Comprehensive genetic selection revealed essential bases in the peptidyl-transferase center

Neuza Satomi Sato*†, Naomi Hirabayashi*, Ilana Agmon‡, Ada Yonath‡§, and Tsutomu Suzuki*§

*Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan;and ‡Department of Structural Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

Comprehensive genetic selection revealed essential bases in the peptidyl-transferase center

Neuza Satomi Sato*†, Naomi Hirabayashi*, Ilana Agmon‡, Ada Yonath‡§, and Tsutomu Suzuki*§

*Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan; and ‡Department of Structural Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

Contributed by Ada Yonath, July 18, 2006

During protein synthesis, the ribosome catalyzes peptide-bond formation. Biochemical and structural studies revealed that conserved nucleotides in the peptidyl-transferase center (PTC) and its proximity may play a key role in peptide-bond formation; the exact mechanism involved remains unclear. To more precisely define the functional importance of the highly conserved residues, we used a systematic genetic method, which we named SSER (systematic selection of functional sequences by enforced replacement), that allowed us to identify essential nucleotides for ribosomal function from randomized rRNA libraries in *Escherichia coli* cells. These libraries were constructed by complete randomization of the critical regions in and around the PTC. The selected variants contained natural rRNA sequences from other organisms and organelles as well as unnatural functional sequences; hence providing insights into the functional roles played by these essential bases and suggesting how the universal catalytic mechanism of peptide-bond formation could evolve in all living organisms. Our results highlight essential bases and interactions, which are shaping the PTC architecture and guiding the motions of the tRNA terminus from the A to the P site, found to be crucial not only for the formation of the peptide bond but also for nascent chain elongation.

nucleotide essentiality | protein biosynthesis | symmetrical region

Ribosomes are universally conserved ribonucleoproteins that translate the genetic information contained in mRNAs into proteins. The large (50S) ribosomal subunit catalyzes peptide-bond formation at the peptidyl-transferase center (PTC) between aminoacyl-tRNA (aa-tRNA) bound to the A site and peptidyl-tRNA (pep-tRNA) at the P site. In the crystal structures of 50S subunits of *Haloarcula marismortui*, H50S (1, 2), *Deinococcus radiodurans*, D50S (3, 4), and 70S ribosomes (5, 6), the PTC is composed solely of 23S rRNA and, hence, acts as a ribozyme, consistent with biochemical findings using deproteinized 50S subunit (7–10) for the formation of a single peptide bond.

The PTC provides the frame for peptide-bond formation (11, 12) and plays a critical role in tRNA and nascent chain release (13–17), and the global ribosomal architecture is crucial for substrate positioning (18, 19). Consistently, the hypothesis, based on structures of H50S complexed with minimum substrates, that the PTC acts as a general acid–base catalyst (2, 20–22), was contradicted by various mutagenesis and biochemical studies (12, 23–28). The main catalytic contribution of the ribosomes, substrates positioning at proper orientation (4, 28, 29), is achieved by remote interactions, accompanied by symmetrical base-pairing of C75 of both tRNAs with G2553 (*Escherichia coli* numbering throughout) and G2251 (Fig. 4*A*, which is published as supporting information on the PNAS web site), respectively (2, 4, 31). The distinction between the rates of peptide-bond formation by full-length tRNAs and minimal substrates is also consistent with the essential role played by the A76 2′ OH of P-tRNA in the reaction formation rate (32).

The PTC is situated within a universal sizable symmetrical region spanning from G2502 to C2610 and from A2058 to C2501 that is divided into two subregions, with the A and P loops residing on the opposite sides in the inner surface of the PTC cavity (18, 29). In each cycle of elongation, the A site tRNA 3′ terminus travels from the A to the P loop by 180° rotatory motion, in concert with the overall mRNA/tRNA sideways shift (4, 29). Protruding bases on the inner surface of the symmetry region that interact with the rotating tRNA 3′ terminus are highly conserved, implying that these interactions are essential for guarding and directing the rotatory motion, thus providing the positional catalysis of peptide-bond formation.

To analyze the functional importance of particular sequences in rRNA genes, we developed a genetic method that we named "systematic selection of functional sequences by enforced replacement" (SSER), which involves the systematic selection from the plasmid library in which a critical region of *E. coli* rRNA is randomized. The results of this study revealed nucleotides important for peptide-bond formation and for protein elongation *in vivo*, thus progressing toward the elucidation of the catalytic mechanism involved in this process.

Results

Comprehensive Genetic Selection of Functional Nucleotides in the PTC. The use of the SSER genetic selection system (detailed description in *Supporting Methods*, which is published as supporting information on the PNAS web site) allowed us to rapidly identify functional sequences in the cell among randomized sequences. Plasmid libraries were constructed by complete randomization of six critical regions in the PTC and its vicinity known to be essential for peptide-bond formation (Fig. 4*B*), namely, the A loop (helix 92), the P loop (helix 80), the conserved A2602-bulge region (helix 93), the 2451 region (residues 2448–2454), the 2585 region (residues 2583–2587), and the 2608 region (residues 2607–2610) in the domain V central loop. Next, we carried out large-scale transformation of *E. coli* NT101 with each member of the library and selected for kanamycin-resistant transformants. If the incorporated plasmid has a toxic sequence that results in a dominant lethal phenotype, a transformant will not be obtained. Because the PTC is critical for ribosomal function, most of the sequences in the library must have been excluded during this step. Transformants on the kanamycin plate produced colonies of different sizes (Fig 5, which is published as supporting information on the PNAS web site), indicating that each cell contains a sequence resulting in altered ribosomal activity. The kanamycin-resistant cells contain both pRB101 and pRB102 from the library. To drive plasmid replacement, each cell was picked and spotted onto selection plates containing kanamycin and sucrose. If the incorporated pRB102 plasmid has a functional se-

Author contributions: T.S. designed research; N.S.S. and N.H. performed research; N.S.S. and T.S. contributed new reagents/analytic tools; N.S.S., I.A., A.Y., and T.S. analyzed data; and A.Y. and T.S. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Abbreviations: aa-tRNA, aminoacyl-tRNA; PTC, peptidyl-transferase center; SSER, systematic selection of functional sequences by enforced replacement; WC, Watson–Crick.

