Nascent peptide in the ribosome exit tunnel affects functional properties of the A-site of the peptidyl transferase center

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Summary

The ability to monitor the nascent peptide structure and to respond functionally to specific nascent peptide sequences is a fundamental property of the ribosome. An extreme manifestation of such response is nascent peptide-dependent ribosome stalling, involved in the regulation of gene expression. The molecular mechanisms of programmed translation arrest are unclear. By analyzing ribosome stalling at the regulatory cistron of the antibiotic resistance gene *ermA*, we uncovered a carefully orchestrated cooperation between the ribosomal exit tunnel and the A-site of the peptidyl transferase center (PTC) in halting translation. The presence of an inducing antibiotic and a specific nascent peptide in the exit tunnel abrogate the ability of the PTC to catalyze peptide bond formation with a particular subset of amino acids. The extent of the conferred A-site selectivity is modulated by the C-terminal segment of the nascent peptide, where the third from last residue plays a critical role.

Introduction

The key reaction catalyzed by the ribosomal peptidyl transferase center (PTC), peptide bond formation, entails the transfer of the growing polypeptide from peptidyl-tRNA bound in the ribosomal P-site to the amino acid residue of aminoacyl-tRNA residing in the A-site. The ribosome accelerates this reaction ca. 10^7-fold (Sievers et al., 2004) by juxtaposing the reaction substrates for an efficient nucleophilic attack of the α-amino group of the aminoacyl residue onto the carbon atom of the ester bond linking the nascent peptide to the tRNA (Beringer et al., 2005; Weinger et al., 2004). Amino acids delivered to the ribosomal A-site by aminoacyl-tRNAs differ significantly in their size, structure, and chemical properties. However, the catalytic center has evolved to allow for efficient transfer of the peptidyl moiety to any of the canonical amino acids placed in the A-site.

The growing amino acid chain leaves the ribosome through the nascent peptide exit tunnel (NPET) that starts at the PTC and spans the body of the large ribosomal subunit. Through the decades of studies of mechanisms of proteins synthesis, it has been largely assumed that the ribosome takes little notice of the nature of the polypeptide it makes. Only recently has it been realized that the ribosome can monitor the structure of the nascent peptide located in the exit tunnel (Tenson and Ehrenberg, 2002). One of the best-recognized manifestations of this intriguing property of the ribosome is nascent peptide–dependent translation arrest, which is at the heart of the regulatory mechanism that controls expression of several important bacterial and eukaryotic genes. In this mechanism, programmed ribosome stalling takes place at an upstream regulatory open reading frame (ORF) and leads to activation or repression of expression of the

downstream gene(s) (Fang et al., 2004; Gong and Yanofsky, 2002; Nakatogawa and Ito, 2002). Stalling ensues when the ribosome has polymerized a critical amino acid sequence encoded in the regulatory ORF. Specific interactions of the nascent peptide with sensory elements of the exit tunnel trigger structural rearrangements in the ribosome, which corrupt its functions and result in formation of a stalled ribosome complex (SRC) (Ito et al., 2010; Morris and Geballe, 2000; Ramu et al., 2009). The general principles of functional interactions between the ribosome and the nascent peptide are fundamental in their nature and may affect regulation of protein synthesis at different levels. However, molecular mechanisms that determine the ribosomal response to specific nascent peptide sequences are essentially unknown.

Clinically relevant *erm* genes in bacteria, which confer resistance to macrolide antibiotics, are regulated by drug- and nascent peptide–dependent ribosome stalling (Weisblum, 1995; Ramu et al., 2009). Macrolides, *e.g.*, erythromycin, bind in the ribosome exit tunnel in the vicinity of the PTC (Schlunzen et al., 2001; Tu et al., 2005). The *erm*-encoded enzyme methylates rRNA in the drug-binding site, reducing the affinity of the antibiotic to its target (Weisblum, 1995). In the best-studied example of an inducible *erm* gene, antibiotic-dependent ribosome stalling at the 9^{th} codon of the 19-codon regulatory ORF *ermCL* activates expression of the downstream resistance gene *ermC* (Gryczan et al., 1980; Horinouchi and Weisblum, 1980; Vazquez-Laslop et al., 2008). The sequence of the four C-terminal amino acids (IFVI) of the ErmCL nascent peptide, which is essential for SRC formation, appears to be recognized by specific rRNA residues located in the PTC-proximal segment of the NPET (Vazquez-Laslop et al., 2010; Vazquez-Laslop et al., 2008).

One of the key unanswered questions about nascent peptide–controlled translation arrest is why the ribosome stalls. In other words, what becomes "broken" in the ribosome in response to the presence of a specific nascent peptide in the exit tunnel? Several lines of evidence point to the importance of the PTC in programmed translation arrest (Gong and Yanofsky, 2002; Muto et al., 2006). For example, the inefficient release by puromycin of the peptides from SRC suggested that stalling results from the impaired activity of the PTC (Gong and Yanofsky, 2002; Muto et al., 2006; Vazquez-Laslop et al., 2008). Furthermore, spatial proximity of the NPET segments critical for stalling to the PTC makes the ribosome catalytic center the primary candidate for the target of the stalling response (Seidelt et al., 2009; Yap and Bernstein, 2009; Bhushan et al., 2010). However, the functions of the PTC, which are highly sensitive to even minute alterations in its structure, can be disrupted in many different ways (Polacek and Mankin, 2005). It remains unknown which aspect of the PTC function becomes corrupted in the SRC.

In this paper, we demonstrate that the presence of a drug and a specific nascent peptide in the ribosome exit tunnel dramatically alters the properties of the normally versatile PTC A-site to render it either selective or restrictive. The inability of the ribosome to efficiently incorporate specific amino acids in the growing polypeptide results in translation arrest. A conceptually similar mechanism may direct drug-independent ribosome stalling. These findings reveal the key role of the ribosomal A-site in SRC formation and clearly show that the functions of the PTC can be directly controlled by the nascent peptide located in the exit tunnel.

Results

ErmAL1 nascent peptide directs programmed translation arrest

To understand the molecular principles of nascent peptide–controlled translation arrest, we investigated SRC formation at the *ermAL1* ORF. The *ermA* methyltransferase gene is preceded by two leader ORFs, *ermAL1* and *ermAL2* (Murphy, 1985) (Figure 1A). The *ermA* transcript has the potential to fold into a secondary structure that sequesters the translation initiation region of *ermAL2* and *ermA* (Murphy, 1985; Sandler and Weisblum, 1988). By analogy with *ermC*, it was suggested that drug-dependent ribosome stalling at the *ermAL1* ORF triggers translation of *ermAL2* and that subsequent drug-dependent stalling at *ermAL2* allows for activation of *ermA* expression (Murphy, 1985; Sandler and Weisblum, 1988). The 19–amino acid ErmAL2 peptide is highly homologous to ErmCL, and the ribosome translating *ermAL2* stalls at the same 9th codon as in the previously investigated *ermCL* regulatory ORF (Vazquez-Laslop et al., 2008) (Supplementary Figure S1A). In contrast, the sequence of the 15–amino acid peptide encoded in *ermAL1* is substantially different from both ErmCL and ErmAL2 (Figure 1A), which made it an attractive model for gaining new insights into the molecular mechanisms of drug- and nascent peptide–dependent ribosome stalling.

We investigated ribosome stalling at the *ermAL1* ORF *in vitro* using primer extension inhibition analysis (Hartz et al., 1988). When the *ermAL1* mRNA is translated *in vitro* in the presence of erythromycin, a strong band appears on the primer extension gel whose position shows that the ribosome comes to a standstill when the 8th (Val) codon of *ermAL1* enters the ribosomal P-site (Figure 1B) (Vazquez-Laslop et al., 2010). To assess the role of the nascent peptide sequence in translation arrest, amino acid

residues 2-8 of ErmAL1 were mutated one at a time to alanine (except for position 6, which is an alanine in the wild-type *ermAL1* sequence and was mutated to a glycine). While changing codons 2-4 had no effect on SRC formation, mutations of codons Ile_5, Ala_6, Val_7, or Val_8 abolished ribosome stalling (Figure 1C). To verify that *ermAL1* mutations prevent translation arrest because of changes in the nascent peptide rather than in the mRNA structure, a total of 9 synonymous mutations were introduced simultaneously at *ermAL1* codons 2-7. Erythromycin-dependent stalling of the ribosome at the mutant mRNA was as prominent as with wild-type mRNA (Supplementary Figure S2B), arguing that it is the amino acid sequence of the ErmAL1 nascent peptide rather than the structure of its mRNA that guides SRC formation.

Stalled ribosome is unable to catalyze peptide bond formation

Translation arrest at the 8th codon of *ermAL1* is compatible with two scenarios. The ribosome may stall because peptide bond formation is impaired; in this case an octapeptide encoded in the first eight codons of *ermAL1* would be esterified to the P-site $tRNA^{Val}$ (Figure 2A). Alternatively, if translation is arrested after the next peptide bond is formed, then a 9–amino acid peptide would be esterified to the A-site $tRNA^{Glu}$, leaving a deacylated $tRNA^{Val}$ in the P-site. To distinguish between these possibilities, the nature of peptidyl-tRNA in the SRC was analyzed by Northern blotting. In the presence of erythromycin, $tRNA_1^{Val}$ decoding the 8th *ermAL1* codon GUA migrated more slowly in the gel, indicating its association with the nascent peptide (Figure 2A). In contrast, the mobility of $tRNA^{Glu}$ corresponding to the *ermAL1* 9th codon remained unchanged. This result shows that the ribosome arrested at the 8th codon of *ermAL1* is unable to catalyze

transfer of the 8–amino acid nascent peptide to the glutamyl moiety of Glu-tRNA^{Glu} decoding the A-site codon.

The nature of the A-site amino acid is critical for stalling

Previous studies of ribosome stalling at the *ermCL* regulatory ORF indicated that mutations of the codon located in the SRC A-site had little effect on stalling (Mayford and Weisblum, 1989; Vazquez-Laslop et al., 2008). In striking contrast to those results, replacement of the Glu_9 codon of *ermAL1* with an Ala (GCA) codon dramatically reduced the efficiency of SRC formation (Figure 1C). This indicates that the identity of the A-site codon and thus the nature of the aminoacyl-tRNA in the A-site are critical for drug- and nascent peptide–dependent translation arrest at *ermAL1*. We further verified this important conclusion by replacing the wild-type Glu_9 codon with codons specifying each of the other 18 conventional amino acids. Only a subset of the tested codons was found to be conducive to SRC formation (Figure 2B). Stalling was especially prominent with codons corresponding to charged amino acids (Glu, Asp, Lys, Arg, His). Codons specifying certain uncharged amino acids (Trp, Ile, Tyr) also strongly promoted translation arrest. In contrast, we found that in addition to the Ala codon, SRC formation was significantly reduced or even completely abolished when Phe, Met, or Cys codons replaced the Glu_9 codon of *ermAL1* (Figure 2B). This unexpected observation that SRC formation at the *ermAL1* ORF critically depends on the nature of the aminoacyl-tRNA specified by the A-site codon emphasized the importance of the ribosomal A-site in the mechanism of drug- and nascent peptide–controlled translation arrest.

Mutations at the A-site codon result in binding of aminoacyl-tRNAs that differ from the wild-type Glu-tRNA^{Glu} both in the structure of the tRNA body and the nature of the acceptor amino acid substrate placed in the PTC. To discern which of these two features is central to the ribosome stalling response, we compared effects of pairs of tRNA isoacceptors differing in the structure of tRNA but delivering the same amino acid. The 9th codon of *ermAL1* was replaced with pairs of synonymous codons decoded by glutamine, serine, leucine, and arginine tRNA isoacceptors (Figure 2C). Primer extension inhibition analysis showed that with each pair of synonymous codons, both isoacceptor aminoacyl-tRNAs were either equally conducive to SRC formation (pairs of tRNA^{Leu} or tRNA^{Arg}) or were similarly inefficient in promoting stalling (tRNA^{Gln} or tRNA^{Ser} pairs). This observation led us to conclude that the structure of tRNA itself had little influence upon translation arrest, which left the amino acid residue delivered to the PTC A-site as the primary determinant for discrimination.

