesis, activities can approach 100% of that of untreated 50S subunits (*37*), and using the "fragment" peptidyl transferase reaction, activities vary between 10 and 20% of that of untreated 50S subunits (*4*, *21*).

Following the success of the *B. stearothermophilus* reconstitution, Nierhaus and Dohme (*5*) successfully reconstituted the *E. coli* 50S subunit from the individual protein and RNA components using a substantially different protocol. R-Proteins were removed from the rRNAs using an acetic acid extraction and were then dialyzed directly against appropriate buffer (containing ammonium as the monovalent cation). The rRNAs were isolated by phenol extraction of the associated proteins (initially in the presence of SSC-EDTA and 10% SDS), ethanol precipitation, and resuspension in buffer. These components were then combined and subjected to a two-step reconstitution procedure; step 1 proceeded at 40 °C for 20 min in 4 mM magnesium, and step 2 proceeded at 50 °C for 90 min in 20 mM magnesium. Single-step reconstitution protocols in the *E. coli* system were extremely inefficient (ca. 3%) when compared with the two-step protocols where up to 50% activity was reported. In the initial study, these authors compared the quality of urea/LiCl- and phenol-extracted rRNAs, and urea/LiCl- and acetic acid-prepared r-proteins, and concluded that the purity of the phenol- and acetic acid-treated components, respectively, exceeded that of the urea/LiCl-treated components, and were equally effective in reconstitution assays (*5*, *21*).

Total reconstitution of the 50S subunit has also been reported for two archael species, *H. mediterranei* and *Sulfolobus solfataricus* (*6*, *7*). While the ionic requirements of the halophile and thermoacidophile are quite distinct from those of the mesophilic and moderately thermophilic eubacteria, in both cases the RNA and protein components were prepared essentially according to the *E. coli* procedures of Nierhaus and Dohme (rRNAs by phenol extraction and TP70 by acetic acid extraction) (*5*). In this study, *B. stearothermophilus* rRNA and r-protein components were prepared both according to the LiCl/urea extraction procedures of Nomura and according to the phenol and acetic acid extraction procedures of Nierhaus. Due to clearer partitioning of RNA and protein components using phenol for RNA isolation and acetic acid for protein extraction, the purity of the components is higher using these procedures, and the components were equally effective in the in vitro reconstitution reactions (Table 1, experiment 2). Due to the extreme insolubility of the rRNAs following the urea/LiCl/magnesium (pH 2.0) treatment, we found the phenol extraction procedure to be more satisfactory for rRNA preparation.

In vitro-transcribed *B. stearothermophilus* 23S rRNA reconstitutes into catalytically active 50S subunits with an efficiency only 3−4-fold lower than that of natural *B. stearothermophilus* 23S rRNA (10 vs 30% of the activity of untreated 50S subunits in the fragment peptidyl transferase reaction). This level of activity can be contrasted with the extremely low levels of activity associated with *E. coli* in vitro-transcribed 23S rRNA (<0.001%) in an analogous in vitro reconstitution (*14*). Thus, the one or more post-transcriptional modifications in *E. coli* 23S rRNA identified as critical to the assembly or function of those 50S subunits do not appear to be essential in the *B. stearothermophilus* system. Notably, while there are clearly post-transcriptional modifications present in the *B. stearothermophilus* 23S rRNA, their location and identity have not been thoroughly characterized (P. F. Crain and J. A. McCloskey, personal communication). And, while it is possible that post-transcriptional modifications might occur during the actual reconstitution reaction with the total protein mixture, in the case of *E. coli*, we have previously shown that no significant level of modification takes place during this incubation (R. Green, P. F. Crain, and J. A. McCloskey, unpublished observations). With few exceptions (including the example of pseudouridine), the known post-transcriptional modifications are generated in biosynthetic reactions requiring the energy of ATP or *S*-adenosylmethionine, reagents not supplied in the in vitro reconstitution reactions. These data are consistent with general observations made in other systems indicating that the post-transcriptional modifications, which are important for fine-tuning the specificity or rates of biological functions, are not essential for RNA function (*38*, *39*). For example, in vitro transcripts of *E. coli* 16S rRNA lacking all 11 post-transcriptional modifications can be reconstituted into 30S subunits with structure and function nearly indistinguishable from those of an untreated particle (*40*). Similarly, in vitro transcripts of various tRNAs are efficiently aminoacylated by their cognate synthetases, are bound in a ternary complex by EF-Tu, and are recognized as substrates by the ribosome (*41*). Our previous results with *E. coli* 23S rRNA transcripts are likely an exception to a general rule which can be explained by the unusually specific requirements of the *E. coli* in vitro reconstitution reaction (*18*). It should be noted that there have been reports of low levels of peptidyl transferase activity (assayed under non-fragment conditions with two intact tRNAs for the aminoacyl and peptidyl substrates) associated with natural and in vitro transcripts of *E. coli* 23S rRNA incubated in the absence of ribosomal proteins and 5S rRNA (*42*, *43*). Using our reaction conditions and substrates, we have not observed any detectable peptidyl transferase activity associated with protein-free rRNAs (data not shown).