†Present address, Institute Adolfo Lutz, Avenida Dr. Arnaldo 355, 10th Floor, 01246-902 Sao Paulo, SP, Brazil.

§To whom correspondence may be addressed. E-mail: ts@chembio.t.u-tokyo.ac.jp or ada.yonath@weizmann.ac.il

quence for ribosomal activity, it rapidly eliminates the pRB101 rescue plasmid, thus yielding sucrose-resistant cells (NT102 derivatives). Besides, if the incorporated plasmid has a nonfunctional or very weak functional sequence for ribosomal activity, the rescue plasmid cannot be replaced by the introduced plasmid, and the transformant becomes sensitive to sucrose because of the *sacB* gene. In each SSER selection, hundreds of colonies on the sucrose–kanamycin plates were picked and sequenced.

Nucleotides Essential for tRNA 3′ End Positioning. Five nucleotides (2552–2556) in the A loop were completely randomized to give 1,024 sequence variations (Fig. 4*B*). Despite the highly conserved sequence of the A loop, nine functional sequences, including the wild-type sequence, were selected from the library (Table 1 and Fig. 1*A*). Although sequence variations were found in four of the five residues of this site, nucleotide G2553 of helix 92 (Fig. 4*A*), which basepairs with C75 of aa-tRNA CCA-end (Fig. 2*A*) (2, 31) was completely conserved in all selected variants, confirming its essentiality for ribosomal function. In addition to UGUUC, which is the conserved sequence found in most organisms, UGCCC, the naturally occurring A loop sequence of archaea, was also selected from the random pool. The functional consensus sequence for the A loop was assigned as DGYYY (2552–2556), indicating that three bases at positions 2554–2556 must be U or C, whereas base 2552 cannot be C [indeed, it exists in only one mitochondrial rRNA (www.rna.icmb.utexas.edu)]. Importantly, most variants had U2552, and variants bearing A2552 (clone 5) or G2552 (clones 6–9) show relatively reduced growth rates (Table 1). The P loop in helix 80 serves as the attachment site for the 3′ terminus of pep-tRNA by base-pairing between its C75/C74 and the universally conserved G2251/G2252 residues (Figs. 2*B* and 4*A*) (2, 4, 29, 30, 33). From the P loop library, wherein the five nucleotides at positions 2250–2254 were completely randomized (Fig. 4*B*), 12 variants were selected as functional sequences (Table 1 and Fig. 1*A*). The two variants GGGGC (clone 1) and GGGGU (clone 4) are naturally occurring sequences found in most organisms and mitochondrial large subunits, respectively. The other 10 variants are unnatural functional sequences. G2251 and G2252 were invariant, supporting the notion that they position the pep-tRNA at the P site. The invariant nucleotides G2250 and D2253 residue (except for the latter's ability to mutate to C) that were also selected may be involved in establishing the P loop proper conformation. Note that all U2253 variants (clones 5–8) show an apparent growth defect compared with the other variants (Table 1). The nucleotide at 2254 was found to be replaceable with the other three bases.

The A2602 Bulge in Helix 93. One of the central features in the architecture of the symmetrical region, as indicated by the binding modes of substrates to D50S, is the extremely flexible nucleotide A2602 (4, 18, 29, 30). Consistently, analysis of crystal structures of H50S complexes (2, 34) suggested that A2602 undergoes a conformational change when the tetrahedral intermediate is established.

A2602, with two adjacent base pairs (2591, 2592, and 2601–2603), was randomized to construct the library (Fig. 4*B*). Eleven sequence variations, all of which bore the conserved A2602 bulge, were selected as functional sequences (Table 1 and Fig. 1*A*), suggesting that the bulging of A2602 plays a critical role in ribosomal function, consistent with biochemical and crystallographic observations (2–4, 14, 29, 30, 34) and supported by our failure to obtain any functional strains after A2602 deletions (data not shown). Among the adjacent base pairs, the functional C2591-G2603 (clone 1) can be replaced with G–C (clones 5–9) or A–U (clones 10 and 11). This result suggests that both Watson–Crick (WC)-type pairs between 2591 and 2603 are allowed and may be influential in establishing one of the functional configurations of the A2602 bulge. For the 2592–2601 pair, four variants (clones 2, 3, 7, and 11) had lost the WC base pair, and one variant (clone 6) had a G–U wobble pair, which showed a slow-growth phenotype (Table 1). In particular, clone 6 suffered a severe growth defect. The result suggests that the 2592–2601 pair is not essential but seems to be required for efficient ribosomal function.

According to phylogenetic search (www.rna.icmb.utexas.edu), G2602 is found in only one archaeal rRNA. All others have A2602.

Table 1. Selected sequences in 23S rRNA as determined by SSER using the randomized rRNA libraries