The ribosome stalls because certain A-site amino acids serve as poor acceptors of the ErmAL1 nascent peptide

The Northern blot analysis (Figure 2A) showed that the ribosome stalled at the 8th codon of *ermAL1* is unable to transfer peptide from peptidyl-tRNA^{Val} to the A-site Glu-tRNA^{Glu}, while the A-site codon mutations revealed that only a subset of aminoacyl-tRNAs are conducive to stalling (Figure 2B). We therefore hypothesized that in the stalled ribosome, some ("stalling") amino acids serve as particularly poor acceptors in the peptidyl transfer reaction, whereas other ("non-stalling") amino acids are still able to function as fairly efficient substrates in the reaction of peptide bond formation. To

directly test this hypothesis, we analyzed transfer of the ErmAL1 N-terminal octapeptide to model A-site substrates CCA-N-Lys or CCA-N-Ala in which the aminoacyl-tRNA 3' end analog CCA is linked via a stable amide bond to a stalling (Lys) or nonstalling (Ala) amino acid. Importantly, because binding of these substrates is codon independent, this experiment directly focuses on the role of the A-site amino acid in the formation of the stalled translation complex.

The *ermAL1* mRNA, truncated after the 8th codon, was translated *in vitro* in the presence of [^{35}S]-methionine and erythromycin. The SRC carrying radiolabeled peptidyl-tRNA was isolated by sucrose gradient centrifugation and allowed to react with an excess (1 mM) of CCA-N-Lys or CCA-N-Ala (Figure 3A). We monitored progression of the reaction by quantifying the amount of unreacted peptidyl-tRNA resolved on a Tricine-SDS polyacrylamide gel (the reaction products CCA-N-nonapeptides were too small to be resolved well enough for direct quantitation). The ErmAL1 nascent peptide in the SRC showed a strikingly different reactivity to the tested aminoacyl-tRNA analogs. The peptide was virtually unreactive with the substrate that contained the stalling amino acid (CCA-N-Lys) as could be judged from the essentially unchanged intensity of the peptidyl-tRNA band even after 30 min of incubation at 37°C. In contrast, the amount of SRC-associated peptidyl-tRNA rapidly decreased on incubation with CCA-N-Ala (Figure 3A), indicating that the ribosome could fairly efficiently catalyze transfer of the ErmAL1 nascent peptide to a non-stalling amino acid. While the tested aminoacyl-tRNA analogs showed a remarkably different reactivity with the peptidyl-tRNA in the SRC, both of them could be readily used as acceptors in the uninhibited reaction of peptide bond formation. When 70S initiation complex carrying fMet-tRNA in the P-site was reacted

with these substrates, transfer of formyl-methionine to either CCA-N-Ala or CCA-N-Lys occurred very quickly: the band of fMet-tRNA completely disappeared after only 30 sec of incubation—the shortest time point we could reliably acquire in our experimental setup (Supplementary Figure 3). Thus, the results of the experiments with the model A-site substrates strongly supported our assertion that the presence of ErmAL1 nascent peptide and erythromycin in the NPET alters properties of the PTC A-site in such a way that peptide bond formation in SRC becomes particularly slow with certain amino acids.

We independently verified this conclusion by analyzing how the nature of the 9th amino acid in the ErmAL1 peptide (the A-site amino acid in the SRC) affects the frequency at which the ribosome can bypass the *ermAL1* stalling site. In the presence of erythromycin, only a small fraction of the translating ribosomes could continue translation beyond the stalling site in the wild-type *ermAL1*: minute amounts of the full-length polypeptide were synthesized and a large amount of peptidyl-tRNA (likely corresponding to peptidyl-tRNA^{Val} in the SRC) accumulated (Figure 3B). When the *ermAL1* 9th (Glu) codon was replaced with a codon of the "non-stalling" amino acid Phe, more than twice the amount of full-length polypeptide was produced with a concomitant decrease in accumulated peptidyl-tRNA. This observation was compatible with the notion that nascent peptide in the SRC could be transferred more efficiently to a non-stalling amino acid as compared with the wild-type (stalling) amino acid.

Altogether, these results illuminated an unexpected selectivity of the PTC A-site in the stalled ribosome, imposed by the presence of an antibiotic and a specific nascent peptide in the NPET. The versatile A-site, which efficiently operates with all types of natural aminoacyl-tRNAs in the "normal" ribosome, becomes highly selective to the

nature of the acceptor substrate in the SRC. As a result, the PTC is unable to catalyze peptide bond formation with a range of natural amino acids.

The properties of the PTC A-site depend on the nascent peptide sequence

The attributes of SRCs formed at the *ermCL* and *ermAL1* regulatory ORFs are substantially different. While the nature of the A-site amino acid dramatically affects the efficiency of stalling at the *ermAL1* ORF (Figure 2B), ribosome stalling at the *ermCL* ORF is much less sensitive to the identity of the codon in the A-site of the stalled ribosome (Mayford and Weisblum, 1989; Vazquez-Laslop et al., 2008) (Supplementary Figure 4). The ribosome that has polymerized the MGIFSIFVI sequence of the ErmCL peptide stalls irrespective of whether the A-site codon is Ser (wild type) or is mutated to Glu (Figure 4). In contrast, the ribosome that has polymerized the eight N-terminal amino acids of the ErmAL1 peptide (MCTSIAVV) stalls when the 9th (A-site) codon is Glu (wild type) but would not stall if it is mutated to Ser (Figure 2B and 4). Because in both cases ribosome stalling is controlled by the same drug (erythromycin) but different nascent peptides, it is most reasonable to think that the PTC A-site properties depend on the structure of the nascent peptide in the NPET. We then asked, which of the critical amino acid residues of the stalling peptide in the tunnel define the properties of the A-site in the PTC?

Although the ErmCL (MGIFSIFVI) and ErmAL1 (MCTSIAVV) stalling nascent peptides are substantially different, the four C-terminal amino acids (IFVI in ErmCL and IAVV in ErmAL1) in both cases are critical for stalling (Figure 1C and Vazquez-Laslop et al., 2008). When we transplanted the C-terminal sequence of the ErmAL1 stalling

peptide to the ErmCL peptide rendering the hybrid sequence MGIFSIAVV, the stalled complex that formed at the 9th codon of the hybrid ORF acquired A-site selectivity characteristic of the ribosome stalled at *ermAL1*: SRC formed with Glu in the A-site but not with Ser (Figure 4). Hence, determinants of the A-site properties reside within the four C-terminal amino acids of the stalling peptide. ErmCL wild type (MGIFSIFVI), insensitive to the A-site codon, and the hybrid peptide (MGIFSIAVV), which shows a clear A-site codon selectivity, differ at only two residues: the 9th amino acid (Ile/Val) that in the SRC esterifies the P-site tRNA and the 7th amino acid (Phe/Ala), at position -2 relative to the nascent peptide C-terminus. Mutation of Ile_9 to Val in ErmCL had little effect on A-site selectivity. However, when Phe_7 of ErmCL was mutated to Ala, the SRC became sensitive to the nature of the A-site codon. If the same residue (Phe_7) was mutated to Gly, stalling was abolished altogether, irrespective of whether the 9th codon was Ser or Glu. Thus, within the context of the ErmCL nascent peptide, the identity of a single amino acid located in the NPET two residues away from the PTC defines the catalytic properties of the PTC active site.

Discussion

Comparison of SRCs that form at regulatory ORFs *ermAL1* and *ermCL* unmasked an important role of the PTC A-site in the mechanism of nascent peptide–controlled translation arrest. Our findings lead to a simple model that accommodates the known facts regarding drug-dependent translation arrest (Figure 5A-C). Depending on the structure of the peptide in the NPET, the A-site can be in different states. During normal translation, the PTC A-site is in the versatile state when it can properly accommodate any of the natural amino acids delivered by aminoacyl-tRNA (Figure 5A). Peptide bond formation with any of the incoming amino acids is efficient. In the presence of an inducing antibiotic and a specific nascent peptide (*e.g.*, ErmAL1), the A-site becomes selective (Figure 5B). Peptide bond formation with certain amino acids (red) becomes very slow; the corresponding A-site codons are conducive to SRC formation. Some peptide sequences (*e.g.*, ErmCL) can impair the A-site even further, rendering it highly restrictive so that almost no amino acids can be comfortably accommodated (Figure 5C); essentially no natural amino acids can be efficiently used as peptide acceptor and the SRC is formed irrespective of the A-site codon. According to this view, the operational state of the PTC A-site can be directly influenced by the nascent peptide in the NPET and the properties of the A-site can be progressively altered depending on the nascent peptide sequence.

This model is not limited to drug-dependent translation arrest but can also account for key results of previous studies of drug-independent nascent peptide–controlled ribosome stalling. SRC formation at the natural or genetically modified *secM* regulatory ORF requires the presence of a proline codon in the A-site of the stalled ribosome (Muto

et al., 2006; Yap and Bernstein, 2009). Similarly, ribosome stalling at the sequences selected from a randomized peptide library requires the presence of a proline codon in the SRC A-site (Tanner et al., 2009). In these cases, the nascent peptides are likely to infringe on the A-site just enough to prevent use of the most structurally constrained amino acid, proline. In another extensively studied example of nascent peptide–dependent translation arrest, the ribosome stalls at the end of the *tnaC* regulatory cistron when either a stop codon or codons specifying Trp, Arg, Lys, or Ile are present in the A-site; however, stalling is diminished with certain other A-site codons (Cruz-Vera et al., 2009; Gong and Yanofsky, 2002). In this case, the A-site appears to be more restrictive because it excludes a broader range of amino acids. Noteworthy is that the A-site codons that promote stalling at *tnaC* match the best-stalling codons we identified with *ermAL1*, suggesting that they are generally the easiest to discriminate against.

Except for proline, which is known to be a fairly inefficient nascent peptide acceptor because of its constrained structure and alkylation of the α-amino group (Pavlov et al., 2009), the exact trend that distinguishes amino acids conducive to SRC formation at *ermAL1* is unclear. Although the nature of the amino acid is known to influence aminoacyl-tRNA binding to the ribosome, indicating that each amino acid interacts with the PTC in a unique way (Fahlman and Uhlenbeck, 2004), little structural information is available about the specifics of placement of different amino acids in the A-site. Only binding of Phe-tRNA or puromycin derivatives in the PTC A-site has been examined so far by high-resolution crystallographic analysis (Bashan et al., 2003; Nissen et al., 2000; Voorhees et al., 2009). In the analyzed complexes, the aromatic side chains of phenylalanine or puromycin are drawn into the hydrophobic crevice formed by the 23S

rRNA residues A2451 and C2452 (Nissen et al., 2000; Voorhees et al., 2009). However, precise molecular contacts of the amino acid side chains may vary depending on their chemical nature, even though the placement of the α-amino group that participates in the nucleophilic attack that drives the reaction of peptide bond formation should remain invariant. Thus, it is conceivable that even small alterations in the orientation of 23S nucleotides that constitute the PTC A-site may have a dramatic effect on the accurate placement of specific amino acids and their activity as peptidyl acceptors.

Our results show that the identity of the amino acid residue at position -2 relative to the C-terminus of ErmAL1 and ErmCL nascent peptides is a key element that influences the properties of the A-site. When this position in the ErmCL nascent peptide in the tunnel is occupied by phenylalanine, the A-site becomes restrictive (the ribosome stalls with either Glu or Ser A-site codons). Replacement of Phe with Ala renders the A-site selective (stalling with Glu but not Ser A-site codons). Finally, when Phe is replaced with Gly, the A-site of the ribosome translating the *ermCL* ORF remains versatile (no stalling). Noteworthy is that the residue at the nascent peptide position -2 apparently also controls ribosome stalling at the *secM* ORF (Yap and Bernstein, 2009), reinforcing our previous notion that the mechanism of drug-dependent ribosome stalling at *ermCL* and *ermAL1* shares many similarities with drug-independent translation arrest at the *secM* ORF (Vazquez-Laslop et al., 2010).

