

SPARK—A Novel Method To Monitor Ribosomal Peptidyl Transferase Activity[†]Norbert Polacek,^{‡,§} Steven Swaney,^{§,||} Dean Shinabarger,^{*,||} and Alexander S. Mankin^{*,‡}

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ABSTRACT: The key enzymatic activity of the ribosome is catalysis of peptide bond formation. This reaction is a target for many clinically important antibiotics. However, the molecular mechanisms of the peptidyl transfer reaction, the catalytic contribution of the ribosome, and the mechanisms of antibiotic action are still poorly understood. Here we describe a novel, simple, convenient, and sensitive method for monitoring peptidyl transferase activity (SPARK). In this method, the ribosomal peptidyl transferase forms a peptide bond between two ligands, one of which is tritiated whereas the other is biotin-tagged. Transpeptidation results in covalent attachment of the biotin moiety to a tritiated compound. The amount of the reaction product is then directly quantified using the scintillation proximity assay technology: binding of the tritiated radioligand to the commercially available streptavidin-coated beads causes excitation of the bead-embedded scintillant, resulting in detection of radioactivity. The reaction is readily inhibited by known antibiotics, inhibitors of peptide bond formation. The method we developed is amenable to simple automation which makes it useful for screening for new antibiotics. The method is useful for different types of ribosomal research. Using this method, we investigated the effect of mutations at a universally conserved nucleotide of the active site of 23S rRNA, A2602 (*Escherichia coli* numbering), on the peptidyl transferase activity of the ribosome. The activities of the in vitro reconstituted mutant subunits, though somewhat reduced, were comparable with those of the subunits assembled with the wild-type 23S rRNA, indicating that A2602 mutations do not abolish the ability of the ribosome to catalyze peptide bond formation. Similar results were obtained with double mutants carrying mutations at A2602 and another universally conserved nucleotide in the peptidyl transferase center, A2451. The obtained results agree with our previous conclusion that the ribosome accelerates peptide bond formation primarily through entropic rather than chemical catalysis.

Solving the crystallographic structure of both ribosomal subunits (1–4) as well as of the whole 70S ribosome (5) brought about a quantum leap in the understanding of the ribosome organization. For the first time, active sites of such an enormously complex multifunctional ribonucleoprotein assembly like the ribosome could be described in detailed molecular terms. The combination of structural information and biochemical data led already to the postulation of atomic models for two fundamental ribosome functions, namely, peptide bond formation and mRNA decoding [reviewed in (6)]. Additionally, crystallographic structures of ribosomal subunits complexed with several ribosome-targeted antibiotics significantly improved our understanding of the mode of action of protein synthesis inhibitors, as well as the mechanisms of drug resistance (7–9). However, to keep up

with the pace of progress in the structural research of the ribosome, new sensitive, fast, and accurate biochemical techniques to assess ribosome functions are needed.

The key enzymatic activity of the ribosome is polymerizing amino acids into polypeptides. Peptide bonds linking amino acids in a protein are formed as the result of the transfer of the growing polypeptide chain from the peptidyl-tRNA bound in the ribosomal P-site to the α -amino group of the aminoacyl-tRNA positioned in the A-site. This reaction takes place in the peptidyl transferase center located in a cleft on the interface side of the large (50S) ribosomal subunit. Though proteins are important for the ribosomal peptidyl transferase activity (10–12), the active site of the peptidyl transferase center is built entirely of rRNA (13, 14); the mechanisms of its function, however, are being debated (13, 15–19).

Several methods have been developed to study the peptidyl transfer reaction. Most of them are based on the use of analogues of peptidyl- and aminoacyl-tRNA. Formyl-methionyl- or *N*-acetyl-phenylalanyl-tRNAs (or their 3'-terminal fragments) are usually used as donor (peptidyl) substrates while puromycin (Pmn),¹ an antibiotic that mimics the 3' end of amino acyl-tRNA, is often employed as an acceptor substrate (20, 21). The reaction leads to the transfer of the radiolabeled *N*-protected aminoacyl residue to puromycin. The product is extracted into ethyl acetate and can be either

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directly detected by scintillation counting or resolved by paper electrophoresis with subsequent radiographic detection (20, 22). In a more recent version of this technique, puromycin-containing oligonucleotides (C-Pmn or CC-Pmn) were used instead of puromycin; this allowed the incorporation of the radioactive label into the acceptor substrate and the use of gel-electrophoresis for the detection of the reaction products (23). Despite important contributions of these techniques to our current knowledge of the mechanisms of peptide bond formation, they have their intrinsic confines including the use of limiting amounts of either donor or acceptor substrate, high error rates due to multiple pipetting steps involved in reaction product detection, and the difficulty in automating the reaction for use in high through-put tests.

Here we describe a new, sensitive, accurate, and convenient way to monitor ribosomal peptidyl transferase activity which is based on the scintillation proximity assay (SPA) technology (24) which utilizes commercially available streptavidin-coated polymeric SPA beads containing embedded scintillant. SPA beads can detect ^3H -radioactivity of the ligand that bind to the beads, but not that of the free radioligand. We applied this technique for measuring the peptidyl transferase activity of the ribosome or isolated large ribosomal subunit. The method was tested with ribosomes from diverse organisms including Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Thermus aquaticus*) bacteria. The high sensitivity and reproducibility of this new technique made it instrumental for studies of in vitro reconstituted large ribosomal subunits containing 23S rRNA mutations in the peptidyl transferase active site. Furthermore, the method is amenable for use in a high through-put format, and can be employed for studies of the mechanisms of antibiotic action and for screening of natural or synthetic compound libraries for new inhibitors of protein synthesis.