Selected seq	Clone	Sequence	Doubling time, min	Percent	Note
A loop 2552–2556	1 (wt)	U**G**UUC	47.5 ± 1.4	100	Conserved seq
	2	U**G**UCC	48.2 ± 2.0	98	
	3	U**G**CUC	56.8 ± 1.9	84	
	4	U**G**CCC	48.3 ± 2.2	97	Archae
	5	A**G**UUC	61.5 ± 5.7	77	
	6	**GG**UUC	60.7 ± 2.0	78	
	7	**GG**UCC	48.2 ± 2.0	98	
	8	**GG**UCU	55.0 ± 1.0	86	
	9	**GG**UUU	60.7 ± 3.3	78	
	Consensus	DGYYY			
P loop 2250–2254	1 (wt)	**GGG**GC	47.4 ± 0.9	100	Conserved seq
	2	**GGG**GG	54.2 ± 0.3	88	
	3	**GGG**GA	45.7 ± 0.5	104	
	4	**GGG**GU	47.7 ± 2.6	99	Mitochondria
	5	**GGG**UC	68.5 ± 4.7	69	
	6	**GGG**UG	60.4 ± 2.1	78	
	7	**GGG**UA	55.6 ± 0.3	85	
	8	**GGG**UU	55.3 ± 1.2	86	
	9	**GGG**AC	50.1 ± 0.5	94	
	10	**GGG**AG	52.1 ± 1.0	91	
	11	**GGG**AA	51.8 ± 0.3	92	
	12	**GGG**AU	48.7 ± 0.3	97	
	Consensus	**GGG**DN			
The 2602 region 2591, 2592, 2601–2603	1 (wt)	CG-C**A**G	47.2 ± 1.3	100	Conserved seq
	2	CC-C**A**G	48.0 ± 1.4	98	
	3	CC-U**A**G	51.6 ± 1.8	87	
	4	CA-U**A**G	49.2 ± 0.4	96	Mitochondria
	5	GG-C**A**C	49.2 ± 1.2	93	
	6	GG-U**A**C	119.9 ± 3.7	17	
	7	GC-U**A**C	99.2 ± 1.3	40	
	8	GC-G**A**C	85.0 ± 1.8	57	
	9	GA-U**A**C	60.1 ± 0.7	77	
	10	AG-C**A**U	64.8 ± 0.9	64	
	11	AC-U**A**U	92.8 ± 0.8	49	
	Consensus	VV-B**A**B			
The 2585 region 2583–2587	1 (wt)	GU**U**UA	47.1 ± 0.9	100	Conserved seq
	2	GU**U**UG	48.5 ± 0.5	97	
	3	GU**U**UU	61.2 ± 1.6	77	
	4	GU**U**UC	55.7 ± 0.5	85	
	5	GU**U**GA	55.2 ± 2.3	85	
	6	GU**U**CA	47.5 ± 0.4	99	*B. subtilis*
	7	GU**U**AA	48.4 ± 0.3	97	
	8	GC**U**UA	57.3 ± 0.9	82	
	9	AU**U**UA	52.3 ± 0.5	89	
	10	AU**U**CA	82.5 ± 0.3	57	
	11	GU**U**AG	99.9 ± 1.3	47	
	12	GU**U**AC	106.8 ± 0.6	44	
	Consensus	RY**U**NN			
The 2451 region 2448–2554	1 (wt)	A(C)**AAC**AG	47.0 ± 2.6	100	Conserved seq
	2	U(C)**AAC**AG	77.1 ± 3.2	61	Protozoan mt
	3	C(C)**AAC**AG	63.6 ± 1.9	74	
	4	G(C)**AAC**AG	62.7 ± 3.1	75	
	5	A(C)**AAC**UG	55.4 ± 1.8	85	Eukaryote
	Consensus	N(C)**AAC**WG			
The triad 2061, 2447, 2451	1 (wt)	**G**-G-**A**	47.3 ± 1.2	100	Conserved seq
	2	**G**-A-**A**	76.8 ± 1.7	62	
	Consensus	**G**-R-**A**			

Invariant or semiconserved residues selected in this study are bold or underlined, respectively. RNA modifications are ignored. The nomenclature used for the mixed bases obeys the rules previously established in the literature (44); R is A or G, Y is U or C, W is A or U, K is U or G, B is all bases except A, D is all bases except C, H is all bases except G, V is all bases except U, and N is all bases (A, U, G, or C). wt, wild type; seq, sequence.

BIOCHEMISTRY

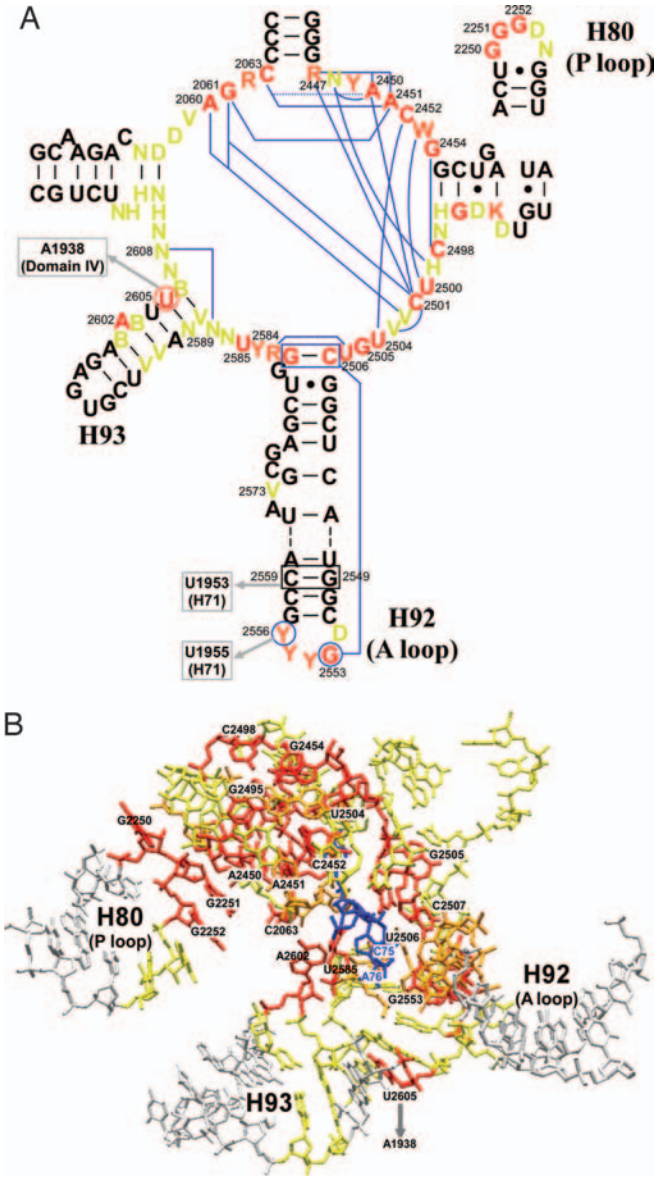

Fig. 1. Genetically selected functional bases in domain V of 23S rRNA responsible for ribosomal function. (*A*) Selected nucleotides with no variation from the wild-type sequence are shown in red. Nucleotides that can be replaced by only one other base are shown in orange. Nucleotides that can be replaced by two or three other bases are shown in yellow. This color code is used also in *B* and in Figs. 2 and 3. The nomenclature used for mixed bases obeys previously established rules (44). The blue lines indicate interactions in the crystal structure (4). (*B*) Location of the functionally essential conserved bases in D50S complexed with acceptor stem-loop (ASM) at the A site. Coordinates were obtained from the Protein Data Bank (PDB ID code 1NJP) (4). The nucleotides found to be essential are indicated with the same color code as described in *A*. The 3′ terminus of ASM is shown in blue.