How is the information about the presence of a stalling nascent peptide sequence and, more specifically, about the nature of the amino acid residue at position -2 communicated to the PTC A-site? In our previous work, we identified several important components of drug-dependent SRC formation. From the ribosome side, the identity of

the 23S rRNA residues A2062 and A2503 located in the NPET were found to be critical for programmed translation arrest at *ermCL* and *ermAL1* (Vazquez-Laslop et al., 2010; Vazquez-Laslop et al., 2008). From the side of an inducing macrolide antibiotic bound in the NPET, the presence of C3-cladinose is essential for stalling (Vazquez-Laslop et al., 2008). If we are to assume that the 9–amino acid ErmCL nascent peptide can thread through the opening of the tunnel constricted by the bound antibiotic, then its Phe_7 (occupying position -2 of the nascent peptide) would be located only a short distance (2-4 Å) from both the cladinose sugar of the inducing antibiotic and A2062 of the 23S rRNA (Figure 5B). Thus, as we proposed earlier (Vazquez-Laslop et al., 2010), the presence of cladinose-containing antibiotic ensures interaction of the nascent peptide (probably specifically the amino acid at position -2) with the highly flexible base of A2062, which serves as the peptide sensor. Reorientation of A2062 can alter the pose of A2503. Displacement of A2062 and A2503 can allosterically, via their immediate neighbors G2061 and U2504, affect the opening of the A2451/C2452 A-site crevice. This signal relay pathway is supported by rigid theory analysis of the statics of the tunnel (Fulle and Gohlke, 2009) and SRC structural studies (Seidelt et al., 2009). Noteworthy, the extent of the A-site impairment in the SRC formed at the *ermCL* ORF conspicuously correlates with the size of the amino acid in position -2 of the nascent peptide: the bulkiest Phe renders the A-site restrictive, the intermediate-sized Ala renders the A-site selective, and the smallest Gly leaves the A-site versatile. In terms of our model, a larger residue at position -2 of the nascent peptide would cause a stronger displacement of the tunnel sensors resulting in a more pronounced A-site distortion. The size of amino acid in position -2 of the peptide is likely not the only characteristic that defines its role in

stalling. Hydrophobicity, charge, and other chemical properties may influence its interactions with the NPET sensors.

The A-site impairment is likely only one component of the stalling mechanism, even though it is critical. The identity of the P-site amino acid also has a direct effect on SRC formation (Gong and Yanofsky, 2002; Muto et al., 2006; Tanner et al., 2009; Vazquez-Laslop et al., 2008). It is generally possible that the selectivity of the A-site in SRC revealed by our data is induced via improper placement of peptidyl-tRNA in the ribosomal P-site, which can be affected by specific interactions of the nascent peptide with the tunnel walls and the presence of additional ligands in the tunnel or PTC. Furthermore, different stalling peptides may affect the A-site properties via different relay pathways (Seidelt et al., 2009; Vazquez-Laslop et al., 2010). Nevertheless, the importance of the nature of the A-site codon for ribosome stalling at a variety of regulatory ORFs (Ramu and Mankin, unpublished data) shows that nascent peptide-induced selectivity of the PTC A-site is a common theme in the mechanism of programmed translation arrest.

Experimental Procedures

Primer extension inhibition assay.

Linear DNA templates for *in vitro* translation were generated by PCR as described (Vazquez-Laslop et al., 2010). Primers are listed in Supplementary Table 1. DNA templates were expressed in a cell-free transcription-translation system (Shimizu et al., 2001) (PURExpress kit, New England BioLabs), and primer extension inhibition analysis was performed as described previously (Vazquez-Laslop et al., 2008).

Identification of peptidyl-tRNA in the *ermAL1* SRC by Northern hybridization.

The *ermAL1* ORF DNA equipped with the T7 promoter was chemically synthesized (BioBasics, Inc.) and introduced into BamHI-ApaI sites of the pUC57 vector to generate plasmid pErmAL. The plasmid was used to direct cell-free transcription-translation reactions (Promega T7 S30 system for circular DNA). 10 μL reactions, assembled according to the manufacturer's protocol and supplemented with 0.8 μg of plasmid, were incubated at 37°C for 30 min and then on ice for 10 min. When needed, the reactions were supplemented with erythromycin (final concentration: 50 μM). SRC was isolated by filtering the reactions through a Microcon YM-100 column (molecular weight cutoff of 100 kDa). The retained material was diluted to 200 μL with 0.3 M sodium acetate, pH 4, and total RNA was extracted with acidic phenol/chloroform followed by precipitation with ethanol. RNA-associated material was subjected to denaturing gel electrophoresis under acidic conditions (Varshney et al., 1991), transferred to a Hybond N+ nylon membrane (GE Healthcare), and probed with radiolabeled DNA oligos complementary to $tRNA^{Val}$ (UAC) or $tRNA^{Glu}$ (UUC) (Table S1 in Supplemental Information). Following hybridization, membranes were air-dried and exposed to phosphorimager screens.

Isolation of $ermAL_8$-SRC and in vitro peptidyl transfer reaction.

Commercially synthesized (Thermo Fisher) transcript encoding *ermAL* truncated at the 8th codon (ATAAGGAGGAAAAAATATGTGCACCAGTATCGCAGTAGTA) was used to direct the S30 cell-free translation reaction (Promega *E.coli* S30 Extract System for Linear Templates). The 50 μL reaction contained 8 μM transcript, 50 μM

erythromycin, 0.4-μCi/μL ^{35}S-Met, and 2.4 U/μL RiboLock RNase Inhibitor (Fermentas) and was carried out for 30 min at 37°C. An equal volume of gradient buffer (20 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 10 mM NH_4Cl, and 2 mM ß-mercaptoethanol) containing 50 μM erythromycin was added to the sample prior to loading onto a 5%-30% sucrose gradient prepared in the same buffer supplemented with 50 μM erythromycin. Gradients were centrifuged in a SW41 rotor at 39,000 rpm for 3 hr at 4°C. Fractions containing 70S complex were pooled, concentrated (Vivaspin100, Sartorious), and stored at -20°C.

Sucrose gradient–purified SRCs (0.6 μM) were combined with CCA-N-Lys or CCA-N-Ala (1 mM) in Pure System Buffer (Shimizu et al., 2001) containing 50 μM erythromycin in a final volume of 22 μL. Reactions were incubated at 37°C and aliquots were removed at 1, 2.5, 5, 15, and 30 min. Reactions were stopped by precipitation with cold acetone, and products were analyzed in a 16% Bis-Tris polyacrylamide gel as described in http://openwetware.org/wiki/Sauer:bis-Tris_SDS-PAGE,_the_very_best based on US patent 6,162,338.

Analysis of translation products of *ermAL* wild-type and mutant ORFs.

To enable visualization of the ErmAL leader peptide by gel electrophoresis, the *ermAL1* ORF in permAL was extended to 72 codons by introducing a frameshift mutation (QuikChange II Site-Directed mutagenesis kit, Stratagene) at codon 12 of *ermAL* using primers ermAL-shift1 and ermAL-shift2 (Table S1) and generating a plasmid ermAL-FS. Subsequently, the ermAL1 9th codon (GAA) encoding glutamic acid was changed to TTC (phenylalanine) using primer permAL.FS-E9F-F, producing plasmid *permAL-FS-E9F*.

Extended *ermAL1* genes were expressed in a cell-free transcription-translation system (Promega T7 S30 system for circular DNA). Reactions (6.4 μL) contained 0.5 μg of plasmid DNA and 0.75 μCi of [^{35}S] methionine (1175 Ci/mmol; when needed, they were supplemented with erythromycin (50μM final concentrations). The reactions were incubated at 37°C for 15 min. Following precipitation with acetone, translation products were fractionated by Tricine-SDS gel electrophoresis (Schagger and von Jagow, 1987).

Additional information is provided in Supplemental Experimental Procedures.

Acknowledgements

We thank Holger Moroder and Jessica Steger for synthesis of some aminoacyl-tRNA analogs, John Zaborske and Tao Pan for advice with experiments and Shannon Sparenga for proofreading the manuscript. This work was supported by grant MCB-0824739 from the National Science Foundation (to ASM and NVL). QD was supported by the SPARK Award from the Chicago Biomedical Consortium.

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Figure legends

Figure 1.

(A) Schematic map of the regulatory region of the *ermA* gene. The amino acid sequences encoded in the regulatory ORFs *ermAL1* and *ermAL2* are shown. The sequence of the peptide encoded in the *ermCL* regulatory ORF is presented for comparison. (B) Primer extension inhibition analysis of the site of ribosome stalling at *ermAL1*. The *ermAL1* ORF was translated *in vitro* (Shimizu et al., 2001) in the absence (-) or presence (+) of erythromycin (Ery). A primer was annealed to the 3' end of mRNA and extended with reverse transcriptase (Vazquez-Laslop et al., 2010; Vazquez-Laslop et al., 2008). The same primer was used to generate sequencing lanes (U, C). The sequence of the *ermAL1* gene and the encoded amino acids are shown to the left of the gel. Reverse transcriptase stops are indicated by arrowheads. The codon located in the P-site of the stalled ribosome is boxed. (C) The effect of mutations of codons 2-10 of *ermAL1* on ribosome stalling. The amino acid changes associated with the codon mutations are indicated in the cartoon and over the corresponding lanes of the gel. The primer extension bands representing the ribosome stalled at the 8th *ermAL1* codon are shown by arrowheads. The lanes representing mutations at codons located in the P- and A-sites of the stalled ribosomes are boxed. In the cartoon, the star represents erythromycin bound in the tunnel.

Figure 2.

The role of the A-site codon in SRC formation. (A) The A-site amino acid is not incorporated in the nascent peptide. The cartoon shows possible versions of peptidyl-tRNA in SRC containing the 8th codon of *ermAL1* in the P-site. The A-site amino acid

(Glu) is shown by a filled circle and the erythromycin molecule bound in the exit tunnel is represented by a star. The gel represents Northern blot analysis of tRNA associated with the stalled ribosome. Positions of aminoacyl-tRNAs and peptidyl-tRNA are indicated. (B) Effects of mutations in the 9th codon of *ermAL1* on ribosome stalling. The control (no erythromycin) lane is shown only for the wild-type *ermAL1* sequence. The bar diagram represents the results of quantitation of the intensity of the "stalled ribosome" bands (an average of three independent experiments). (C) Testing the effects of A-site codons decoded by different tRNA isoacceptors on ribosome stalling. Note that a single nucleotide shift in the position of the band observed with tRNA^{Arg} isoacceptors apparently reflects change in the ribosome geometry in response to binding of different tRNAs which affects the precise site where reverse transcriptase stops. This effect was also seen when binding of different tRNAs was directed to the A-site (see gel in panel B).

Figure 3.

Differential acceptor activity of stalling and nonstalling amino acids in the reaction of peptide bond formation in the SRC. (A) The ribosome stalled at the end of the truncated *ermAL1* mRNA was allowed to react for a specified time at 37°C with 1 mM CCA-N-Ala or CCA-N-Lys, and the remaining unreacted peptidyl-tRNA was resolved by gel electrophoresis. The first two lanes in the gel show samples incubated for 0 or 30 min in the absence of aminoacyl-tRNA analogs. The graph below the gel represents the results of quantitation of the amount of radioactivity in the peptidyl-tRNA bands. (B) Translation, in the presence of erythromycin, of an extended *ermAL1* ORF containing a mutation of the wild-type 9th codon (Glu) to the nonstalling Phe. A frameshift mutation

downstream from the stalling site extends the ORF to 72 codons. The codons located in the P- and A-sites of the SRC are boxed. The position of gel bands representing a 72–amino acid full-size translation product and peptidyl-tRNA esterified by an 8–amino acid nascent peptide are marked by filled and contoured arrowheads, respectively. Four-fold less material was loaded onto the no-erythromycin lanes compared with the erythromycin lanes. The bar diagram represents the results of quantitation of the amount of radioactivity in the gel bands in the samples containing erythromycin.

Figure 4.