EXPERIMENTAL PROCEDURES

tRNA Substrates. Formyl- ^3H Met-tRNA (14 300 or 54 000 dpm/pmol) was purchased from NEN Life Science Products (Boston, MA). Phe-tRNA was prepared by aminoacylation of *E. coli* tRNA^{Phe} or total *E. coli* tRNA in a buffer containing 20 mM Hepes/KOH (pH 7.6), 8 mM MgOAc₂, 150 mM NH₄Cl, 4 mM β -mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine as described by Triana-Alonso et al. (25). Biotinylation of Phe-tRNA was performed by incubating 1300–1700 pmol of Phe-tRNA^{Phe} in 200 μL of 50 mM NaHCO₃ (pH 8.2) with 100 μL of a 7 mg/mL solution of sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce) for 1 h on ice. Aminoacylated formyl- ^3H Met-tRNA and Phe-tRNA as well as biotinylated Phe-tRNA were HPLC-purified, ethanol-precipitated, and resuspended in H₂O.

Biotinylated Lys-tRNA was purchased from Promega.

SPA Beads. SPA beads were obtained from Amersham Pharmacia Biotech (catalog number RPNQ0007). In control

experiments designed to elucidate the counting efficiency of SPA beads in the reaction buffer, 120 μL of SPA beads containing Stop solution [10 mM phosphate buffer, pH 7.4, 137 mM KCl, 2.7 mM NaCl, 125 mM EDTA, 0.45 mg of SPA beads (binding capacity: 127 pmol/mg)] was added to 5 pmol of biotin- ^3H Met-tRNA in 60 μL of reaction buffer containing 20 mM Hepes/KOH (pH 7.6), 6 mM MgOAc₂, 150 mM NH₄Cl, 4 mM β -mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine. SPA beads registered 35% of radioactivity (cpm) compared to conventional liquid scintillation counting.

SPARK-Pmn. The reaction can be performed either in 96-well plates or in 1.5 mL Eppendorf tubes. Typically, 15–24 pmol of *E. coli* or *S. aureus* 70S was programmed with 76 pmol of the synthetic mRNA AAGGAGAUUAACAAUGGGU or AAGGAGAUUAACAAUGAAA (Dharmacon Research, Inc.), and incubated with 48 pmol of formyl- ^3H Met-tRNA (14 300 dpm/pmol; 5900 cpm/pmol) for 10 min at 37 °C in SA buffer containing 10 mM Tris-HCl, pH 7.6, 30 mM MgCl₂, and 50 mM NH₄Cl. The reaction was initiated by the addition of 60 pmol of biotin-puromycin (^{biot}Pmn) and performed at 37 °C in a final volume of 60 μL for the indicated period of time. The reaction was stopped by the addition of 120 μL of Stop solution and incubated at room temperature for at least 1 h to allow complete binding of the reaction product to the beads. Subsequently, the radioactivity of the reaction product was counted in a 96-well plate scintillation counter (Wallac) (if the reaction was performed in 96-well plates) or in Beckman scintillation counter LS 6000IC (if the reaction was done in Eppendorf tubes). The background values of experimental samples which did not contain ribosomes or ^{biot}Pmn (usually 250–500 dpm) were subtracted from all the readings. SPARK-Pmn with *E. coli* or *T. aquaticus* was performed as above but in a polyamine buffer (26) [20 mM Hepes/KOH (pH 7.6), 150 mM NH₄Cl, 4 mM β -mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine] containing 30 mM MgOAc₂. SPARK-Pmn with reassociated 70S ribosomes containing in vitro reconstituted 50S subunits was performed as described (17).

Two tRNA-SPARK with 70S Ribosomes (SPARK-2Ta, SPARK-2Tb). SPARK-2Ta was performed under the same conditions as SPARK-Pmn in the SA buffer with the exception that ^{biot}Pmn was replaced with 30 pmol of biotinylated Lys-tRNA as the acceptor and 76 pmol of AAGGAGAUUAACAAUGAAA was used as an mRNA analogue.

In SPARK-2Tb, 7 pmol of *E. coli* 70S ribosomes was incubated with 5.7 pmol of ^{biot}Phe-tRNA^{Phe} and 40 μg of poly(U) in the polyamine buffer (26) containing 6 mM MgOAc₂ for 15 min at 37 °C. Subsequently, 5.2 pmol of ^3H -Phe-tRNA^{Phe} (6000 cpm/pmol) was added, and the reaction was performed at 37 °C for the indicated period of time in a final volume of 23.3 μL . The reaction was terminated by the addition of 47 μL of Stop solution.

Two tRNA-SPARK with Isolated 50S Subunits (SPARK-50S). Reactions with *E. coli* or *T. aquaticus* 50S subunits were performed in the same polyamine buffer containing 30 mM MgOAc₂. P-site binding was performed by incubating 14 pmol of 50S subunits with 11 pmol of ^{biot}Phe-tRNA^{Phe} for 10 min at 37 °C. After the addition of 12 pmol of ^3H -Phe-tRNA^{Phe}, the reaction was started by the addition of 15

¹ Abbreviations: SPARK, scintillation proximity assay for ribosomal kinetics; Pmn, puromycin; ^{biot}Pmn, biotin-puromycin containing a biotin group attached to the C5' of puromycin via an 18 atom linker; Lys^{biot}-tRNA, Lys-tRNA carrying the biotin tag at the ϵ -amino group of lysine; ^{biot}Phe-tRNA and ^{biot}Met-tRNA, N-biotinylated Phe-tRNA and Met-tRNA, respectively.