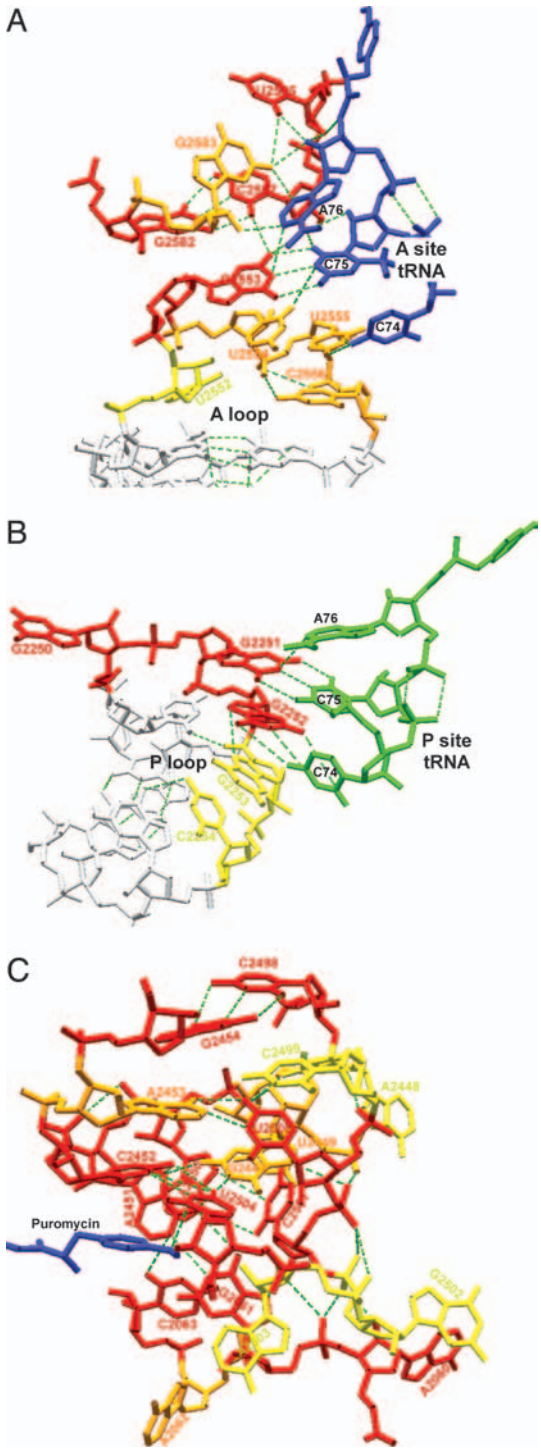

Fig. 2. Essential bases and H bond networks responsible for proper tRNA positioning and ribosomal function. (*A*) The CCA terminus of A site tRNA is fixed by binding to G2553 in the A loop and by interacting with the essential G2582–C2507 pair. A76 of A site tRNA makes a contact with the essential U2506. (*B*) The derived P site tRNA 3′ end (4) in relation to G2251/G2252. (C) The cluster of essential bases in the PTC that is constituted by three regions in the domain V central loop, 2060–2063, 2447–2454, and 2498–2504. Also shown is the complex H bond network involving these nucleotides. Coordinates were obtained from 1NJP (4).

Some compensatory mutations might be required for replacement of A2602 by G2602 in *E. coli* 23S rRNA.

Essential Nucleotides in the Domain V Central Loop. The connecting loop between helices 92 and 93, which includes U2585, is located near the aa-tRNA 3′ terminus regardless of whether it is a minimal or a longer substrate analog (4, 34, 35), in a hydrogen-bonding distance from 2′ OH of A76 tRNA. A library with five nucleotides (2583–2587) was randomized (Fig. 4*B*) and subjected to SSER selection. Twelve sequence variations, all including the conserved U2585, were selected as functional sequences (Table 1 and Fig. 1*A*). Purines at position 2583 and pyrimidines at position 2584 were selected as functional bases, whereas all types are allowed at

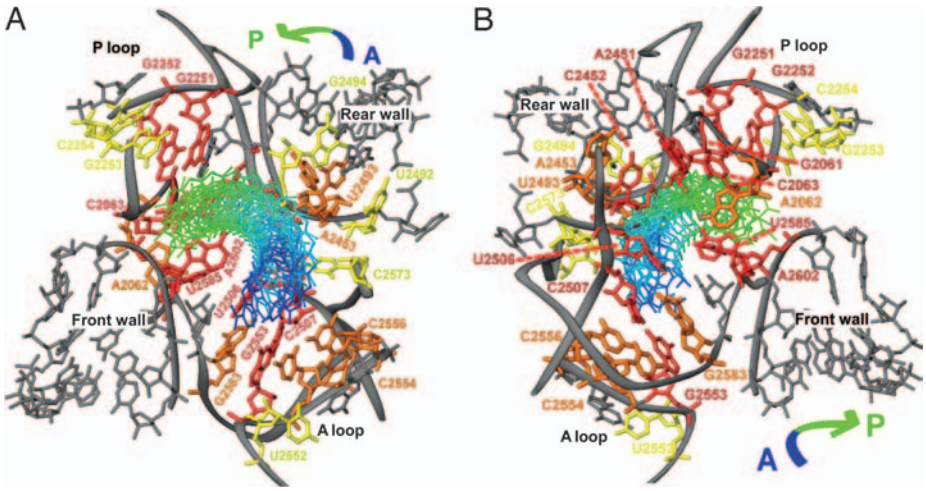

Fig. 3. Essentiality of highly conserved bases in the PTC arched void responsible for rotatory motion of tRNA 3′ end. (*A* and *B*) PTC arched void with snapshots of intermediate stages in the motion of the tRNA 3′ end from the A to the P site (blue to green), viewed down (*A*) and up (*B*) the axis of the rotation. The essentiality for each nucleotide is indicated by the color code as in Fig. 1. Coordinates were obtained from 1NJP (5).

positions 2586 and 2587. The variant with GUUUA (clone 1) is the conserved sequence of most organisms, whereas the variant with GUUCA (clone 6) exists only in *Bacillus subtilis*. The other 10 variants are functional sequences that have never been found in any organisms.