Effects of the nascent peptide sequence on the properties of the PTC A-site. The gels show the primer extension inhibition signal (bold arrowheads) representing SRC formation at different ORFs (erythromycin was present in all the samples). Bands corresponding to translation initiation sites at the *erm* ORFs are shown for reference and are indicated by thin arrows. The amino acid sequences corresponding to the ErmCL peptide are red, and those representing the ErmAL1 peptide are blue. The Gly mutation at position 7 of ErmCL is shown in green, and the A-site amino acid is black. Amino acids located in the A-site of the PTC in the stalled ribosome are boxed with solid lines. The amino acid position -2 relative to the nascent peptide C-terminus is boxed with a dashed line.

Figure 5.

Nascent peptide controls properties of the PTC A-site. (A) During normal translation, the PTC A-site (orange) is in the versatile state. (B) In the presence of an inducing antibiotic (ery) and a specific nascent peptide (*e.g.*, ErmAL1), the A-site becomes selective. Peptide bond formation with certain amino acids (red) becomes very slow; the corresponding A-site codons are conducive to SRC formation. (C) Certain peptide sequences (*e.g.*, ErmCL) can render the A-site even more restrictive; the SRC is formed irrespective of the A-site codon. The ErmAL1 and ErmCL sequences essential for stalling are shown in cyan; the amino acid residue in position -2, which apparently controls the A-site properties, is blue. (D) A possible signal relay pathway communicating the information from the ribosomal exit tunnel to the PTC A-site. The 8-amino acid ErmAL1 nascent peptide, attached to the P-site tRNA, was modeled in the structure of *Thermus thermophilus* 70S ribosome (PDB accession number 2WDL (Voorhees et al., 2009)). C-terminal amino acids critical for stalling are colored in cyan; the residue at position -2 is shown in blue. The 23S rRNA residues A2451 and C2452 forming the A-site crevice are orange. Erythromycin shape (ery) is sown as violet mesh with the cladinose residue highlighted in red. Mutations of residues A2062 and A2503 (purple) prevent staling. Neighboring residues, G2061 and U2504 (pale blue) may participate in communicating the stalling signal to the A-site crevice. The conformational flexibility of the rRNA residues putatively involved in relaying the stalling signal from the exit tunnel to the PTC is illustrated by their varying placement in different ribosomal complexes (Schmeing et al., 2005; Gurel et al., 2009; Schuwirth et al., 2005; Jenner et al., 2005; Petry et al., 2005).

Highlights (MC-D-10-01262)

In the presence of erythromycin, the ribosome stalls at the 8th codon of *ermAL1*

Stalled ribosome is unable to catalyze peptide bond formation with the 9th amino acid

The nature of the A-site codon is critical for stalling

Nascent peptide sequence renders the A-site selective to the nature of aminoacyl-tRNA

Graphical Abstract

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Nascent peptide in the ribosome exit tunnel affects functional properties of the A-site of the peptidyl transferase center

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Summary

The ability to monitor the nascent peptide structure and to respond functionally to specific nascent peptide sequences is a fundamental property of the ribosome. An extreme manifestation of such response is nascent peptide-dependent ribosome stalling, involved in the regulation of gene expression. The molecular mechanisms of programmed translation arrest are unclear. By analyzing ribosome stalling at the regulatory cistron of the antibiotic resistance gene *ermA*, we uncovered a carefully orchestrated cooperation between the ribosomal exit tunnel and the A-site of the peptidyl transferase center (PTC) in halting translation. The presence of an inducing antibiotic and a specific nascent peptide in the exit tunnel abrogate the ability of the PTC to catalyze peptide bond formation with a particular subset of amino acids. The extent of the conferred A-site selectivity is modulated by the C-terminal segment of the nascent peptide, where the third from last residue plays a critical role.

Introduction

The key reaction catalyzed by the ribosomal peptidyl transferase center (PTC), peptide bond formation, entails the transfer of the growing polypeptide from peptidyl-tRNA bound in the ribosomal P-site to the amino acid residue of aminoacyl-tRNA residing in the A-site. The ribosome accelerates this reaction ca. 10^7-fold (Sievers et al., 2004) by juxtaposing the reaction substrates for an efficient nucleophilic attack of the α-amino group of the aminoacyl residue onto the carbon atom of the ester bond linking the nascent peptide to the tRNA (Beringer et al., 2005; Weinger et al., 2004). Amino acids delivered to the ribosomal A-site by aminoacyl-tRNAs differ significantly in their size, structure, and chemical properties. However, the catalytic center has evolved to allow for efficient transfer of the peptidyl moiety to any of the canonical amino acids placed in the A-site.

The growing amino acid chain leaves the ribosome through the nascent peptide exit tunnel (NPET) that starts at the PTC and spans the body of the large ribosomal subunit. Through the decades of studies of mechanisms of proteins synthesis, it has been largely assumed that the ribosome takes little notice of the nature of the polypeptide it makes. Only recently has it been realized that the ribosome can monitor the structure of the nascent peptide located in the exit tunnel (Tenson and Ehrenberg, 2002). One of the best-recognized manifestations of this intriguing property of the ribosome is nascent peptide–dependent translation arrest, which is at the heart of the regulatory mechanism that controls expression of several important bacterial and eukaryotic genes. In this mechanism, programmed ribosome stalling takes place at an upstream regulatory open reading frame (ORF) and leads to activation or repression of expression of the

downstream gene(s) (Fang et al., 2004; Gong and Yanofsky, 2002; Nakatogawa and Ito, 2002). Stalling ensues when the ribosome has polymerized a critical amino acid sequence encoded in the regulatory ORF. Specific interactions of the nascent peptide with sensory elements of the exit tunnel trigger structural rearrangements in the ribosome, which corrupt its functions and result in formation of a stalled ribosome complex (SRC) (Ito et al., 2010; Morris and Geballe, 2000; Ramu et al., 2009). The general principles of functional interactions between the ribosome and the nascent peptide are fundamental in their nature and may affect regulation of protein synthesis at different levels. However, molecular mechanisms that determine the ribosomal response to specific nascent peptide sequences are essentially unknown.

Clinically relevant *erm* genes in bacteria, which confer resistance to macrolide antibiotics, are regulated by drug- and nascent peptide–dependent ribosome stalling (Weisblum, 1995; Ramu et al., 2009). Macrolides, *e.g.*, erythromycin, bind in the ribosome exit tunnel in the vicinity of the PTC (Schlunzen et al., 2001; Tu et al., 2005). The *erm*-encoded enzyme methylates rRNA in the drug-binding site, reducing the affinity of the antibiotic to its target (Weisblum, 1995). In the best-studied example of an inducible *erm* gene, antibiotic-dependent ribosome stalling at the 9^{th} codon of the 19-codon regulatory ORF *ermCL* activates expression of the downstream resistance gene *ermC* (Gryczan et al., 1980; Horinouchi and Weisblum, 1980; Vazquez-Laslop et al., 2008). The sequence of the four C-terminal amino acids (IFVI) of the ErmCL nascent peptide, which is essential for SRC formation, appears to be recognized by specific rRNA residues located in the PTC-proximal segment of the NPET (Vazquez-Laslop et al., 2010; Vazquez-Laslop et al., 2008).

One of the key unanswered questions about nascent peptide–controlled translation arrest is why the ribosome stalls. In other words, what becomes "broken" in the ribosome in response to the presence of a specific nascent peptide in the exit tunnel? Several lines of evidence point to the importance of the PTC in programmed translation arrest (Gong and Yanofsky, 2002; Muto et al., 2006). For example, the inefficient release by puromycin of the peptides from SRC suggested that stalling results from the impaired activity of the PTC (Gong and Yanofsky, 2002; Muto et al., 2006; Vazquez-Laslop et al., 2008). Furthermore, spatial proximity of the NPET segments critical for stalling to the PTC makes the ribosome catalytic center the primary candidate for the target of the stalling response (Seidelt et al., 2009; Yap and Bernstein, 2009; Bhushan et al., 2010). However, the functions of the PTC, which are highly sensitive to even minute alterations in its structure, can be disrupted in many different ways (Polacek and Mankin, 2005). It remains unknown which aspect of the PTC function becomes corrupted in the SRC.

In this paper, we demonstrate that the presence of a drug and a specific nascent peptide in the ribosome exit tunnel dramatically alters the properties of the normally versatile PTC A-site to render it either selective or restrictive. The inability of the ribosome to efficiently incorporate specific amino acids in the growing polypeptide results in translation arrest. A conceptually similar mechanism may direct drug-independent ribosome stalling. These findings reveal the key role of the ribosomal A-site in SRC formation and clearly show that the functions of the PTC can be directly controlled by the nascent peptide located in the exit tunnel.

Results

ErmAL1 nascent peptide directs programmed translation arrest

To understand the molecular principles of nascent peptide–controlled translation arrest, we investigated SRC formation at the *ermAL1* ORF. The *ermA* methyltransferase gene is preceded by two leader ORFs, *ermAL1* and *ermAL2* (Murphy, 1985) (Figure 1A). The *ermA* transcript has the potential to fold into a secondary structure that sequesters the translation initiation region of *ermAL2* and *ermA* (Murphy, 1985; Sandler and Weisblum, 1988). By analogy with *ermC*, it was suggested that drug-dependent ribosome stalling at the *ermAL1* ORF triggers translation of *ermAL2* and that subsequent drug-dependent stalling at *ermAL2* allows for activation of *ermA* expression (Murphy, 1985; Sandler and Weisblum, 1988). The 19–amino acid ErmAL2 peptide is highly homologous to ErmCL, and the ribosome translating *ermAL2* stalls at the same 9th codon as in the previously investigated *ermCL* regulatory ORF (Vazquez-Laslop et al., 2008) (Supplementary Figure S1A). In contrast, the sequence of the 15–amino acid peptide encoded in *ermAL1* is substantially different from both ErmCL and ErmAL2 (Figure 1A), which made it an attractive model for gaining new insights into the molecular mechanisms of drug- and nascent peptide–dependent ribosome stalling.

We investigated ribosome stalling at the *ermAL1* ORF *in vitro* using primer extension inhibition analysis (Hartz et al., 1988). When the *ermAL1* mRNA is translated *in vitro* in the presence of erythromycin, a strong band appears on the primer extension gel whose position shows that the ribosome comes to a standstill when the 8th (Val) codon of *ermAL1* enters the ribosomal P-site (Figure 1B) (Vazquez-Laslop et al., 2010). To assess the role of the nascent peptide sequence in translation arrest, amino acid

residues 2-8 of ErmAL1 were mutated one at a time to alanine (except for position 6, which is an alanine in the wild-type *ermAL1* sequence and was mutated to a glycine). While changing codons 2-4 had no effect on SRC formation, mutations of codons Ile_5, Ala_6, Val_7, or Val_8 abolished ribosome stalling (Figure 1C). To verify that *ermAL1* mutations prevent translation arrest because of changes in the nascent peptide rather than in the mRNA structure, a total of 9 synonymous mutations were introduced simultaneously at *ermAL1* codons 2-7. Erythromycin-dependent stalling of the ribosome at the mutant mRNA was as prominent as with wild-type mRNA (Supplementary Figure S2B), arguing that it is the amino acid sequence of the ErmAL1 nascent peptide rather than the structure of its mRNA that guides SRC formation.

Stalled ribosome is unable to catalyze peptide bond formation

Translation arrest at the 8th codon of *ermAL1* is compatible with two scenarios. The ribosome may stall because peptide bond formation is impaired; in this case an octapeptide encoded in the first eight codons of *ermAL1* would be esterified to the P-site $tRNA^{Val}$ (Figure 2A). Alternatively, if translation is arrested after the next peptide bond is formed, then a 9–amino acid peptide would be esterified to the A-site $tRNA^{Glu}$, leaving a deacylated $tRNA^{Val}$ in the P-site. To distinguish between these possibilities, the nature of peptidyl-tRNA in the SRC was analyzed by Northern blotting. In the presence of erythromycin, $tRNA_1^{Val}$ decoding the 8th *ermAL1* codon GUA migrated more slowly in the gel, indicating its association with the nascent peptide (Figure 2A). In contrast, the mobility of $tRNA^{Glu}$ corresponding to the *ermAL1* 9th codon remained unchanged. This result shows that the ribosome arrested at the 8th codon of *ermAL1* is unable to catalyze

transfer of the 8–amino acid nascent peptide to the glutamyl moiety of Glu-tRNA^{Glu} decoding the A-site codon.