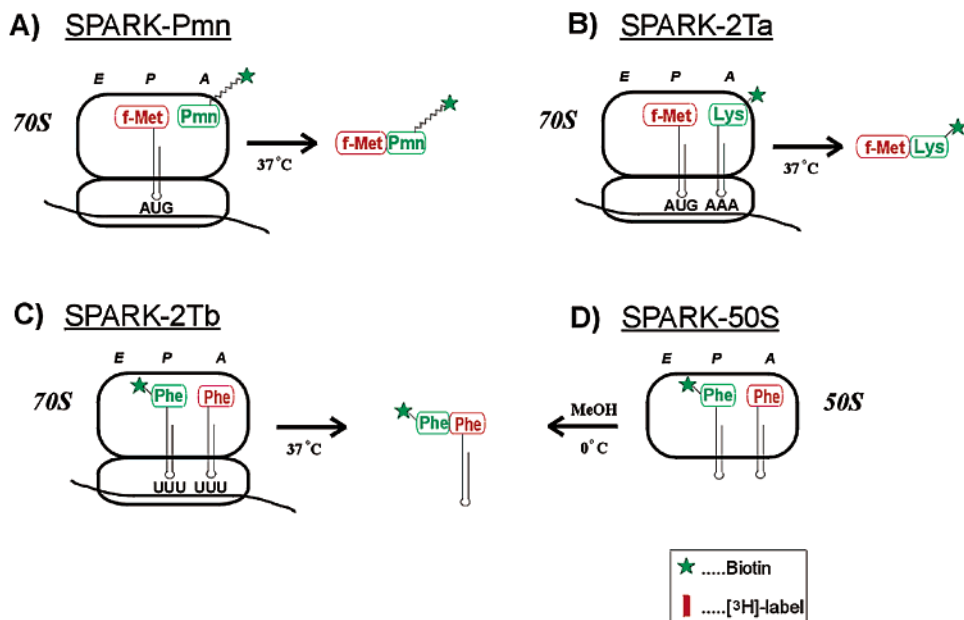


FIGURE 1: Versions of SPARK. ^3H -Labeled substrates are shown in red and biotinylated ligands in green. (A) SPARK-Pmn is performed with 70S ribosomes carrying a ^3H -labeled formyl-Met-tRNA (f-Met) at the P-site and biotin-puromycin (Pmn) at the A-site. (B) In SPARK-2Ta, Lys-tRNA biotinylated at the ϵ -amino group of lysine (Lys^{biot}-tRNA) serves as the acceptor (A-site) substrate, while formyl- ^3H Met-tRNA functions as the donor in the reaction performed by 70S ribosomes. (C) SPARK-2Tb employs unlabeled N-biotinylated Phe-tRNA and ^3H -labeled Phe-tRNA as P- and A-site substrates for poly(U)-programmed 70S ribosomes. (D) The peptidyl transfer reaction in SPARK-50S is promoted by the isolated large ribosomal subunit in the presence of N-biotinylated Phe-tRNA and ^3H -labeled Phe-tRNA as donor and acceptor substrates, respectively. The reaction product, diphenylalanyl-tRNA, is the same in SPARK-2Tb and SPARK-50S.

μL of cold methanol and incubated on ice for the specified time indicated in Figure 5 or overnight (in antibiotic experiments). The final reaction volume before addition of methanol was 30 μL . In antibiotic studies, 100 μM drug was added before methanol addition. The reactions were terminated by the addition of 90 μL of Stop solution containing 0.3 mg of streptavidin-coated SPA beads.

Reconstitution of *T. aquaticus* Mutant 50S Subunits. The mutations at position 2602 of the cloned *T. aquaticus* 23S rRNA gene (27) were engineered by oligonucleotide-based PCR mutagenesis as previously described (17). The double mutants were constructed by introducing the A2451T mutation into the construct carrying the A2602G mutation. Wild-type or mutant 23S RNAs were in vitro transcribed and assembled into 50S subunits as described (27).

RESULTS

The Principle of SPARK. We named the new peptidyl transferase assay SPARK for Scintillation Proximity Assay for Ribosomal Kinetics. In the SPARK technique, one of the substrates of the peptidyl transfer reaction, a peptidyl- or aminoacyl-tRNA analogue, carries the ^3H -labeled aminoacyl moiety while the second substrate is tagged with biotin. The formation of a peptide bond between the reaction substrates results in the appearance of a product that carries both the ^3H -label and a biotin tag. The product can bind to the streptavidin-coated scintillant-containing SPA beads. This brings the radioligand into a close enough proximity with the scintillant to enable detection of radioactivity. The unreacted radioactive substrate does not carry a biotin group and, therefore, does not bind to the beads. Consequently, it will not be detected because its radioactive emission is quenched by the solution before reaching the scintillant which is embedded in the SPA beads. The main advantage of

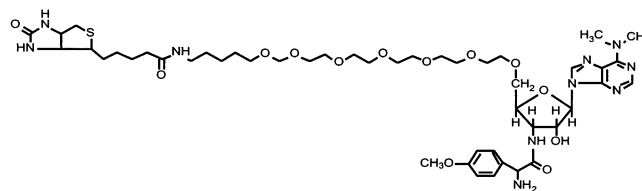


FIGURE 2: Structure of biotin-puromycin used in SPARK-Pmn.

SPARK is that, in contrast to all the previously used methods, no product purification is required: the SPA beads are added directly into the reaction mixture, together with the stop solution, which facilitates the release of the reaction product from the ribosome.

We have designed several different formats of SPARK which can find application in different types of ribosomal studies (Figure 1).

SPARK-Pmn: Measuring the Peptidyl Transferase Activity of the 70S Ribosome Using Peptidyl-tRNA and Biotin-puromycin. In this version of SPARK, formyl- ^3H Met-tRNA serves as a donor substrate of the peptidyl transfer reaction while biotin-puromycin (^{biot}Pmn), which contains a biotin group attached to the C5' of puromycin via an 18 atom linker (Figure 2), is the acceptor. Preincubation of ribosomes with formyl- ^3H Met-tRNA and synthetic mRNA (AAG-GAGAUAUAACAAUGGGU or AAGGAGAUAUAACA-AUGAAA) containing a Shine-Dalgarno sequence GGAG and a unique AUG codon places formyl-Met-tRNA in the ribosomal P-site. The reaction is then initiated by the addition of ^{biot}Pmn and later terminated by the addition of the EDTA-containing Stop buffer and SPA beads.