The 2608-region (2607–2610) between helices 73 and 93 was randomized and subjected to SSER selection (Fig. 4*B*). Although G2608 is conserved in >98% of the large subunit rRNAs of the three domains of life (Fig. 4*A*), consensus sequences and essential bases could not be obtained (Fig. 1*A*). Thus, it appears that the conservation of bases in this region is not important for ribosomal function. The 2451 region (2448–2454) in the domain V central loop is highly conserved in all living organisms (Fig. 4*A*). This region is also known to be a target site of several antibiotics and a tRNA cross-linking site (36), suggesting its possible involvement in ribosomal function, consistent with its central location in the PTC of all known crystal structures of 50S complexes (2, 4, 29, 30, 34, 35). Six nucleotides (2448 and 2450–2454) in this region were completely randomized, yielding 4,096 sequence variations (Fig. 4*B*) that were subjected to SSER selection. Because U2449 can be replaced by C without any phenotype (37), U2449C served as the specific mutation that discriminated the selected sequence from template contamination. Only five sequence variations, which include the wild-type sequence, were selected as functional sequences (Table 1 and Fig. 1*A*), demonstrating the functional importance of the conserved bases in this region and indicates that A2450, A2451, C2452, and G2454 are likely to be involved in ribosomal functions. In support, although A2448 could be replaced by all three of the other bases, its variants showed an apparent growth defect. Notably, U2448 exists in certain protozoan mitochondrial rRNAs. Clone 5, which bears the healthy mutation U2453, is also a natural sequence of eukaryotic rRNAs. Thus, the consensus sequence in this region is NYAACWG (2448–2454) (Table 1).

To shed light on the high conservation of the 2451 region of bacterial rRNA, a competition growth experiment with the five variants obtained by SSER selection was carried out. Thus, equal cell numbers of the five variants were mixed and cocultivated for 48 h (see *Supporting Methods*). As shown in Fig. 6, which is published as supporting information on the PNAS web site, only the eukaryotic U2453 variant survived, along with the cell bearing the wild-type sequence after 24 h of cultivation. The U2453 variant was subsequently overtaken by the wild type and disappeared after 48 h. This result may explain why the wild-type sequence is highly conserved in bacterial rRNA, despite the fact that four other functional sequences are allowed in this region.

The proposed catalytic triad, A2451–G2447–G2061 (2, 22, 34, 35), was randomized to give 64 sequence variations (Fig. 4*B*). SSER showed that, besides the wild-type triad (A2451–G2447–G2061), only A2451–A2447–G2061 was selected as a functional variant, as a slow-growth phenotype (Table 1), consistent with previous observations that G in 2447 can be replaceable to A (25). The only deviation from our finding is the viable A2451T found in *Mycobacterium smegmatis* (26).

The 10-base connecting loop 2497–2506 is a highly conserved region in the domain V central loop (Fig. 4*A*). The SSER selection method is not suitable for testing the functionality of these residues, owing to the enormous library required to test all possible variations (4^{10}) and because the host cell was shown not to be particularly competent. Thus, we adopted another technique to browse through this target region and named it *B*-scan analysis. Here, each nucleotide position is replaced by the other three bases by PCR mutagenesis using a mixed primer. The subsequent steps followed the SSER method, namely, the pRB102-derived library consisting of three mutations at each position was introduced into NT101, and the functional variants were selected on sucrose plates. If a target base is essential for ribosomal function, no variants are obtained. *B*-scan analysis was used to identify essential bases at positions 2056–2063, 2492–2507, 2573, 2582, 2588, 2589, and 2605–2614 (Fig. 4*B*). Although many functional variations were selected, A2060, G2061, C2063, C2495, C2498, U2500, C2501, U2504, G2505, U2506, C2507, G2582, and U2605 were retrieved as essential residues for ribosomal function (Fig. 1*A*).

Nucleotides Responsible for the Rotatory Motion of A Site tRNA 3′ End. During translocation, the A site tRNA 3′ terminus travels from A to P loop by a rotatory motion while contacting PTC nucleotides (4, 18, 29). Some of those, G2061, C2063, A2451, C2452, U2585, and A2602, which seem to control the motion, were shown to be essential (Fig. 3). At 30° rotation, C2573 interacts with C75 and A76 of the rotating tRNA. When C2573 was deleted or replaced with U, no functional variant could be obtained (Fig. 7, which is published as supporting information on the PNAS web site), consistent with the suggestion that this nucleotide is involved in guiding the rotating tRNA. C2573 could be replaced with A or G, but these variants showed a slow-growth phenotype (Fig 7), indicating that they may guide the rotating tRNA but may cause some steric effect. On the other hand, no essential bases at positions 2492–2494 were detected, in accord with the suggestion that the phosphate backbone of G2494 interacts with the rotating C74 and C75 at 60° rotation.

Discussion

The advanced genetic selection system (SSER) allows identification and selection of nucleotides or sequences essential for ribosomal function from a randomized rRNA library in *E. coli* cells. Its main

BIOCHEMISTRY

advantage is that it can be designed to test *in vivo* large number of functional sequences and that it permits neutral genetic selection of functional sequences, as it excludes researcher arbitrariness and bias. Moreover, if several sequences are functional yet bear low homology to each other, the SSER method can identify them, whereas other approaches using conventional site-directed mutagenesis might miss a few of these functional sequences. Importantly, the nucleotides in 23S rRNA found to be essential for ribosome functions include wild-type and natural sequences of other organisms as well as unnatural but functional sequences that may have been excluded by the process of evolution. Noteworthy are studies performed elsewhere that examine a randomized library and have isolated functional sequences in 16S rRNA (38, 39). Likewise, a PCR-based random mutagenesis performed by Garret's group (40) identified 21 single-site mutations at the PTC.