The nature of the A-site amino acid is critical for stalling

Previous studies of ribosome stalling at the *ermCL* regulatory ORF indicated that mutations of the codon located in the SRC A-site had little effect on stalling (Mayford and Weisblum, 1989; Vazquez-Laslop et al., 2008). In striking contrast to those results, replacement of the Glu_9 codon of *ermAL1* with an Ala (GCA) codon dramatically reduced the efficiency of SRC formation (Figure 1C). This indicates that the identity of the A-site codon and thus the nature of the aminoacyl-tRNA in the A-site are critical for drug- and nascent peptide–dependent translation arrest at *ermAL1*. We further verified this important conclusion by replacing the wild-type Glu_9 codon with codons specifying each of the other 18 conventional amino acids. Only a subset of the tested codons was found to be conducive to SRC formation (Figure 2B). Stalling was especially prominent with codons corresponding to charged amino acids (Glu, Asp, Lys, Arg, His). Codons specifying certain uncharged amino acids (Trp, Ile, Tyr) also strongly promoted translation arrest. In contrast, we found that in addition to the Ala codon, SRC formation was significantly reduced or even completely abolished when Phe, Met, or Cys codons replaced the Glu_9 codon of *ermAL1* (Figure 2B). This unexpected observation that SRC formation at the *ermAL1* ORF critically depends on the nature of the aminoacyl-tRNA specified by the A-site codon emphasized the importance of the ribosomal A-site in the mechanism of drug- and nascent peptide–controlled translation arrest.

Mutations at the A-site codon result in binding of aminoacyl-tRNAs that differ from the wild-type Glu-tRNA^{Glu} both in the structure of the tRNA body and the nature of the acceptor amino acid substrate placed in the PTC. To discern which of these two features is central to the ribosome stalling response, we compared effects of pairs of tRNA isoacceptors differing in the structure of tRNA but delivering the same amino acid. The 9th codon of *ermAL1* was replaced with pairs of synonymous codons decoded by glutamine, serine, leucine, and arginine tRNA isoacceptors (Figure 2C). Primer extension inhibition analysis showed that with each pair of synonymous codons, both isoacceptor aminoacyl-tRNAs were either equally conducive to SRC formation (pairs of tRNA^{Leu} or tRNA^{Arg}) or were similarly inefficient in promoting stalling (tRNA^{Gln} or tRNA^{Ser} pairs). This observation led us to conclude that the structure of tRNA itself had little influence upon translation arrest, which left the amino acid residue delivered to the PTC A-site as the primary determinant for discrimination.

The ribosome stalls because certain A-site amino acids serve as poor acceptors of the ErmAL1 nascent peptide

The Northern blot analysis (Figure 2A) showed that the ribosome stalled at the 8th codon of *ermAL1* is unable to transfer peptide from peptidyl-tRNA^{Val} to the A-site Glu-tRNA^{Glu}, while the A-site codon mutations revealed that only a subset of aminoacyl-tRNAs are conducive to stalling (Figure 2B). We therefore hypothesized that in the stalled ribosome, some ("stalling") amino acids serve as particularly poor acceptors in the peptidyl transfer reaction, whereas other ("non-stalling") amino acids are still able to function as fairly efficient substrates in the reaction of peptide bond formation. To

directly test this hypothesis, we analyzed transfer of the ErmAL1 N-terminal octapeptide to model A-site substrates CCA-N-Lys or CCA-N-Ala in which the aminoacyl-tRNA 3' end analog CCA is linked via a stable amide bond to a stalling (Lys) or nonstalling (Ala) amino acid. Importantly, because binding of these substrates is codon independent, this experiment directly focuses on the role of the A-site amino acid in the formation of the stalled translation complex.

The *ermAL1* mRNA, truncated after the 8th codon, was translated *in vitro* in the presence of [^{35}S]-methionine and erythromycin. The SRC carrying radiolabeled peptidyl-tRNA was isolated by sucrose gradient centrifugation and allowed to react with an excess (1 mM) of CCA-N-Lys or CCA-N-Ala (Figure 3A). We monitored progression of the reaction by quantifying the amount of unreacted peptidyl-tRNA resolved on a Tricine-SDS polyacrylamide gel (the reaction products CCA-N-nonapeptides were too small to be resolved well enough for direct quantitation). The ErmAL1 nascent peptide in the SRC showed a strikingly different reactivity to the tested aminoacyl-tRNA analogs. The peptide was virtually unreactive with the substrate that contained the stalling amino acid (CCA-N-Lys) as could be judged from the essentially unchanged intensity of the peptidyl-tRNA band even after 30 min of incubation at 37°C. In contrast, the amount of SRC-associated peptidyl-tRNA rapidly decreased on incubation with CCA-N-Ala (Figure 3A), indicating that the ribosome could fairly efficiently catalyze transfer of the ErmAL1 nascent peptide to a non-stalling amino acid. While the tested aminoacyl-tRNA analogs showed a remarkably different reactivity with the peptidyl-tRNA in the SRC, both of them could be readily used as acceptors in the uninhibited reaction of peptide bond formation. When 70S initiation complex carrying fMet-tRNA in the P-site was reacted

with these substrates, transfer of formyl-methionine to either CCA-N-Ala or CCA-N-Lys occurred very quickly: the band of fMet-tRNA completely disappeared after only 30 sec of incubation—the shortest time point we could reliably acquire in our experimental setup (Supplementary Figure 3). Thus, the results of the experiments with the model A-site substrates strongly supported our assertion that the presence of ErmAL1 nascent peptide and erythromycin in the NPET alters properties of the PTC A-site in such a way that peptide bond formation in SRC becomes particularly slow with certain amino acids.

We independently verified this conclusion by analyzing how the nature of the 9^{th} amino acid in the ErmAL1 peptide (the A-site amino acid in the SRC) affects the frequency at which the ribosome can bypass the *ermAL1* stalling site. In the presence of erythromycin, only a small fraction of the translating ribosomes could continue translation beyond the stalling site in the wild-type *ermAL1*: minute amounts of the full-length polypeptide were synthesized and a large amount of peptidyl-tRNA (likely corresponding to peptidyl-$tRNA^{Val}$ in the SRC) accumulated (Figure 3B). When the *ermAL1* 9^{th} (Glu) codon was replaced with a codon of the "non-stalling" amino acid Phe, more than twice the amount of full-length polypeptide was produced with a concomitant decrease in accumulated peptidyl-tRNA. This observation was compatible with the notion that nascent peptide in the SRC could be transferred more efficiently to a non-stalling amino acid as compared with the wild-type (stalling) amino acid.

Altogether, these results illuminated an unexpected selectivity of the PTC A-site in the stalled ribosome, imposed by the presence of an antibiotic and a specific nascent peptide in the NPET. The versatile A-site, which efficiently operates with all types of natural aminoacyl-tRNAs in the "normal" ribosome, becomes highly selective to the

nature of the acceptor substrate in the SRC. As a result, the PTC is unable to catalyze peptide bond formation with a range of natural amino acids.

The properties of the PTC A-site depend on the nascent peptide sequence

The attributes of SRCs formed at the *ermCL* and *ermAL1* regulatory ORFs are substantially different. While the nature of the A-site amino acid dramatically affects the efficiency of stalling at the *ermAL1* ORF (Figure 2B), ribosome stalling at the *ermCL* ORF is much less sensitive to the identity of the codon in the A-site of the stalled ribosome (Mayford and Weisblum, 1989; Vazquez-Laslop et al., 2008) (Supplementary Figure 4). The ribosome that has polymerized the MGIFSIFVI sequence of the ErmCL peptide stalls irrespective of whether the A-site codon is Ser (wild type) or is mutated to Glu (Figure 4). In contrast, the ribosome that has polymerized the eight N-terminal amino acids of the ErmAL1 peptide (MCTSIAVV) stalls when the 9th (A-site) codon is Glu (wild type) but would not stall if it is mutated to Ser (Figure 2B and 4). Because in both cases ribosome stalling is controlled by the same drug (erythromycin) but different nascent peptides, it is most reasonable to think that the PTC A-site properties depend on the structure of the nascent peptide in the NPET. We then asked, which of the critical amino acid residues of the stalling peptide in the tunnel define the properties of the A-site in the PTC?

Although the ErmCL (MGIFSIFVI) and ErmAL1 (MCTSIAVV) stalling nascent peptides are substantially different, the four C-terminal amino acids (IFVI in ErmCL and IAVV in ErmAL1) in both cases are critical for stalling (Figure 1C and Vazquez-Laslop et al., 2008). When we transplanted the C-terminal sequence of the ErmAL1 stalling

peptide to the ErmCL peptide rendering the hybrid sequence MGIFSIAVV, the stalled complex that formed at the 9th codon of the hybrid ORF acquired A-site selectivity characteristic of the ribosome stalled at *ermAL1*: SRC formed with Glu in the A-site but not with Ser (Figure 4). Hence, determinants of the A-site properties reside within the four C-terminal amino acids of the stalling peptide. ErmCL wild type (MGIFSIFVI), insensitive to the A-site codon, and the hybrid peptide (MGIFSIAVV), which shows a clear A-site codon selectivity, differ at only two residues: the 9th amino acid (Ile/Val) that in the SRC esterifies the P-site tRNA and the 7th amino acid (Phe/Ala), at position -2 relative to the nascent peptide C-terminus. Mutation of Ile_9 to Val in ErmCL had little effect on A-site selectivity. However, when Phe_7 of ErmCL was mutated to Ala, the SRC became sensitive to the nature of the A-site codon. If the same residue (Phe_7) was mutated to Gly, stalling was abolished altogether, irrespective of whether the 9th codon was Ser or Glu. Thus, within the context of the ErmCL nascent peptide, the identity of a single amino acid located in the NPET two residues away from the PTC defines the catalytic properties of the PTC active site.

Discussion

Comparison of SRCs that form at regulatory ORFs *ermAL1* and *ermCL* unmasked an important role of the PTC A-site in the mechanism of nascent peptide–controlled translation arrest. Our findings lead to a simple model that accommodates the known facts regarding drug-dependent translation arrest (Figure 5A-C). Depending on the structure of the peptide in the NPET, the A-site can be in different states. During normal translation, the PTC A-site is in the versatile state when it can properly accommodate any of the natural amino acids delivered by aminoacyl-tRNA (Figure 5A). Peptide bond formation with any of the incoming amino acids is efficient. In the presence of an inducing antibiotic and a specific nascent peptide (*e.g.*, ErmAL1), the A-site becomes selective (Figure 5B). Peptide bond formation with certain amino acids (red) becomes very slow; the corresponding A-site codons are conducive to SRC formation. Some peptide sequences (*e.g.*, ErmCL) can impair the A-site even further, rendering it highly restrictive so that almost no amino acids can be comfortably accommodated (Figure 5C); essentially no natural amino acids can be efficiently used as peptide acceptor and the SRC is formed irrespective of the A-site codon. According to this view, the operational state of the PTC A-site can be directly influenced by the nascent peptide in the NPET and the properties of the A-site can be progressively altered depending on the nascent peptide sequence.

This model is not limited to drug-dependent translation arrest but can also account for key results of previous studies of drug-independent nascent peptide–controlled ribosome stalling. SRC formation at the natural or genetically modified *secM* regulatory ORF requires the presence of a proline codon in the A-site of the stalled ribosome (Muto

et al., 2006; Yap and Bernstein, 2009). Similarly, ribosome stalling at the sequences selected from a randomized peptide library requires the presence of a proline codon in the SRC A-site (Tanner et al., 2009). In these cases, the nascent peptides are likely to infringe on the A-site just enough to prevent use of the most structurally constrained amino acid, proline. In another extensively studied example of nascent peptide–dependent translation arrest, the ribosome stalls at the end of the *tnaC* regulatory cistron when either a stop codon or codons specifying Trp, Arg, Lys, or Ile are present in the A-site; however, stalling is diminished with certain other A-site codons (Cruz-Vera et al., 2009; Gong and Yanofsky, 2002). In this case, the A-site appears to be more restrictive because it excludes a broader range of amino acids. Noteworthy is that the A-site codons that promote stalling at *tnaC* match the best-stalling codons we identified with *ermAL1*, suggesting that they are generally the easiest to discriminate against.