Conditions for this assay were originally optimized for 70S ribosomes from *S. aureus* and subsequently attuned for *E. coli* and *T. aquaticus* ribosomes. The reaction has a relatively sharp Mg^{2+} optimum of 30 mM (Figure 3A). Such

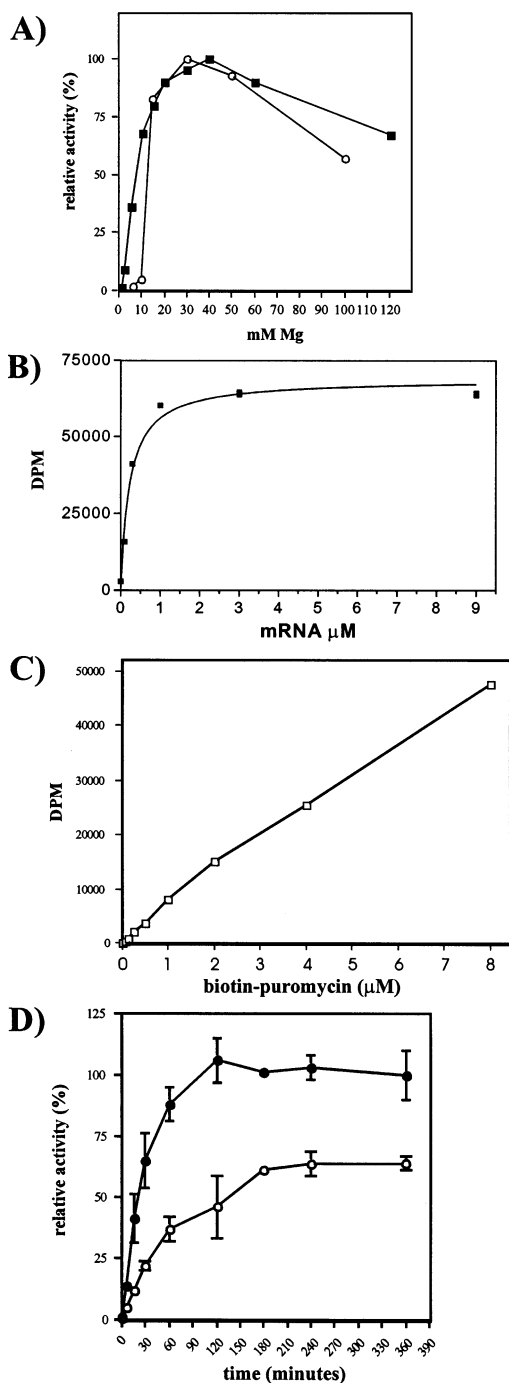


FIGURE 3: Optimization of SPARK-Pmn conditions. (A) Mg-dependence. *S. aureus* (closed symbols) or *E. coli* (open symbols) ribosomes in SA buffer (10 mM Tris-HCl, pH 7.6, 50 mM NH_4Cl) with variable concentrations of MgCl_2 . (B) mRNA-dependence. *S. aureus* ribosomes in SA buffer containing 30 mM MgCl_2 ; varying concentrations of a synthetic mRNA analogue, AAGGAGAUAA-CAAUGAAA, were used in reactions that were performed at 37 °C for 30 min. (C) Biotin-puromycin (biotPmn) dependence. Reactions with *S. aureus* ribosomes in SA buffer containing 30 mM MgCl_2 were performed at 37 °C for 5 min using formyl-[^3H] Met-tRNA (54 000 dpm/pmol) as a donor. (D) Comparison of the SA buffer (open symbols) with the polyamine buffer (20 mM Hepes/KOH, pH 7.6, 30 mM MgOAc_2 , 150 mM NH_4Cl , 2 mM spermidine, 0.05 mM spermine, and 4 mM β -mercaptoethanol) (closed symbols) in time course experiments using 24 pmol of *E. coli* 70S ribosomes, 48 pmol of formyl-[^3H] Met-tRNA (14 300 dpm/pmol), and 1 μM biotPmn . The curves represent the mean of 3–4 independent time course experiments, whereas the amount of product at 360 min in the polyamine buffer was taken as 100%.

a Mg^{2+} concentration is slightly higher than that used in other ribosomal assays and might be required for the proper binding of the biotinylated puromycin to the ribosome. The reaction, which is performed with a 2-fold excess of formyl-[^3H] Met-tRNA over the ribosome, critically depended on the presence of mRNA (Figure 3B), demonstrating that proper binding of formyl-Met-tRNA in the ribosomal P-site is required for an efficient reaction. The yield of the reaction product increases linearly within the biotPmn concentration range of 0.25–8 μM (Figure 3C). However, the actual amount of biotPmn that can be conveniently used in the reaction is limited by the binding capacity of SPA beads; therefore, the standard assay is performed with the final biotPmn concentration of 1 μM .

In the conditions optimized for *S. aureus* ribosomes, the Pmn-SPARK is performed in 60 μL of the SA reaction buffer which contains 10 mM Tris-HCl, pH 7.6, 30 mM MgCl_2 , and 50 mM NH_4Cl . Ribosomes present at 0.2–0.4 μM are preincubated at 37 °C with 1.27 mM synthetic mRNA and 0.8 μM *E. coli* formyl-[^3H] Met-tRNA, and the peptidyl transfer reaction is initiated by the addition of biotPmn to the final concentration of 1 μM .

Time course experiments revealed that under these conditions, a plateau in product formation is reached after about 3 h of incubation at 37 °C (Figure 3D). Though we have not specifically studied this question, the reason for the slow kinetics may be related to the use of derivatized puromycin or, alternatively, slow release of the reaction product, formyl-Met- biotPmn , from the ribosome. However, in experiments with *E. coli* ribosomes, we noticed that replacing the SA buffer optimized for *S. aureus* with a polyamine buffer developed by Nierhaus and co-workers (26) for *E. coli* ribosomes not only significantly accelerated the reaction (ca. 3-fold) but also increased the yield of product formation (Figure 3D). Nevertheless, the reaction still required a high (30 mM) Mg^{2+} concentration (data not shown). Therefore, all the SPARK-Pmn experiments with *E. coli* and *T. aquaticus* ribosomes were performed using the polyamine buffer supplemented with 30 mM Mg^{2+} .