Independent of the biological significance of post-transcriptional modifications in functional RNAs, the reconstitution system dependent on in vitro transcripts of 23S rRNA described here yields functionally active 50S subunits, and has tremendous potential for addressing questions regarding the role of 23S rRNA in its function. Similar success was recently obtained in the reconstitution of *Thermus aquaticus* 50S subunits from in vitro-transcribed 23S rRNA (*44*). Interestingly, in the *B. stearothermophilus* system, there was only a 1.5−2-fold stimulation of reconstituted peptidyl transferase activity by 5S rRNA whereas absolute dependence was observed in the *T. aquaticus* system. The modest effect of 5S rRNA observed in the *B. stearothermophilus* reconstitution system is consistent with our similar analysis in *E. coli* (R. Green and H. F. Noller, unpublished data).

The utility of such an in vitro reconstitution approach was demonstrated with the construction of several 23S rRNA site-directed mutants at positions potentially involved in donor tRNA substrate binding or catalytic function (*29*) (nucleotides G2252, U2506, U2584, and A2602 according to *E. coli* numbering). Mutation at G2252 (U) resulted in substantial decreases in peptidyl transferase activity, consistent with earlier studies (*16*). Neither mutation at U2584 (A or C) had a substantial effect on peptidyl transferase activity, whereas the G mutation, but not the A or C, at position U2506 had a substantial (20-fold) effect. No detectable changes in specificity for A76 mutant tRNA substrates were observed for the mutations at U2506 and U2584; these data are consistent with earlier results of Porse et al. (*32*). Last, mutation at A2602 (C) resulted in a substantial decrease in peptidyl transferase activity (ca. 10-fold), but not one consistent with a fundamental role for this nucleotide in the chemistry of peptide bond formation. Apparently, these and other (*15*, *32*, *45*) highly conserved and highly accessible nucleotides in domain V of 23S rRNA are not involved in simple suppressible Watson−Crick interactions with the CCA end of P-site tRNA, nor do they appear to be essential to catalysis of peptidyl transfer. More sophisticated genetic approaches may be required to identify such nucleotides in the peptidyl transferase center of the 50S subunit.