Except for proline, which is known to be a fairly inefficient nascent peptide acceptor because of its constrained structure and alkylation of the α-amino group (Pavlov et al., 2009), the exact trend that distinguishes amino acids conducive to SRC formation at *ermAL1* is unclear. Although the nature of the amino acid is known to influence aminoacyl-tRNA binding to the ribosome, indicating that each amino acid interacts with the PTC in a unique way (Fahlman and Uhlenbeck, 2004), little structural information is available about the specifics of placement of different amino acids in the A-site. Only binding of Phe-tRNA or puromycin derivatives in the PTC A-site has been examined so far by high-resolution crystallographic analysis (Bashan et al., 2003; Nissen et al., 2000; Voorhees et al., 2009). In the analyzed complexes, the aromatic side chains of phenylalanine or puromycin are drawn into the hydrophobic crevice formed by the 23S

rRNA residues A2451 and C2452 (Nissen et al., 2000; Voorhees et al., 2009). However, precise molecular contacts of the amino acid side chains may vary depending on their chemical nature, even though the placement of the α-amino group that participates in the nucleophilic attack that drives the reaction of peptide bond formation should remain invariant. Thus, it is conceivable that even small alterations in the orientation of 23S nucleotides that constitute the PTC A-site may have a dramatic effect on the accurate placement of specific amino acids and their activity as peptidyl acceptors.

Our results show that the identity of the amino acid residue at position -2 relative to the C-terminus of ErmAL1 and ErmCL nascent peptides is a key element that influences the properties of the A-site. When this position in the ErmCL nascent peptide in the tunnel is occupied by phenylalanine, the A-site becomes restrictive (the ribosome stalls with either Glu or Ser A-site codons). Replacement of Phe with Ala renders the A-site selective (stalling with Glu but not Ser A-site codons). Finally, when Phe is replaced with Gly, the A-site of the ribosome translating the *ermCL* ORF remains versatile (no stalling). Noteworthy is that the residue at the nascent peptide position -2 apparently also controls ribosome stalling at the *secM* ORF (Yap and Bernstein, 2009), reinforcing our previous notion that the mechanism of drug-dependent ribosome stalling at *ermCL* and *ermAL1* shares many similarities with drug-independent translation arrest at the *secM* ORF (Vazquez-Laslop et al., 2010).

How is the information about the presence of a stalling nascent peptide sequence and, more specifically, about the nature of the amino acid residue at position -2 communicated to the PTC A-site? In our previous work, we identified several important components of drug-dependent SRC formation. From the ribosome side, the identity of

the 23S rRNA residues A2062 and A2503 located in the NPET were found to be critical for programmed translation arrest at *ermCL* and *ermAL1* (Vazquez-Laslop et al., 2010; Vazquez-Laslop et al., 2008). From the side of an inducing macrolide antibiotic bound in the NPET, the presence of C3-cladinose is essential for stalling (Vazquez-Laslop et al., 2008). If we are to assume that the 9–amino acid ErmCL nascent peptide can thread through the opening of the tunnel constricted by the bound antibiotic, then its Phe_7 (occupying position -2 of the nascent peptide) would be located only a short distance (2-4 Å) from both the cladinose sugar of the inducing antibiotic and A2062 of the 23S rRNA (Figure 5B). Thus, as we proposed earlier (Vazquez-Laslop et al., 2010), the presence of cladinose-containing antibiotic ensures interaction of the nascent peptide (probably specifically the amino acid at position -2) with the highly flexible base of A2062, which serves as the peptide sensor. Reorientation of A2062 can alter the pose of A2503. Displacement of A2062 and A2503 can allosterically, via their immediate neighbors G2061 and U2504, affect the opening of the A2451/C2452 A-site crevice. This signal relay pathway is supported by rigid theory analysis of the statics of the tunnel (Fulle and Gohlke, 2009) and SRC structural studies (Seidelt et al., 2009). Noteworthy, the extent of the A-site impairment in the SRC formed at the *ermCL* ORF conspicuously correlates with the size of the amino acid in position -2 of the nascent peptide: the bulkiest Phe renders the A-site restrictive, the intermediate-sized Ala renders the A-site selective, and the smallest Gly leaves the A-site versatile. In terms of our model, a larger residue at position -2 of the nascent peptide would cause a stronger displacement of the tunnel sensors resulting in a more pronounced A-site distortion. The size of amino acid in position -2 of the peptide is likely not the only characteristic that defines its role in

stalling. Hydrophobicity, charge, and other chemical properties may influence its interactions with the NPET sensors.

The A-site impairment is likely only one component of the stalling mechanism, even though it is critical. The identity of the P-site amino acid also has a direct effect on SRC formation (Gong and Yanofsky, 2002; Muto et al., 2006; Tanner et al., 2009; Vazquez-Laslop et al., 2008). It is generally possible that the selectivity of the A-site in SRC revealed by our data is induced via improper placement of peptidyl-tRNA in the ribosomal P-site, which can be affected by specific interactions of the nascent peptide with the tunnel walls and the presence of additional ligands in the tunnel or PTC. Furthermore, different stalling peptides may affect the A-site properties via different relay pathways (Seidelt et al., 2009; Vazquez-Laslop et al., 2010). Nevertheless, the importance of the nature of the A-site codon for ribosome stalling at a variety of regulatory ORFs (Ramu and Mankin, unpublished data) shows that nascent peptide-induced selectivity of the PTC A-site is a common theme in the mechanism of programmed translation arrest.

Experimental Procedures

Primer extension inhibition assay.

Linear DNA templates for *in vitro* translation were generated by PCR as described (Vazquez-Laslop et al., 2010). Primers are listed in Supplementary Table 1. DNA templates were expressed in a cell-free transcription-translation system (Shimizu et al., 2001) (PURExpress kit, New England BioLabs), and primer extension inhibition analysis was performed as described previously (Vazquez-Laslop et al., 2008).

Identification of peptidyl-tRNA in the *ermAL1* SRC by Northern hybridization.

The *ermAL1* ORF DNA equipped with the T7 promoter was chemically synthesized (BioBasics, Inc.) and introduced into BamHI-ApaI sites of the pUC57 vector to generate plasmid pErmAL. The plasmid was used to direct cell-free transcription-translation reactions (Promega T7 S30 system for circular DNA). 10 μL reactions, assembled according to the manufacturer's protocol and supplemented with 0.8 μg of plasmid, were incubated at 37°C for 30 min and then on ice for 10 min. When needed, the reactions were supplemented with erythromycin (final concentration: 50 μM). SRC was isolated by filtering the reactions through a Microcon YM-100 column (molecular weight cutoff of 100 kDa). The retained material was diluted to 200 μL with 0.3 M sodium acetate, pH 4, and total RNA was extracted with acidic phenol/chloroform followed by precipitation with ethanol. RNA-associated material was subjected to denaturing gel electrophoresis under acidic conditions (Varshney et al., 1991), transferred to a Hybond N+ nylon membrane (GE Healthcare), and probed with radiolabeled DNA oligos complementary to $tRNA^{Val}$ (UAC) or $tRNA^{Glu}$ (UUC) (Table S1 in Supplemental Information). Following hybridization, membranes were air-dried and exposed to phosphorimager screens.

Isolation of $ermAL_8$-SRC and in vitro peptidyl transfer reaction.

Commercially synthesized (Thermo Fisher) transcript encoding *ermAL* truncated at the 8th codon (ATAAGGAGGAAAAAATATGTGCACCAGTATCGCAGTAGTA) was used to direct the S30 cell-free translation reaction (Promega *E.coli* S30 Extract System for Linear Templates). The 50 μL reaction contained 8 μM transcript, 50 μM

erythromycin, 0.4-μCi/μL ^{35}S-Met, and 2.4 U/μL RiboLock RNase Inhibitor (Fermentas) and was carried out for 30 min at 37°C. An equal volume of gradient buffer (20 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 10 mM NH_4Cl, and 2 mM ß-mercaptoethanol) containing 50 μM erythromycin was added to the sample prior to loading onto a 5%-30% sucrose gradient prepared in the same buffer supplemented with 50 μM erythromycin. Gradients were centrifuged in a SW41 rotor at 39,000 rpm for 3 hr at 4°C. Fractions containing 70S complex were pooled, concentrated (Vivaspin100, Sartorious), and stored at -20°C.

Sucrose gradient–purified SRCs (0.6 μM) were combined with CCA-N-Lys or CCA-N-Ala (1 mM) in Pure System Buffer (Shimizu et al., 2001) containing 50 μM erythromycin in a final volume of 22 μL. Reactions were incubated at 37°C and aliquots were removed at 1, 2.5, 5, 15, and 30 min. Reactions were stopped by precipitation with cold acetone, and products were analyzed in a 16% Bis-Tris polyacrylamide gel as described in http://openwetware.org/wiki/Sauer:bis-Tris_SDS-PAGE,_the_very_best based on US patent 6,162,338.

Analysis of translation products of *ermAL* wild-type and mutant ORFs.

To enable visualization of the ErmAL leader peptide by gel electrophoresis, the *ermAL1* ORF in permAL was extended to 72 codons by introducing a frameshift mutation (QuikChange II Site-Directed mutagenesis kit, Stratagene) at codon 12 of *ermAL* using primers ermAL-shift1 and ermAL-shift2 (Table S1) and generating a plasmid ermAL-FS. Subsequently, the ermAL1 9th codon (GAA) encoding glutamic acid was changed to TTC (phenylalanine) using primer permAL.FS-E9F-F, producing plasmid *permAL-FS-E9F*.

Extended *ermAL1* genes were expressed in a cell-free transcription-translation system (Promega T7 S30 system for circular DNA). Reactions (6.4 μL) contained 0.5 μg of plasmid DNA and 0.75 μCi of [^{35}S] methionine (1175 Ci/mmol; when needed, they were supplemented with erythromycin (50μM final concentrations). The reactions were incubated at 37°C for 15 min. Following precipitation with acetone, translation products were fractionated by Tricine-SDS gel electrophoresis (Schagger and von Jagow, 1987).

Additional information is provided in Supplemental Experimental Procedures.

Acknowledgements

We thank Holger Moroder and Jessica Steger for synthesis of some aminoacyl-tRNA analogs, John Zaborske and Tao Pan for advice with experiments and Shannon Sparenga for proofreading the manuscript. This work was supported by grant MCB-0824739 from the National Science Foundation (to ASM and NVL). QD was supported by the SPARK Award from the Chicago Biomedical Consortium.

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Figure legends

Figure 1.

(A) Schematic map of the regulatory region of the *ermA* gene. The amino acid sequences encoded in the regulatory ORFs *ermAL1* and *ermAL2* are shown. The sequence of the peptide encoded in the *ermCL* regulatory ORF is presented for comparison. (B) Primer extension inhibition analysis of the site of ribosome stalling at *ermAL1*. The *ermAL1* ORF was translated *in vitro* (Shimizu et al., 2001) in the absence (-) or presence (+) of erythromycin (Ery). A primer was annealed to the 3' end of mRNA and extended with reverse transcriptase (Vazquez-Laslop et al., 2010; Vazquez-Laslop et al., 2008). The same primer was used to generate sequencing lanes (U, C). The sequence of the *ermAL1* gene and the encoded amino acids are shown to the left of the gel. Reverse transcriptase stops are indicated by arrowheads. The codon located in the P-site of the stalled ribosome is boxed. (C) The effect of mutations of codons 2-10 of *ermAL1* on ribosome stalling. The amino acid changes associated with the codon mutations are indicated in the cartoon and over the corresponding lanes of the gel. The primer extension bands representing the ribosome stalled at the 8th *ermAL1* codon are shown by arrowheads. The lanes representing mutations at codons located in the P- and A-sites of the stalled ribosomes are boxed. In the cartoon, the star represents erythromycin bound in the tunnel.

Figure 2.