The SPARK-Pmn proved to be a very sensitive method for measuring peptidyl transferase activity: control experiments lacking either biotPmn or ribosomes showed that the radioactivity of unreacted formyl-[^3H] Met-tRNA is essentially not registered. The background values measured in these controls were on average 2 orders of magnitude lower than the counts obtained in the full reaction (see Experimental Procedures). This made the assay suitable for measuring even very low levels of peptidyl transferase activity, for example, those obtained with ribosomes reconstituted in vitro with mutant 23S rRNA (see below).

SPARK-2T: Measuring the Activity of 70S Ribosomes Using Peptidyl- and Aminoacyl-tRNAs. To better mimic the in vivo conditions for peptide bond formation, in the other versions of SPARK, biotPmn was replaced with aminoacyl-tRNA, so that both P- and A-sites carry tRNA substrates. We designed two different formats of two-tRNA SPARK to be used with the 70S ribosome. In one version, SPARK-2Ta, formyl-[^3H] Met-tRNA was used as a donor while commercially available biotinylated Lys-tRNA carrying the biotin tag at the ϵ -amino group of lysine (Lys^{biot} -tRNA) was employed as an acceptor (Figure 1B). Synthetic RNA oligonucleotide AAGGAGAUUAACAAUGAAA was used

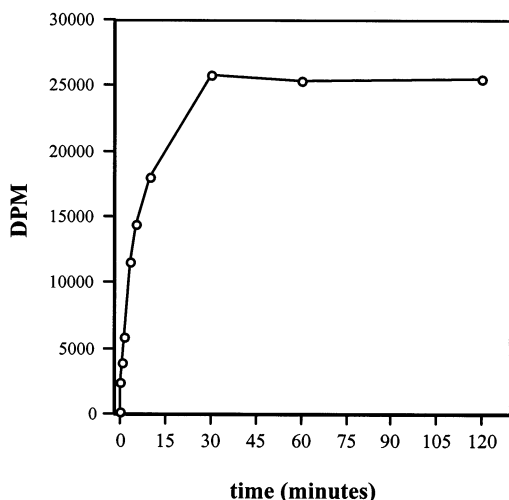


FIGURE 4: SPARK-2Tb reaction kinetics of poly(U)-programmed *E. coli* 70S using the polyamine buffer of Figure 3D at 6 mM MgOAc₂. Reactions contained 7 pmol of 70S ribosomes, 5.7 pmol of biotPhe-tRNA, and 5.2 pmol of [³H]-Phe-tRNA (6000 cpm/pmol) and were performed at 37 °C.

as mRNA. In the SA buffer, *S. aureus* ribosomes showed faster reaction rates in SPARK-2Ta than in SPARK-Pmn, which probably reflected the enhanced binding of Lys^{biot}-tRNA compared to biotPmn to the ribosomal A-site (data not shown).

In another version of the two-tRNA SPARK (SPARK-2Tb), unlabeled N-biotin-Phe-tRNA (biotPhe-tRNA) served as the peptidyl donor whereas a natural aminoacyl-tRNA, [³H]-Phe-tRNA, was an acceptor while poly(U) was used as mRNA (Figure 1C). The tritiated ligand and the biotin-tagged substrate in this version of SPARK were switched compared to SPARK-Pmn and SPARK-2Ta designs. When *E. coli* and *T. aquaticus* ribosomes were tested in SPARK-2Tb in SA buffer (see above), the reaction was slow and, similar to SPARK-Pmn, reached the plateau after about 4 h (data not shown). However, both *E. coli* and *T. aquaticus* ribosomes exhibited significantly faster reaction rates and product yields in the polyamine buffer system of Blaha et al. (26) at 6 mM Mg²⁺ (Figure 4) which is thought to closely mimic the in vivo ionic conditions. Under these conditions, the peptidyl transferase reaction proceeded essentially to completion since the entire [³H]-Phe-tRNA substrate was converted into the dipeptidyl-tRNA product after 30 min of incubation (5.0 pmol of dipeptidyl-tRNA formed when 5.7 pmol of donor and 5.2 mol of acceptor substrate were used). The turnover rate for the SPARK-2Tb with *E. coli* 70S was 0.23 min⁻¹ with an initial rate of 1.62 pmol/min.

The total (plateau) amount of biotPhe-[³H]Phe-tRNA product formed in SPARK-2Tb with 70S ribosomes from *T. aquaticus* under identical conditions was notably reduced (1.43 pmol) compared to *E. coli* ribosome. This indicates that the ribosome preparation contained inactive particles capable of irreversible binding of one of the substrates in a nonproductive ribosome-tRNA complex. However, the reaction catalyzed by the active ribosomes in the preparation was fairly fast, with an initial rate of 0.63 pmol/min, and reached the plateau after 30 min (data not shown).

SPARK-50S: Using SPARK To Monitor the Peptidyl Transferase Activity of Individual Large Ribosomal Subunits. The use of 70S ribosome might not always be optimal for

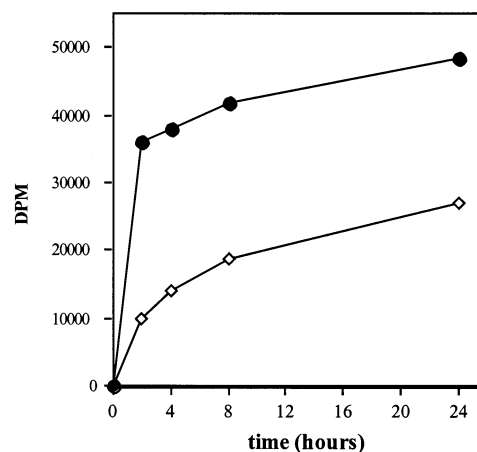


FIGURE 5: SPARK-50S time course experiment employing 14 pmol of 50S subunits from *E. coli* (closed symbols) or *T. aquaticus* (open symbols). Reactions were performed in the polyamine buffer described in Figure 3D. Reactions contained 11 pmol of biotPhe-tRNA and 12 pmol of [³H]-Phe-tRNA (6000 cpm/pmol) and were started by addition of 33% methanol and proceeded at 0 °C for the indicated periods of time.

studies that focus specifically on the activity of the peptidyl transferase center located on the large ribosomal subunit. Indeed, SPARK-Pmn, SPARK-2Ta, and SPARK-2Tb critically depend on the properties of the small ribosomal subunit and its interactions with mRNA and tRNA. To improve SPARK versatility for functional studies of the ribosomal peptidyl transferase center, we modified the two tRNA-SPARK (SPARK-2Tb version) for use with isolated large ribosomal subunits (SPARK-50S) (Figure 1D).