In this study, 66 bases in domain V were randomized and subjected to genetic selection by SSER and *B*-scan methods. In these methods, each functional sequence in the randomized library is isolated independently as a single colony without having to struggle against other variants for survival. Consequently, these methods can test the functionality of every sequence in the library, provided that sufficient numbers of transformants are supplied.

We obtained various mutants differing in their viability, as shown by the differently sized transformants on the kanamycin plates (Fig 5). Moreover, the doubling time of selected sucrose-resistant cells ranged from 47 to 120 min (Table 1). We found that all essential bases (red in Fig. 1*A*) selected in this study are almost fully conserved in all living organisms (Fig. 4*A*). In contrast, nonconsensus sequences were selected in the nonconserved regions (Figs. 1*A* and 4*A*). However, several conserved bases (yellow in Fig. 1*A*) were shown to be replaceable by other bases; G2253D, A2448N, G2502V, A2503V, U2552D, A2587N, G2608N, and A2059V (Fig. 1*A*). Most of these are unnatural, yet functional, variations. Thus, these results narrow the focus on bases and interactions in the PTC that are essential for determining its various mechanisms in protein synthesis.

Fig. 1*B* shows locations of essential bases in D50S complexed with acceptor stem loop (ASM) in the A site, including the WC base pair between its C75 with G2553 of the A loop (Fig. 2*A*). In this structure, G2553 also interacts with the essential G2582–C2507 pair, whereas the 2′ OH and O2 of C75 in the tRNA form H bonds with the 2′ OH of C2507. This base quartet seems to play a key role in stabilizing the positioning of the tRNA 3′ end in the A site. In addition, the A76 base in the A site interacts with U2506 and G2583 (4); the 2′ OH of A76 forms H bonds with 2′ OH (U2506), O2 (U2506), and N2 (G2583), whereas N1 and N3 of A76 form H bonds with the 2′ OH and N2 of G2583 (Fig. 2*A*). Because G2583 can be replaced by A (clone 9 Table 1), the H bonds N2(G2583)–N3(A76) and N2(G2583)–2′ OH(A76) are not essential. However, it is intriguing that U2506 cannot be replaced with C; nevertheless, C2506 does not abolish any H bonds with A76. C2506 might induce local structural change in the A site by forming WC-type pairing with G2583, thereby affecting the precise positioning of the aa-tRNA. Supporting this suggestion is pre-steady-state kinetic analysis of the U2506C mutant ribosome, which has shown that its rate constant of peptide-bond formation is significantly decreased when minimal substrate is used, whereas the rate constant is unaffected when the substrates are intact aa-tRNAs (13).

The precise orientation of the A loop (H92) is important for aa-tRNA positioning. Long-range interactions between H92 and H71 in domain IV may be involved in the placement of the A loop. An H bond between the N4 of C2556 in the A loop (H92) and the O4 of U1955 in H71 is seen in crystal structures (1, 3). C2556 cannot be replaced with purine bases (Fig. 1*A* and Table 1), because it may induce steric hindrance that disrupts the interaction between H92 and H71. A minor interaction between A1953 and G2549–C2559 pairing may have an important function in supporting the interaction between H92 and H71 (Fig. 1*A*).

The P loop (helix 80) serves as the attachment site for the CCA terminus of P site-bound pep-tRNA, and G2250, G2251, and G2252 were selected as essential (Table 1). In the structure of D50S complexed with a derived P site substrate (Fig. 2*B*) (4), the essential G2251 and G2252 make WC-pairing with C75 and C74 of the pep-tRNA, respectively. The essential G2250 bulges out from the P loop, hence it is likely to be involved in stabilizing the P loop (29).

The conserved A2602 that bulges from helix H93 and lies between the CCA termini of both tRNAs is involved in several ribosomal functions, including the release of the nascent peptide (2–4, 14) and undergoes conformational changes while binding substrates and inhibitors (4, 29, 30). To test its critical role in ribosomal function, A2602 was randomized along with adjacent base pairs (2591, 2592, and 2601–2603) (Fig. 4*B*). In all of 11 functional variants, only the A2602 bulge was conserved (Fig. 1*A* and Table 1). With regard to the adjacent base pairs, based on our results, we suggest that the 2591–2603 pair could be replaced with other WC-type pairs, whereas the 2592–2601 pair could be replaced with a G–U wobble pair or nonpairing bases. Kinetic analysis of peptide-bond formation by the 2602 mutant ribosome and intact aa-tRNA has shown unaffected reaction rate (13). However, the 2602 mutation severely affected the peptide-release reaction (13, 15), consistent with its suggested activity.

U2605 was also selected as essential for ribosomal function (Fig. 1*A*). In *E. coli* cells, it is modified to pseudouridine (Ψ). However, its partner base A2589 could be replaced by all other, suggesting that base-pairing between Ψ2605 and A2589 is not essential for ribosomal function. The critical role of Ψ2605 may be explained by the fact that G1938 in domain IV forms H bonds with U2605 (1, 3). Thus, it is possible that A1938 in *E. coli* 23S rRNA forms an A minor interaction with Ψ2605 and that this interaction may play an important function in adjusting the configuration of H93.

The cluster of essential PTC nucleotides, composed of 2451, 2500, and 2602 regions in the central loop of domain V (Figs. 1*B* and 2*C*) is an intriguing functional site. The complex H bond network between essential bases consisting of G2061, C2063, A2450, A2451, C2452, C2501, and U2504 may provide the molecular stage for protein synthesis. It has been reported that G2447 can be replaced with A or C in *E. coli* Δ*rrn7* strain (25). G2447 forms a connection with A2451 and A2450. We obtained the G2447A variant but not G2447C (Fig. 1*A*, Table 1). This discrepancy might be explained by the use a high-copy plasmid with ColE1 origin to rescue the *E. coli* Δ*rrn7* strain (25), whereas we used a low-copy (≈5 copies) plasmid with a pSC101 origin (41), thus requiring a high level of ribosomal functionality. Supporting this view is the apparent reduction of viability (relative growth rate of only 62%, Table 1) shown by our G2447A variant, in contrast to the G2447A mutant that has a similar growth phenotype to that of wild-type cells (25). This observation may, therefore, indicate that G2447 plays a role in maintaining the critical H bond network in the PTC.