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REFERENCES

1. Nierhaus, K. H., Bordasch, K., and Homann, H. E. (1973) *J. Mol. Biol. 74*, 587−97.
2. Srivastava, A. K., and Schlessinger, D. (1990) *Annu. Rev. Microbiol. 44*, 105−29.
3. Nomura, M., and Erdmann, V. A. (1970) *Nature 228*, 744−8.
4. Fahnestock, S., Erdmann, V., and Nomura, M. (1973) *Biochemistry 12*, 220−4.
5. Nierhaus, K. H., and Dohme, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4713−7.
6. Londei, P., Teixido, J., Acca, M., Cammarano, P., and Amils, R. (1986) *Nucleic Acids Res. 14*, 2269−85.
7. Sanchez, M. E., Urena, D., Amils, R., and Londei, P. (1990) *Biochemistry 29*, 9256−61.
8. Montesano-Roditis, L., Glitz, D. G., Perrault, A. R., and Cooperman, B. S. (1997) *J. Biol. Chem. 272*, 8695−703.
9. Cooperman, B. S., Wooten, T., Romero, D. P., and Traut, R. R. (1995) *Biochem. Cell Biol. 73*, 1087−94.
10. Nowotny, P., Ruhl, M., Nowotny, V., May, R. P., Burkhardt, N., Voss, H., and Nierhaus, K. H. (1994) *Biophys. Chem. 53*, 115−22.
11. Schulze, H., and Nierhaus, K. H. (1982) *EMBO J. 1*, 609−13.
12. Auron, P. E., Erdelsky, K. J., and Fahnestock, S. R. (1978) *J. Biol. Chem. 253*, 6893−900.
13. Dohme, F., and Fahnestock, S. R. (1979) *J. Mol. Biol. 129*, 63−81.
14. Green, R., and Noller, H. F. (1996) *RNA 2*, 1011−21.
15. Green, R., Samaha, R. R., and Noller, H. F. (1997) *J. Mol. Biol. 266*, 40−50.
16. Samaha, R. R., Green, R., and Noller, H. F. (1995) *Nature 377*, 309−14.
17. Sirum-Connolly, K., Peltier, J. M., Crain, P. F., McCloskey, J. A., and Mason, T. L. (1995) *Biochimie 77*, 30−9.
18. Amils, R., Matthews, E. A., and Cantor, C. R. (1978) *Nucleic Acids Res. 5*, 2455−70.
19. Fahnestock, S., Erdmann, V., and Nomura, M. (1974) *Methods Enzymol. 30*, 554−62.
20. Kop, J., Wheaton, V., Gupta, R., Woese, C. R., and Noller, H. F. (1984) *DNA 3*, 347−57.
21. Cohlberg, J. A., and Nomura, M. (1976) *J. Biol. Chem. 251*, 209−21.
22. Dohme, F., and Nierhaus, K. H. (1976) *J. Mol. Biol. 107*, 585−99.
23. Milligan, J. F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783−98.
24. Monro, R. E., and Marcker, K. A. (1967) *J. Mol. Biol. 25*, 347−50.
25. Nowotny, V., Rheinberger, H. J., Nierhaus, K., Tesche, B., and Amils, R. (1980) *Nucleic Acids Res. 8*, 989−98.
26. Nierhaus, K. (1990) in *Ribosomes and Protein Synthesis: A Practical Approach* (Spedding, G., Ed.) pp 161−89, IRL Press, Oxford, U.K.
27. Wrede, P., and Erdmann, V. A. (1973) *FEBS Lett. 33*, 315−9.
28. Hartmann, R. K., Vogel, D. W., Walker, R. T., and Erdmann, V. A. (1988) *Nucleic Acids Res. 16*, 3511−24.
29. Moazed, D., and Noller, H. F. (1989) *Cell 57*, 585−97.
30. Steiner, G., Kuechler, E., and Barta, A. (1988) *EMBO J. 7*, 3949−55.
31. Moazed, D., and Noller, H. F. (1989) *Nature 342*, 142−8.
32. Porse, B. T., Thi-Ngoc, H. P., and Garrett, R. A. (1996) *J. Mol. Biol. 264*, 472−83.
33. Triman, K., Becker, E., Dammel, C., Katz, J., Mori, H., Douthwaite, S., Yapijakis, C., Yoast, S., and Noller, H. F. (1989) *J. Mol. Biol. 209*, 645−53.
34. Mankin, A. S., Zyrianova, I. M., Kagramanova, V. K., and Garrett, R. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6535−9.
35. Nierhaus, K. H., Beyer, D., Dabrowski, M., Schafer, M. A., Spahn, C. M., Wadzack, J., Bittner, J. U., Burkhardt, N., Diedrich, G., Junemann, R., et al. (1995) *Biochem. Cell Biol. 73*, 1011−21.
36. Porse, B. T., and Garrett, R. A. (1995) *J. Mol. Biol. 249*, 1−10.
37. Fahnestock, S. R. (1977) *Arch. Biochem. Biophys. 182*, 497−505.
38. Persson, B. C., Gustafsson, C., Berg, D. E., and Bjork, G. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3995−8.
39. Conrad, J., Sun, D., Englund, N., and Ofengand, J. (1998) *J. Biol. Chem. 273*, 18562−6.
40. Krzyzosiak, W., Denman, R., Nurse, K., Hellmann, W., Boublik, M., Gehrke, C. W., Agris, P. F., and Ofengand, J. (1987) *Biochemistry 26*, 2353−64.
41. Sampson, J. R., and Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033−7.
42. Nitta, I., Kamada, Y., Noda, H., Ueda, T., and Watanabe, K. (1998) *Science 281*, 666−9.
43. Nitta, I., Ueda, T., and Watanabe, K. (1998) *RNA 4*, 257−67.
44. Khaitovich, P., Tenson, T., Kloss, P., and Mankin, A. S. (1999) *Biochemistry 38*, 1780−1788.
45. Saarma, U., Spahn, C. M., Nierhaus, K. H., and Remme, J. (1998) *RNA 4*, 189−94.

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