The peptidyl transferase assay conventionally used with 50S subunits ("fragment reaction") is performed in the presence of 33% methanol (28), which is thought to be required for binding of peptidyl-tRNA analogues to the P-site of 50S subunits (29, 30). Although binding of biotPhe-tRNA or its 3' fragment to the P-site of 50S subunits does not require methanol [(31) and our unpublished results], the activity of isolated 50S subunits in the SPARK-50S still strictly depended on the presence of methanol possibly due to stimulation of the binding of the acceptor substrate, [³H]-Phe-tRNA, to the A-site.

In SPARK-50S, the P-site of the peptidyl transferase center is filled by preincubation of the large ribosomal subunits (0.47 μM) with biotPhe-tRNA (0.37 μM) for 15 min at 37 °C in the polyamine buffer containing 30 mM Mg²⁺. The reaction is then initiated by the addition of [³H]-Phe-tRNA (0.4 μM) and methanol (33%) and incubated on ice. The reaction yields and the kinetics of biotPhe-[³H]Phe-tRNA product formation by *E. coli* or *T. aquaticus* 50S are shown in Figure 5 and were similar to those seen with poly(U)-programmed *E. coli* 70S ribosomes under similar incubation conditions (0 °C, polyamine buffer at 6 mM Mg²⁺; data not shown). Replacement of the polyamine buffer with the one usually used in the fragment reaction (28) (e.g., 20 mM Tris-HCl, pH 8.0, 30–60 mM MgCl₂, 400 mM KCl) did not affect notably the reaction yield or rate. Similar to SPARK-2Tb, 50S subunits prepared from *T. aquaticus* were less active than *E. coli* 50S subunits (Figure 5). Nevertheless, even such reduced activity was more than sufficient for most practical purposes.

Table 1: Effect of Antibiotics on the Formation of Biotin-Phe-[³H]Phe-tRNA in SPARK-50S and SPARK-2Tb^a

antibiotic ^b	50S subunits (% activity)	70S ribosomes (% activity)
none	100	100
chloramphenicol	50 ± 28	39 ± 30
sparsomycin	18 ± 1	49 ± 5
hygromycin A	32 ± 2	23 ± 1
carbomycin	44 ± 10	40 ± 18
erythromycin	101 ± 19	79 ± 8
tetracycline	113 ± 5	62 ± 7

^a SPARK-2Tb reactions were performed with poly(U)-programmed *E. coli* 70S at the same incubation temperature (0 °C) as the SPARK-50S. ^b Final concentration of the added drugs was 100 μM each. Values are an average of 2 or 3 independent experiments.

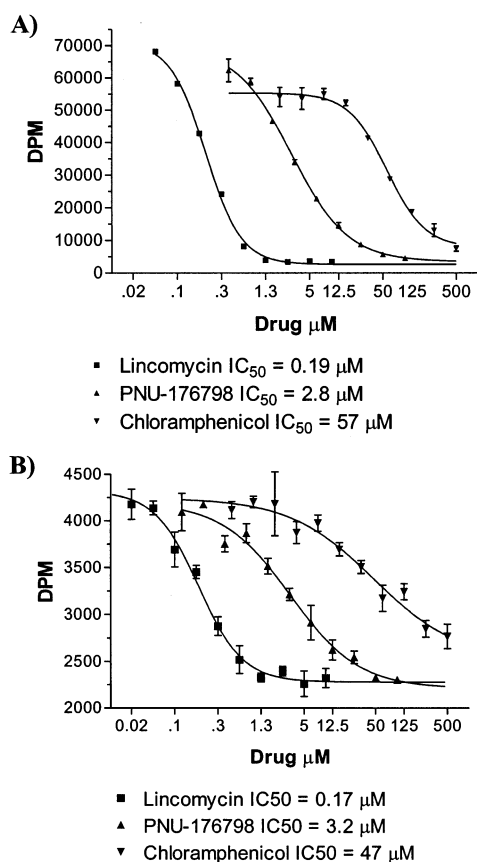


FIGURE 6: Antibiotic sensitivity of SPARK. SPARK-Pmn (A) and SPARK-2Ta (B) were performed with *S. aureus* 70S in the standard conditions using formyl-[³H] Met-tRNA (54 000 dpm/pmol) as a donor in the presence of varying concentrations of peptidyl transferase inhibitors. The measured IC₅₀ values for each drug, obtained from averaging at least three independent experiments, are indicated.

Sensitivity of SPARK to Antibiotics. Many antibiotics, inhibitors of protein synthesis, interfere with functions of the ribosomal peptidyl transferase (32, 33). As anticipated for the reaction catalyzed by the ribosomal peptidyl transferase center, all versions of SPARK were sensitive to antibiotics, inhibitors of peptide bond formation (Table 1, Figure 6 and our unpublished results). All the tested peptidyl transferase inhibitors (chloramphenicol, sparsomycin, hygromycin A, and carbomycin tested with *E. coli* ribosome, as well as lincomycin, PNU-176798, and chloramphenicol tested with *S. aureus* ribosomes) inhibited formation of the reaction product. Conversely, erythromycin, a drug which