It has been proposed that the 3′ terminus of the A site tRNA travels from the A to the P loop by a spiral rotatory motion (4, 29, 30) within an arched void confined by highly conserved PTC nucleotides: G2061, C2063, G2251, A2451, C2452, A2453, U2492, U2493, and G2494 of the rear wall; U2585, A2602, and U2506 from the front; and the A and P loops from the side: A2062, C2507, G2553, U2555, and C2573 (Fig. 3). According to this study, 8 of 12 nucleotides involved in the rotatory motion were found to be essential. The replacement of A at position 2453 by U (Table 1) is in accord with the interaction of this nucleotide with the rotating aa-tRNA 3′ end through the backbone phosphate (29), as are the three remaining nucleotides (U2492–G2494) of which the phosphates rather than the bases are the functional parts. From the front wall of the PTC, the essential A2602 and U2585, respectively, contact with A73 and 2′ OH of A76 of the tRNA throughout the rotatory motion, suggesting their pivotal role in the rotation.

Similarly, the rear wall nucleotide C2573 has been implicated only with the rotatory motion guidance (29) by its backbone phosphate. Indeed, when C2573 was deleted or replaced with U, no functional variants could be obtained (Fig 7), supporting the notion that C2573 guides the rotating tRNA in addition to its putative participation in tRNA accommodation (42). Interestingly, ribosome variants with A2573 or G2573 could be obtained but showed severe growth phenotype (Fig 7), indicating that the purine base at this position may affect the rotatory motion because of steric problems.

Materials and Methods

The SSER Method. The method is outlined in *Supporting Methods*. Initially, we defined specific, nondeleterious mutations that could be introduced near the sites in 23S rRNA in pRB102 that were targeted for randomization; these sites would then be used as markers to distinguish the transformants from the wild-type templates after SSER. These specific mutations were introduced into pRB102 by QuikChange site-directed mutagenesis (Stratagene) according to the manufacturer's instructions. The resulting plasmids were checked to ensure that the mutations were not dominant lethal by replacing them into NT101 (by the process described below). The pRB102 derivatives bearing specific and nonlethal mutations were then subjected to hypermethylation *in vitro* (*Supporting Methods*; see Table 2, which is published as supporting information on the PNAS web site) and used as the template plasmid for the subsequent PCR randomization process. The primer sets used in the PCR randomization are shown in Table 3, which is published as supporting information on the PNAS web site. In the first PCR, a set of primers complementary to sequences just outside the target sequence was used to generate gapped pRB102 (Fig 5), enhancing the efficiency of PCR randomization and reducing the background for SSER selection. The gapped pRB102 was then gel-purified and used as the template for the next series of PCRs. These reactions involved a pool of N primers for each region in domain V; these primers contained four to six randomized bases flanked by the 5′ and 3′ adjacent regions of the target site, including the specific point mutations that had been introduced previously. The reverse primer was complementary to the opposite direction of the 5′ side of the N primer pool. This PCR randomization was performed in a 50-μl reaction mixture containing 150 ng of gapped pRB102 as the template, 12.5 pmol of each N primer and the reverse primer, 200 μM each dNTP, 1× Pfu Turbo buffer, and 2.5 units Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). PCR was carried out by using the thermal cycler (PTC 200; MJ Research, Tokyo, Japan) by the following program: preincubation at 95°C for 30 sec, followed by 18 cycles of a denaturing step at 95°C for 30 sec, an annealing step at 55°C for 1 min, and an extension step at 68°C for 20 min. To obtain enough of the randomized library, this reaction was performed in 10–20 tubes. After the PCR, the reaction mixture (50 μl) was treated with DpnI (10 units; New England Biolabs, Ipswich, MA) and λ exonuclease (5 units; New England Biolabs) at 37°C for 90 min to digest the template plasmid, cleaned up by QIAquick (Qiagen, Valencia, CA), and checked by agarose gel electrophoresis. The distribution of the 4–6 nt at each randomized position was confirmed by direct sequencing of the plasmid library (Fig. 5) before the selection. NT101 was then transformed by the randomized library and inoculated onto LB plates containing Spc/Km. For each 50-μl tube of the library, 1 ml of competent cells prepared by the modified Hanahan method (43) were used. Transformant colonies were picked to make a suspension in LB broth and then spotted onto LB plates containing Spc/Km/sucrose to drive plasmid replacement. When variants with a slow-growth phenotype were picked, their resulting cell suspensions were first spotted onto LB plates containing Spc/Km (instead of being spotted onto sucrose plates) and then transferred to LB sucrose plates. Sucrose-resistant colonies were then cultured in 2× LB broth for miniprep of the plasmid for sequencing. To check the plasmid replacement, each transformant was spotted onto two LB plates containing Spc/Amp and Spc/Km/sucrose. No growth of the spotted cells on the Amp plate demonstrates complete plasmid replacement.

We thank Suzuki group members, especially T. Komoda, K. Kitahara, T. Yokoyama, and M. Nagaoka, for fruitful discussions; The Weizmann Institute ribosomal crystallography group, especially A. Bashan, for active participation; and Dr. Catherine L. Squires (Tufts University, Medford, MA) for providing a valuable strain. Special thanks are due to Dr. Kimitsuna Watanabe (National Institute of Advanced Industrial Science and Technology, Tokyo, Japan) for continuous support and encouragement. Diffraction data were collected at ID19 beamline, Structural Biology Center Advanced Photon Source, Argonne National Laboratory and the ID14 beamline, European Synchrotron Radiation Facility. This work was supported in part by grants-in-aid for scientific research on priority areas from the Japan Ministry of Education, Science, Sports, and Culture; Human Frontier Science Program Grant RGY23/2003 (to T.S.); National Institutes of Health Grant GM34360; and the Kimmelman Center for Macromolecular Assemblies (A.Y.). A.Y. holds the Martin and Helen Kimmel Professorial Chair.