The role of the A-site codon in SRC formation. (A) The A-site amino acid is not incorporated in the nascent peptide. The cartoon shows possible versions of peptidyl-tRNA in SRC containing the 8th codon of *ermAL1* in the P-site. The A-site amino acid

(Glu) is shown by a filled circle and the erythromycin molecule bound in the exit tunnel is represented by a star. The gel represents Northern blot analysis of tRNA associated with the stalled ribosome. Positions of aminoacyl-tRNAs and peptidyl-tRNA are indicated. (B) Effects of mutations in the 9th codon of *ermAL1* on ribosome stalling. The control (no erythromycin) lane is shown only for the wild-type *ermAL1* sequence. The bar diagram represents the results of quantitation of the intensity of the "stalled ribosome" bands (an average of three independent experiments). (C) Testing the effects of A-site codons decoded by different tRNA isoacceptors on ribosome stalling. Note that a single nucleotide shift in the position of the band observed with tRNA^{Arg} isoacceptors apparently reflects change in the ribosome geometry in response to binding of different tRNAs which affects the precise site where reverse transcriptase stops. This effect was also seen when binding of different tRNAs was directed to the A-site (see gel in panel B).

Figure 3.

Differential acceptor activity of stalling and nonstalling amino acids in the reaction of peptide bond formation in the SRC. (A) The ribosome stalled at the end of the truncated *ermAL1* mRNA was allowed to react for a specified time at 37°C with 1 mM CCA-N-Ala or CCA-N-Lys, and the remaining unreacted peptidyl-tRNA was resolved by gel electrophoresis. The first two lanes in the gel show samples incubated for 0 or 30 min in the absence of aminoacyl-tRNA analogs. The graph below the gel represents the results of quantitation of the amount of radioactivity in the peptidyl-tRNA bands. (B) Translation, in the presence of erythromycin, of an extended *ermAL1* ORF containing a mutation of the wild-type 9th codon (Glu) to the nonstalling Phe. A frameshift mutation

downstream from the stalling site extends the ORF to 72 codons. The codons located in the P- and A-sites of the SRC are boxed. The position of gel bands representing a 72–amino acid full-size translation product and peptidyl-tRNA esterified by an 8–amino acid nascent peptide are marked by filled and contoured arrowheads, respectively. Four-fold less material was loaded onto the no-erythromycin lanes compared with the erythromycin lanes. The bar diagram represents the results of quantitation of the amount of radioactivity in the gel bands in the samples containing erythromycin.

Figure 4.

Effects of the nascent peptide sequence on the properties of the PTC A-site. The gels show the primer extension inhibition signal (bold arrowheads) representing SRC formation at different ORFs (erythromycin was present in all the samples). Bands corresponding to translation initiation sites at the *erm* ORFs are shown for reference and are indicated by thin arrows. The amino acid sequences corresponding to the ErmCL peptide are red, and those representing the ErmAL1 peptide are blue. The Gly mutation at position 7 of ErmCL is shown in green, and the A-site amino acid is black. Amino acids located in the A-site of the PTC in the stalled ribosome are boxed with solid lines. The amino acid position -2 relative to the nascent peptide C-terminus is boxed with a dashed line.

Figure 5.

Nascent peptide controls properties of the PTC A-site. (A) During normal translation, the PTC A-site (orange) is in the versatile state. (B) In the presence of an inducing antibiotic (ery) and a specific nascent peptide (*e.g.*, ErmAL1), the A-site becomes selective. Peptide bond formation with certain amino acids (red) becomes very slow; the corresponding A-site codons are conducive to SRC formation. (C) Certain peptide sequences (*e.g.*, ErmCL) can render the A-site even more restrictive; the SRC is formed irrespective of the A-site codon. The ErmAL1 and ErmCL sequences essential for stalling are shown in cyan; the amino acid residue in position -2, which apparently controls the A-site properties, is blue. (D) A possible signal relay pathway communicating the information from the ribosomal exit tunnel to the PTC A-site. The 8-amino acid ErmAL1 nascent peptide, attached to the P-site tRNA, was modeled in the structure of *Thermus thermophilus* 70S ribosome (PDB accession number 2WDL (Voorhees et al., 2009)). C-terminal amino acids critical for stalling are colored in cyan; the residue at position -2 is shown in blue. The 23S rRNA residues A2451 and C2452 forming the A-site crevice are orange. Erythromycin shape (ery) is sown as violet mesh with the cladinose residue highlighted in red. Mutations of residues A2062 and A2503 (purple) prevent staling. Neighboring residues, G2061 and U2504 (pale blue) may participate in communicating the stalling signal to the A-site crevice. The conformational flexibility of the rRNA residues putatively involved in relaying the stalling signal from the exit tunnel to the PTC is illustrated by their varying placement in different ribosomal complexes (Schmeing et al., 2005; Gurel et al., 2009; Schuwirth et al., 2005; Jenner et al., 2005; Petry et al., 2005).

Figure 1

Click here to download high resolution image

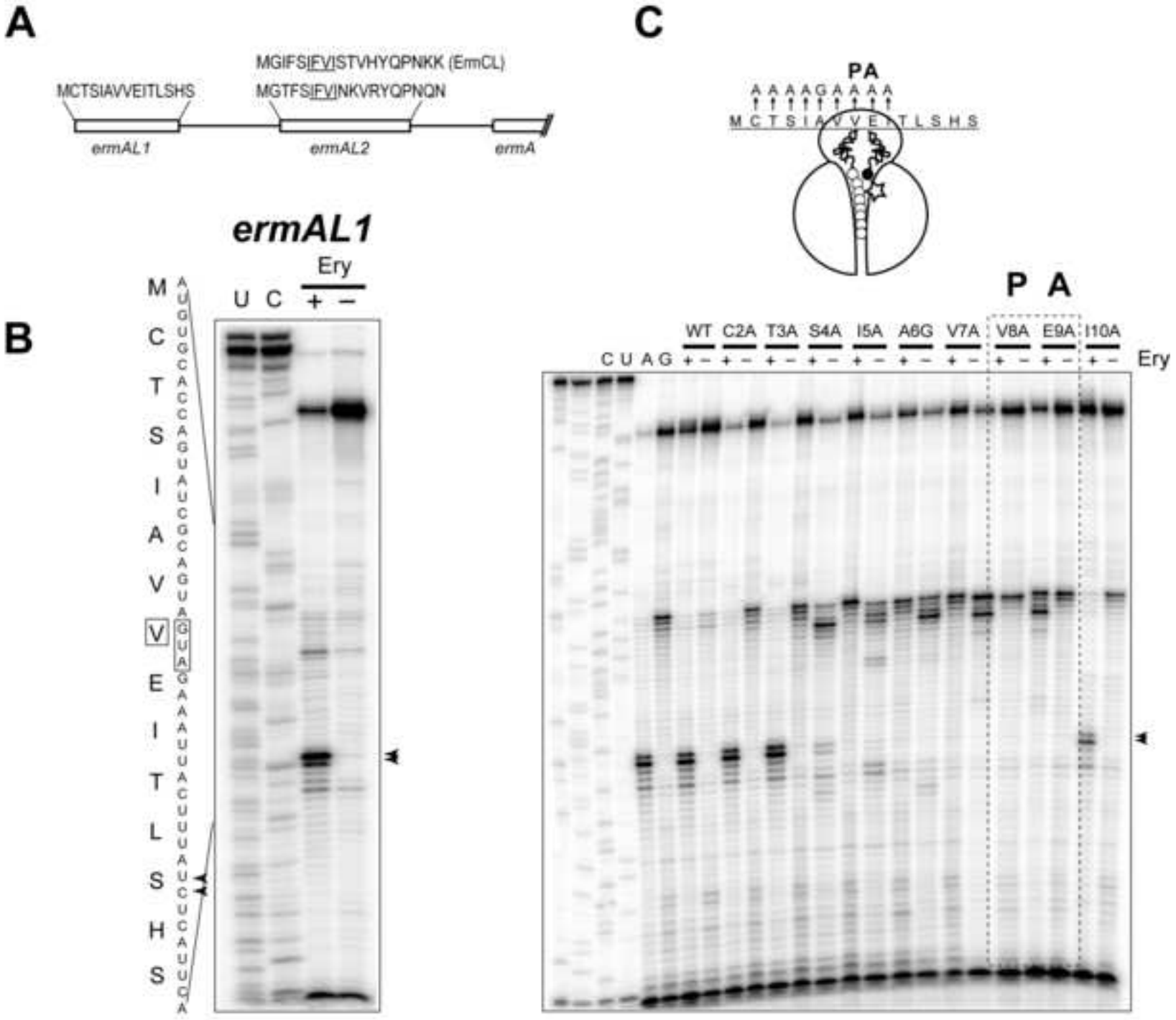

Figure 2

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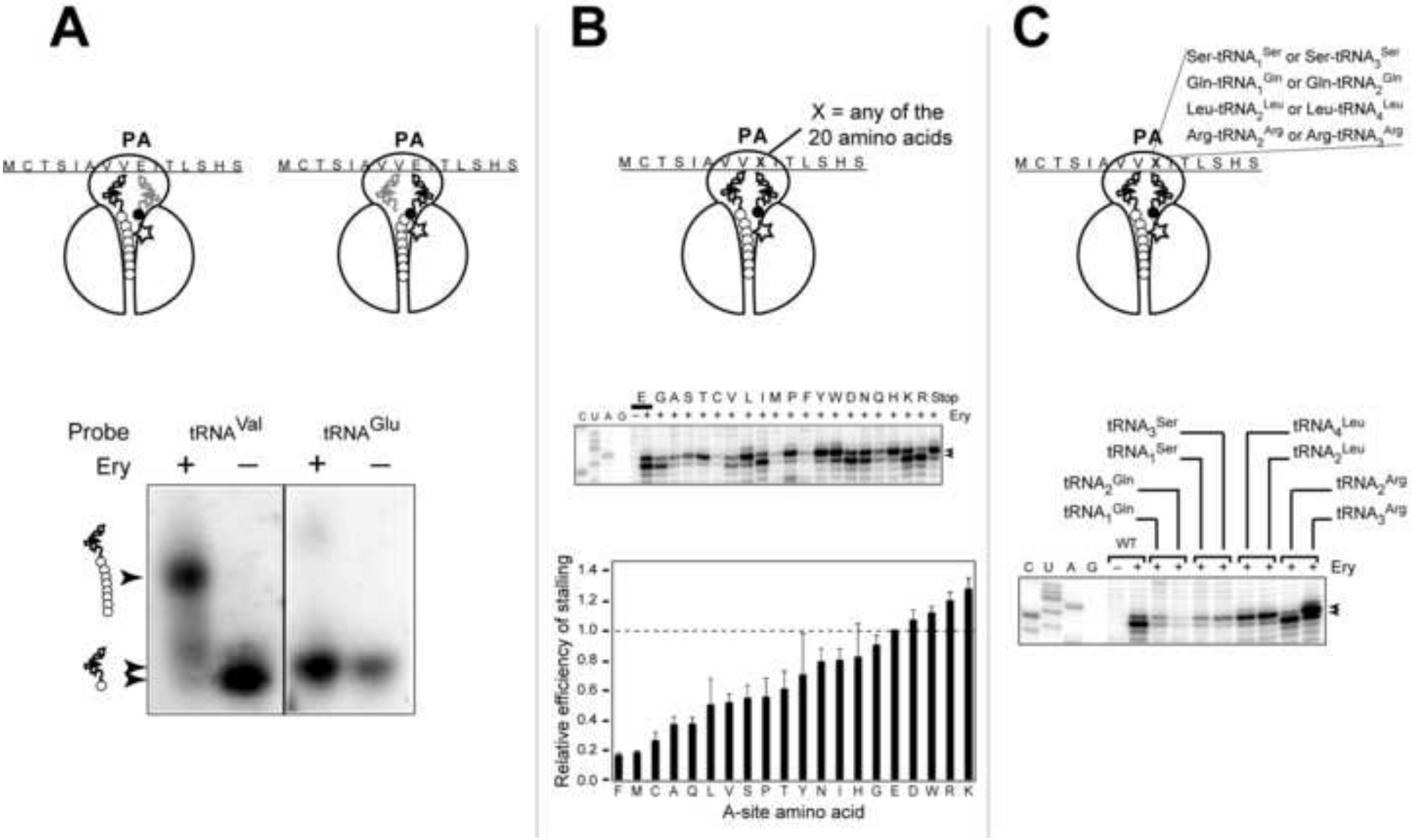