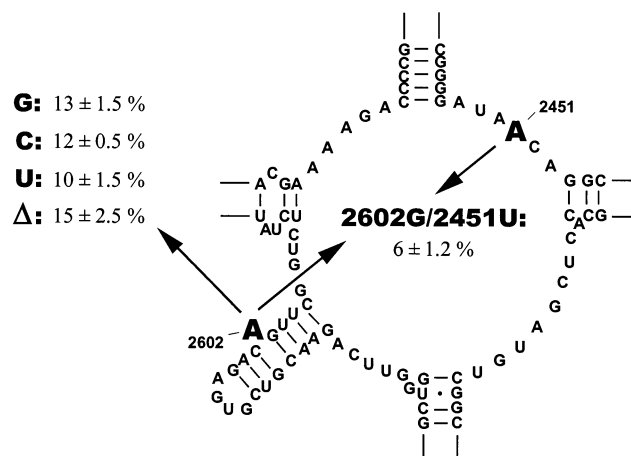


FIGURE 7: Effect of 23S rRNA mutations on the peptidyl transferase activity. The location and the nature of the engineered mutations at the two highly conserved adenines at positions 2602 and 2451 are shown on the secondary structure diagram of the central loop of domain V of *T. aquaticus* 23S rRNA (41). The relative activities in the SPARK-Pmn of reassociated *T. aquaticus* 70S ribosomes composed of in vitro reconstituted 50S subunits (harboring 23S rRNA base changes at 2602 and 2451) and native 30S subunits are indicated. The values shown are averages of 2–3 independent SPARK-Pmn experiments stopped after 240 min of incubation at 37 °C.

binds to the large ribosomal subunit in the vicinity of the peptidyl transferase center but does not directly inhibit transpeptidation (7, 34), had little or no effect. Tetracycline, a drug which interferes with the binding of tRNA to the A-site of the small subunit (8, 35–37), inhibited only the activity of 70S ribosomes in SPARK-2Tb, but had no effect on the isolated large ribosomal subunits in SPARK-50S (Table 1).

Using SPARK To Test the Effects of 23S rRNA Mutations on the Peptidyl Transferase Activity of in Vitro Reconstituted Large Ribosomal Subunits. High sensitivity and reproducibility of SPARK makes it a convenient method for studying functions of ribosomal peptidyl transferase. As a proof of principle, we used SPARK-Pmn to test effects of mutations at one of the important nucleotides in the ribosomal peptidyl transferase center on the catalytic activity of the ribosome.

In vitro transcribed *T. aquaticus* 23S rRNA can be reconstituted with 5S rRNA and the total protein fraction of the large ribosomal subunit (TP50) into functionally active 50S subunits (27). This technique allows the use of in vitro genetics for studying the functional consequences of mutations which may have lethal or deleterious in vivo phenotypes. Previously, we studied the transpeptidation activity of reconstituted 50S subunits harboring base changes at positions A2451 and G2447 (*E. coli* numbering) in the peptidyl transferase active site (17).

One of the intriguing nucleotides in the ribosomal peptidyl transferase center is A2602. This universally conserved adenine base bulges out from helix 93 of 23S rRNA (Figure 7) and is located in close proximity to the peptidyl-tRNA ester carbonyl carbon atom of the transition-state intermediate (13). To investigate if the base identity at position 2602 of 23S rRNA is critical for the transpeptidation reaction, all three base changes were introduced in the in vitro transcribed 23S rRNA, and the activities of the assembled subunits were tested in SPARK-Pmn. Neither of the A2602 mutations

eliminated peptidyl transferase activity of the in vitro reconstituted 50S subunits (G, $13 \pm 1.5\%$; C, $12 \pm 0.5\%$; U, $10 \pm 1.5\%$) (Figure 7). Notably, even the deletion of the universally conserved A2602 (the Δ mutant) or the double mutant 2602G/2451U, which combines in the same 23S rRNA molecule two different base changes that confer in vivo lethality, still showed considerable in vitro transpeptidation activity (Δ , $15 \pm 2.5\%$; 2602G/2451U, $6 \pm 1.2\%$).

DISCUSSION

In this report we present SPARK, a new, fast, and convenient method for measuring peptidyl transferase activity of ribosomes and isolated large ribosomal subunits. This can be used in various applications including studying protein synthesis, elucidating mechanisms of antibiotic action, and screening for new ribosome-targeted drugs. The central principle of the assay remains the same in all presented versions of SPARK: the ribosomal peptidyl transferase forms a peptide bond between two ligands closely resembling natural ribosomal substrates, one of which is tritiated and the other biotin-tagged. Transpeptidation results in the covalent attachment of the biotin moiety to a tritiated compound, and the amount of the reaction product can be directly quantified using commercially available SPA beads.

The big advantage of SPARK compared to the previously used method of measuring peptidyl transferase activity is its simplicity. Once the reaction has started, only one pipetting step (adding the SPA beads together with the Stop buffer) is required before the samples can be placed in a scintillation counter. No product purification is required. The combination of this simplicity with the possibility of performing SPARK in a small reaction volume, plus the availability of 96-well scintillation counters, makes SPARK amenable to simple automation for use in high through-put screens of natural or synthetic libraries in search for new antibiotics. Another important benefit of SPARK is its high sensitivity, with the reaction activity values being 2 orders of magnitude higher than the background. Using in vitro reconstituted 50S containing mutant 23S rRNA, transpeptidation activities as low as 0.5% could be measured compared to ribosomes assembled from wild-type 23S rRNA transcripts. This combination of high reproducibility and sensitivity makes SPARK instrumental for addressing important questions on the mechanism of peptide bond catalysis.

Of course each of the SPARK versions has its intrinsic limitations. At least one of the reaction substrates has to carry a biotin group which might affect the interaction of the ligand with the ribosome. The limited capacity of the SPA beads prevents the use of saturating amounts of the biotinylated substrate. Furthermore, since the SPA beads possess only ~35% counting efficiency compared to a regular liquid scintillant, substrates with relatively high specific activities have to be used. In addition, the reaction rates in the SPARK, though comparable or exceeding those obtained in the most "popular" fragment reaction, still do not reach the in vivo rates of peptide bond formation. Nevertheless, the ease of using SPARK as well as its high sensitivity and reproducibility makes it an excellent technique for various types of in vitro studies of the ribosome.