1. Ban N, Nissen P, Hansen J, Moore PB, Steitz TA (2000) *Science* 289:905–920.
2. Nissen P, Hansen J, Ban N, Moore PB, Steitz TA (2000) *Science* 289:920–930.
3. Harms J, Schluenzen F, Zarivach R, Bashan A, Gat S, Agmon I, Bartels H, Franceschi F, Yonath A (2001) *Cell* 107:679–688.
4. Bashan A, Agmon I, Zarivach R, Schluenzen F, Harms J, Berisio R, Bartels H, Franceschi F, Auerbach T, Hansen HA, *et al.* (2003) *Mol Cell* 11:91–102.
5. Yusupov MM, Yusupova GZ, Baucom A, Lieberman K, Earnest TN, Cate JH, Noller HF (2001) *Science* 292:883–896.
6. Schuwirth BS, Borovinskaya MA, Hau CW, Zhang W, Vila-Sanjurjo A, Holton JM, Cate JH (2005) *Science* 310:827–834.
7. Noller HF, Hoffarth V, Zimniak L (1992) *Science* 256:1416–1419.
8. Khaitovich P, Mankin AS, Green R, Lancaster L, Noller HF (1999) *Proc Natl Acad Sci USA* 96:85–90.
9. Green R, Noller HF (1997) *Annu Rev Biochem* 66:679–716.
10. Garrett R, Rodriguez-Fonseca C (1996) *Ribosomal RNA: Structure, Evolution, Processing and Function in Protein Biosynthesis* (CRC, Boca Raton, FL).
11. Gregory ST, Dahlberg AE (2004) *Nat Struct Mol Biol* 11:586–587.
12. Sievers A, Beringer M, Rodnina MV, Wolfenden R (2004) *Proc Natl Acad Sci USA* 101:7897–7901.
13. Youngman EM, Brunelle JL, Kochaniak AB, Green R (2004) *Cell* 117:589–599.
14. Polacek N, Gomez MJ, Ito K, Xiong L, Nakamura Y, Mankin A (2003) *Mol Cell* 11:103–112.
15. Nakamura Y, Ito K, Isaksson LA (1996) *Cell* 87:147–150.
16. Petry S, Brodersen DE, Murphy FVT, Dunham CM, Selmer M, Tarry MJ, Kelley AC, Ramakrishnan V (2005) *Cell* 123:1255–1266.
17. Schmeing TM, Huang KS, Strobel SA, Steitz TA (2005) *Nature* 438.520–524.
18. Agmon I, Auerbach T, Baram D, Bartels H, Bashan A, Berisio R, Fucini P, Hansen HA, Harms J, Kessler M, *et al.* (2003) *Eur J Biochem* 270:2543–2556.
19. Yonath A (2003) *Biol Chem* 384:1411–1419.
20. Katunin VI, Muth GW, Strobel SA, Wintermeyer W, Rodnina MV (2002) *Mol Cell* 10:339–346.
21. Rodnina MV, Wintermeyer W (2003) *Curr Opin Struct Biol* 13:334–340.
22. Muth GW, Ortoleva-Donnelly L, Strobel SA (2000) *Science* 289:947–950.
23. Polacek N, Gaynor M, Yassin A, Mankin AS (2001) *Nature* 411:498–501.
24. Erlacher MD, Lang K, Shankaran N, Wotzel B, Huttenhofer A, Micura R, Mankin AS, Polacek N (2005) *Nucleic Acids Res* 33:1618–1627.
25. Thompson J, Kim DF, O'Connor M, Lieberman KR, Bayfield MA, Gregory ST, Green R, Noller HF, Dahlberg AE (2001) *Proc Natl Acad Sci USA* 98:9002–9007.
26. Beringer M, Bruell C, Xiong L, Pfister P, Bieling P, Katunin VI, Mankin AS, Bottger EC, Rodnina MV (2005) *J Biol Chem* 29:15–19.
27. Barta A, Dorner S, Polacek N (2001) *Science* 291:203.
28. Parnell KM, Seila AC, Strobel SA (2002) *Proc Natl Acad Sci USA* 99:11658–11663.
29. Agmon I, Bashan A, Zarivach R, Yonath A (2005) *Biol Chem* 386:833–844.
30. Yonath A (2005) *Annu Rev Biochem* 74:649–679.
31. Kim DF, Green R (1999) *Mol Cell* 4:859–864.
32. Weinger JS, Parnell KM, Dorner S, Green R, Strobel SA (2004) *Nat Struct Mol Biol* 11:1101–1106.
33. Samaha RR, Green R, Noller HF (1995) *Nature* 377:309–314.
34. Hansen JL, Ippolito JA, Ban N, Nissen P, Moore PB, Steitz TA (2002) *Mol Cell* 10:117–128.
35. Schmeing TM, Seila AC, Hansen JL, Freeborn B, Soukup JK, Scaringe SA, Strobel SA, Moore PB, Steitz TA (2002) *Nat Struct Biol* 9:225–230.
36. Raue HA, Musters W, Rutgers CA, Van't Riet J, Planta RJ (1990) *The Ribosome, Structure, Function, and Evolution* (Am Soc Microbiol, Washington, DC).
37. O'Connor M, Lee WM, Mankad A, Squires CL, Dahlberg AE (2001) *Nucleic Acids Res* 29:710–715.
38. Morosyuk SV, Lee K, SantaLucia J, Jr, Cunningham PR (2000) *J Mol Biol* 300:113–126.
39. Yassin A, Fredrick K, Mankin AS (2005) *Proc Natl Acad Sci USA* 102:16620–16625.
40. Porse BT, Garrett RA (1995) *J Mol Biol* 249:1–10.
41. Bernardi A, Bernardi F (1984) *Nucleic Acids Res* 12:9415–9426.
42. Sanbonmatsu KY, Joseph S, Tung CS (2005) *Proc Natl Acad Sci USA* 102:15854–15859.
43. Jain C, Belasco JG (2000) *Methods Enzymol* 318:309–332.
44. Cornish-Bowden A (1985) *Nucleic Acids Res* 13:3021–3030.