During the optimization of SPARK conditions, a general tendency was clear: the more the experimental setup

resembled the physiological situation in respect to the substrates or ionic conditions, the faster the reaction kinetics and the higher the product yields. Replacing puromycin used in SPARK-Pmn with a complete aminoacyl-tRNA as the acceptor substrate in SPARK-2Ta or SPARK-2Tb clearly improved the reaction performance. Thus, while a high Mg^{2+} concentration (30 mM) was required for efficient product formation in the puromycin-based SPARK-Pmn, a significantly lower concentration of Mg^{2+} (6 mM) was sufficient for the aminoacyl tRNA-based assay, SPARK-2Ta and SPARK-2Tb. The difference in Mg^{2+} dependence could reflect better A-site binding of aminoacyl-tRNAs compared to puromycin. The reaction kinetics were also notably faster with aminoacyl-tRNA as an acceptor substrate (compare Figures 3D and 4) compared to puromycin. Not only Mg^{2+} concentration but also the general buffer composition had a significant influence on the reaction. With *E. coli* and *T. aquaticus* ribosomes, the best rates and product yields were found in a polyamine buffer which is thought to closely mimic the in vivo conditions (26).

Different versions of SPARK can find applications in different types of ribosomal studies (Figure 1). A relatively inexpensive SPARK-Pmn, which utilizes commercially available ^{biot}Pmn, is suitable for large library screening projects. Somewhat more expensive, but also more physiologically feasible, two tRNA-SPARKs, SPARK-2Ta and SPARK-2Tb, can be used for studying interactions of tRNA substrates with their binding site in the peptidyl transferase center as well as for investigating the mechanism of catalysis. Finally, large ribosomal subunit-based SPARK-50S can be used to address more specific questions on the mechanism of peptide bond formation and antibiotic action.

Peptide bond synthesis in all versions of SPARK could be efficiently inhibited by peptidyl transferase-targeted antibiotics (Figure 6, Table 1, and our unpublished data). These data provided strong evidence that the formation of dipeptides in various versions of SPARK was indeed catalyzed by the peptidyl transferase center. Conversely, the drugs that bind to the ribosome but do not inhibit peptidyl transferase directly, erythromycin, for example, did not affect SPARK. Therefore, SPARK can be used for studying mechanisms of antibiotic action, as well as for searching for new antibiotics in libraries of synthetic or natural compounds. The possibility of using SPARK with isolated large 50S ribosomal subunits (SPARK-50S) makes such screens even better targeted because it enables the screening for drugs that directly act on the peptidyl transferase center of the large subunit while ignoring compounds that may affect mRNA or tRNA interactions with the small subunits. The latter compounds, which may also represent important leads for drug development, may be identified by comparing the results of the 50S subunit-based assay (SPARK-50S) with the result of the 70S ribosome-based tests (for example, SPARK-2Tb). This idea is demonstrated by our experiments with tetracycline, which efficiently inhibited SPARK-2Tb, but not SPARK-50S (Table 1). Indeed, tetracycline is known to bind to the 30S subunit and interfere with the binding of aminoacyl-tRNA in the A-site (8, 35–37).

We believe that SPARK can be useful for studies of the ribosomal peptidyl transferase. As an example, we used SPARK to study the effects of mutations of one of the central nucleotides in the ribosomal peptidyl transferase center. The

mechanism of transpeptidation, the main catalytic activity of the ribosome, remains enigmatic despite the availability of the high-resolution crystal structure of the large ribosomal subunit (1, 3). In one of the proposed mechanisms, the universally conserved nucleotide A2451 in domain V of the 23S rRNA serves as a general acid/base in catalysis of peptide bond formation (13). Further experiments showed, however, that mutations at position 2451 do not abolish transpeptidation activity (17, 19), indicating that the ribosome may promote peptide bond formation primarily through positional, rather than chemical, catalysis (17, 38).

The proposed models do not exclude alternative catalytic scenarios implicating other nucleotides located in close vicinity of the peptidyl transferase active site. One of the conspicuous nucleotides is A2602. Not only is this nucleotide located within the peptidyl transferase active site, but also its precise orientation is dramatically affected by the presence of peptidyl transferase substrates (13, 39). This suggests that A2602 may play an essential functional role. In vivo, the mutations of A2602 have a profound lethal effect (Lieberman and Dahlberg, personal communication).

We used a combination of in vitro genetics and SPARK to test how critical the base identity of A2602 is for the ability of the ribosome to promote transpeptidation. The in vitro transcribed *T. aquaticus* 23S rRNA, containing either the three possible nucleotide substitutions or the deletion of A2602, was assembled into the 50S subunits whose activity was analyzed using SPARK-Pmn. Though the activity of the mutant 50S subunits was diminished compared to the wild type, they all were readily capable of forming peptide bonds (Figure 7). Similar activities employing the fragment reaction were seen with the A2602C mutant in studies using the *B. stearothermophilus* reconstitution system (40). We further tested a double mutant, which combines the two in vivo lethal base changes, one at A2602 and another at A2451. Again, we found that this mutant exhibited significant peptidyl transferase activity in SPARK-Pmn. This result, as well as the previously studied combination of a 2451G mutation with mutations at position 2447 (17), is important for understanding the contribution of these nucleotides to chemical catalysis of peptide bond formation. Since transpeptidation is not the rate-limiting step in the overall reaction of protein synthesis, even significant reduction of the catalytic rate of transpeptidation can have limited impact on the total performance of the reaction. Thus, the "first" mutation (e.g., at positions 2602 or 2447) affecting any of the nucleotides possibly important for the catalysis may not have a decisive negative effect on the apparent reaction rate. However, since such mutant ribosomes exhibit somewhat reduced rates of the reaction, transpeptidation in the mutant has apparently become rate-limiting. Therefore, if chemical catalysis is critical for a dramatic acceleration of the peptidyl transfer, any second mutation that may affect the chemical component of catalysis (e.g., mutations at position 2451) should have a devastating effect on the rate of catalysis. The lack of such strong effects of the second mutation in the double mutants 2602/2451 and 2447/2451 is more compatible, in our view, with a relatively small acceleration of peptidyl transfer due to chemical catalysis by any of the investigated nucleotides. This reveals positional (entropic) catalysis as the main catalytic factor of the ribosomal peptidyl transferase as proposed earlier (17, 38).

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