Figure 3

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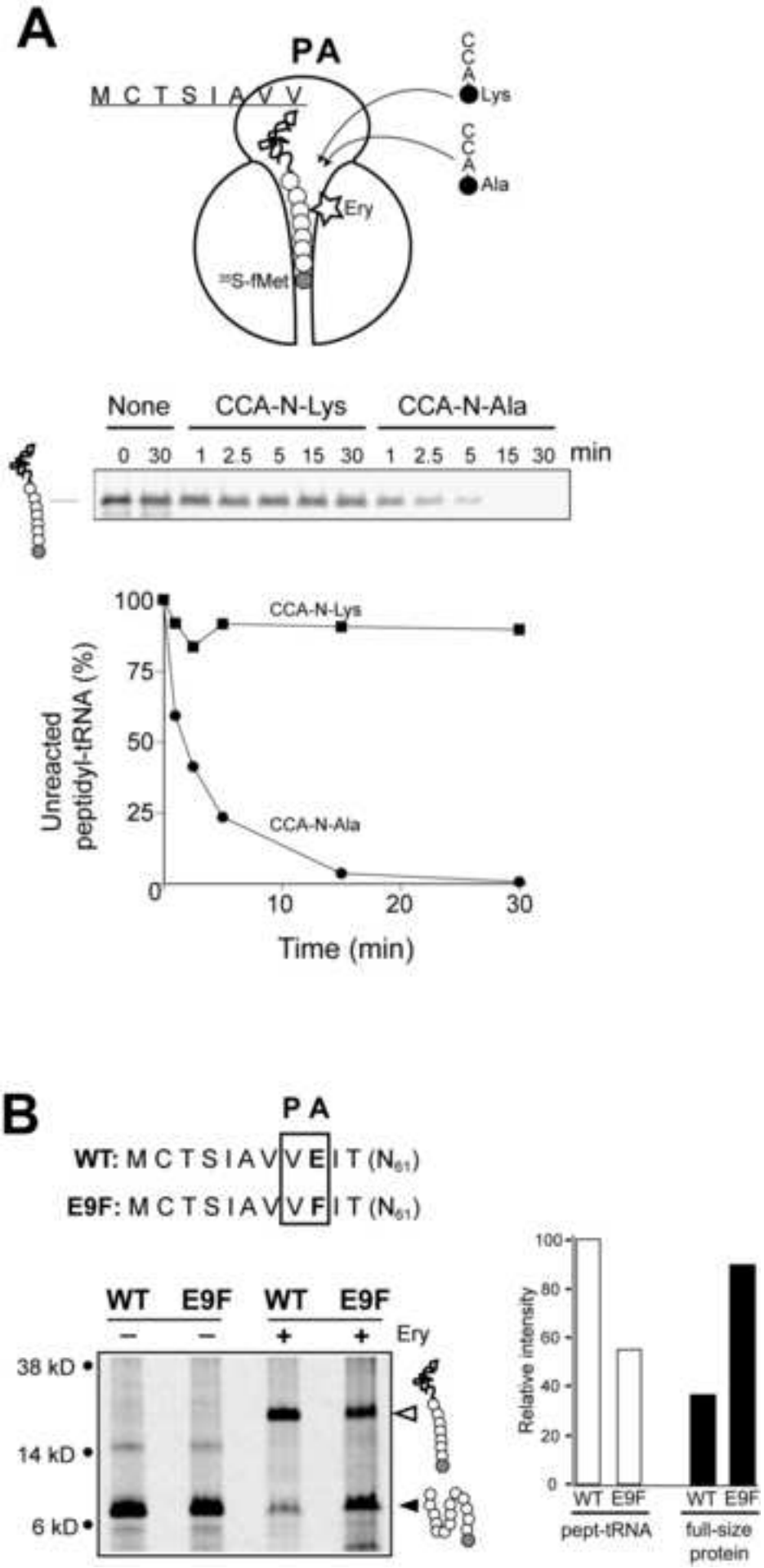

Figure 4

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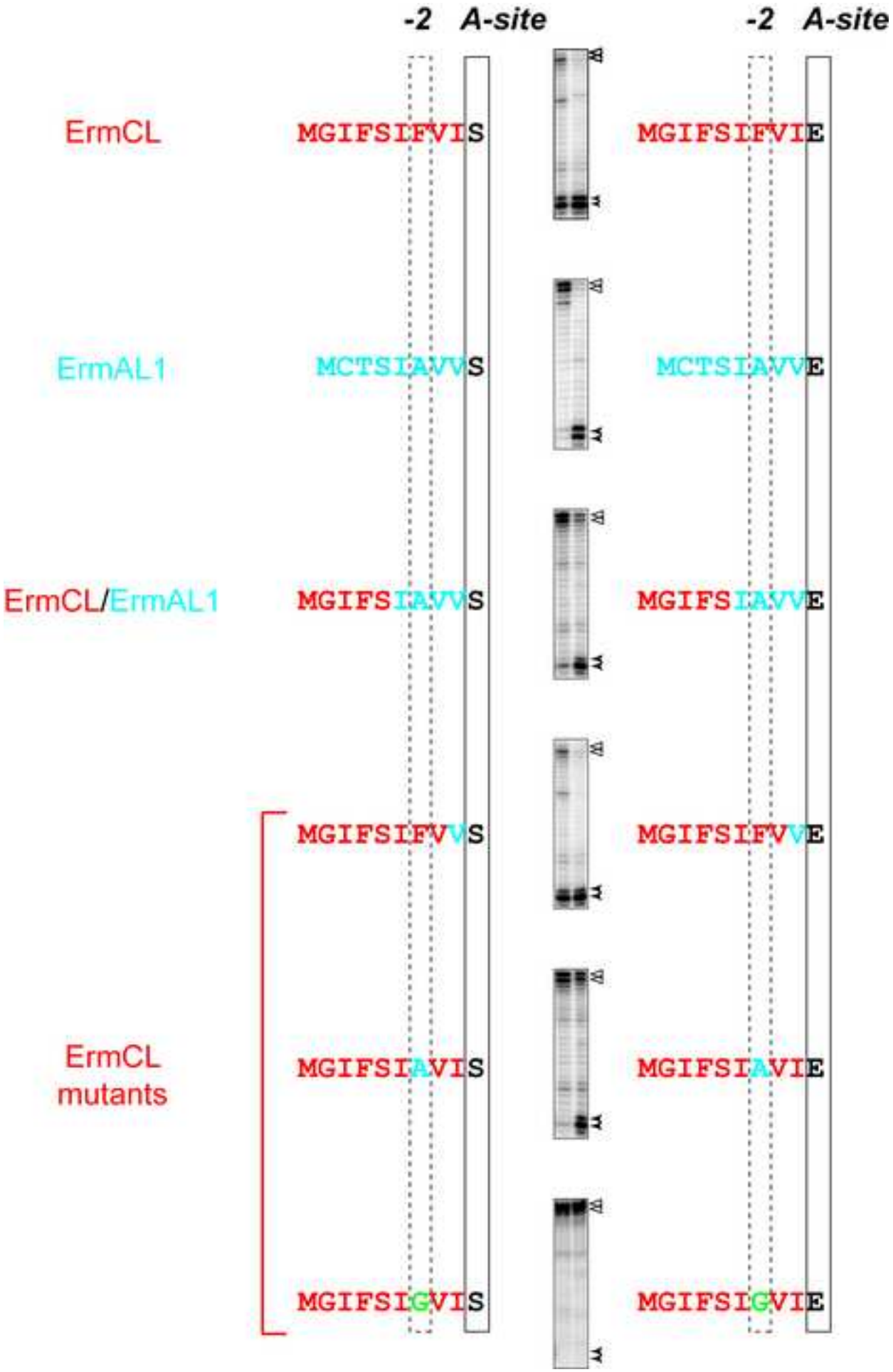

Figure 5

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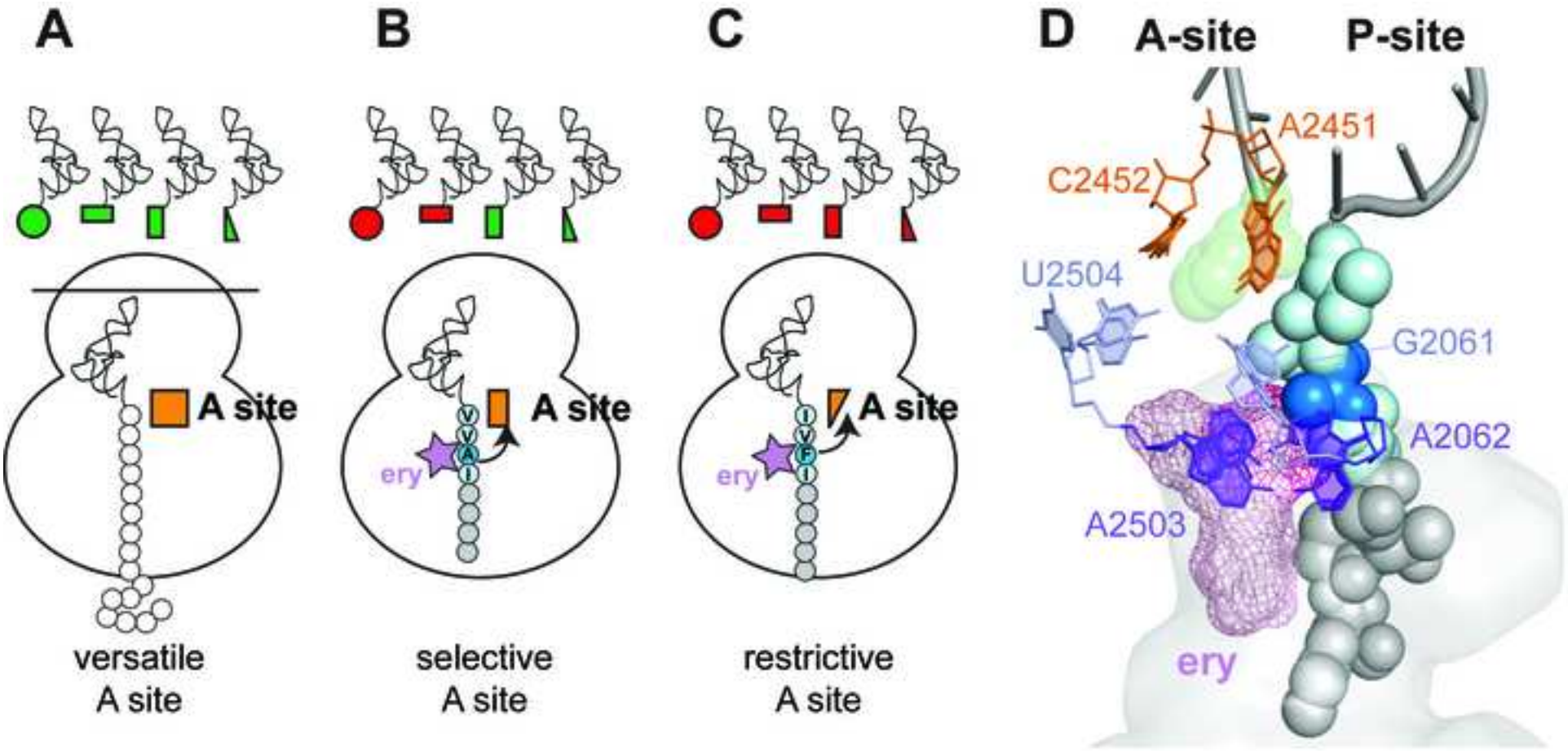


Inventory of Supplemental Information (Molecular-Cell D-10-01262)

Supplemental Data:

Figure S1A is related to Figure 1.

Figure S2 is related to Figure 3A

Figure S3 is related to Figure 2B

Supplemental Table 1 is related to Experimental Procedures

Supplemental Experimental Procedures
(one section which includes the chemical synthesis scheme)

Supplemental references
One reference related to supplemental experimental procedures.

Supplemental Data

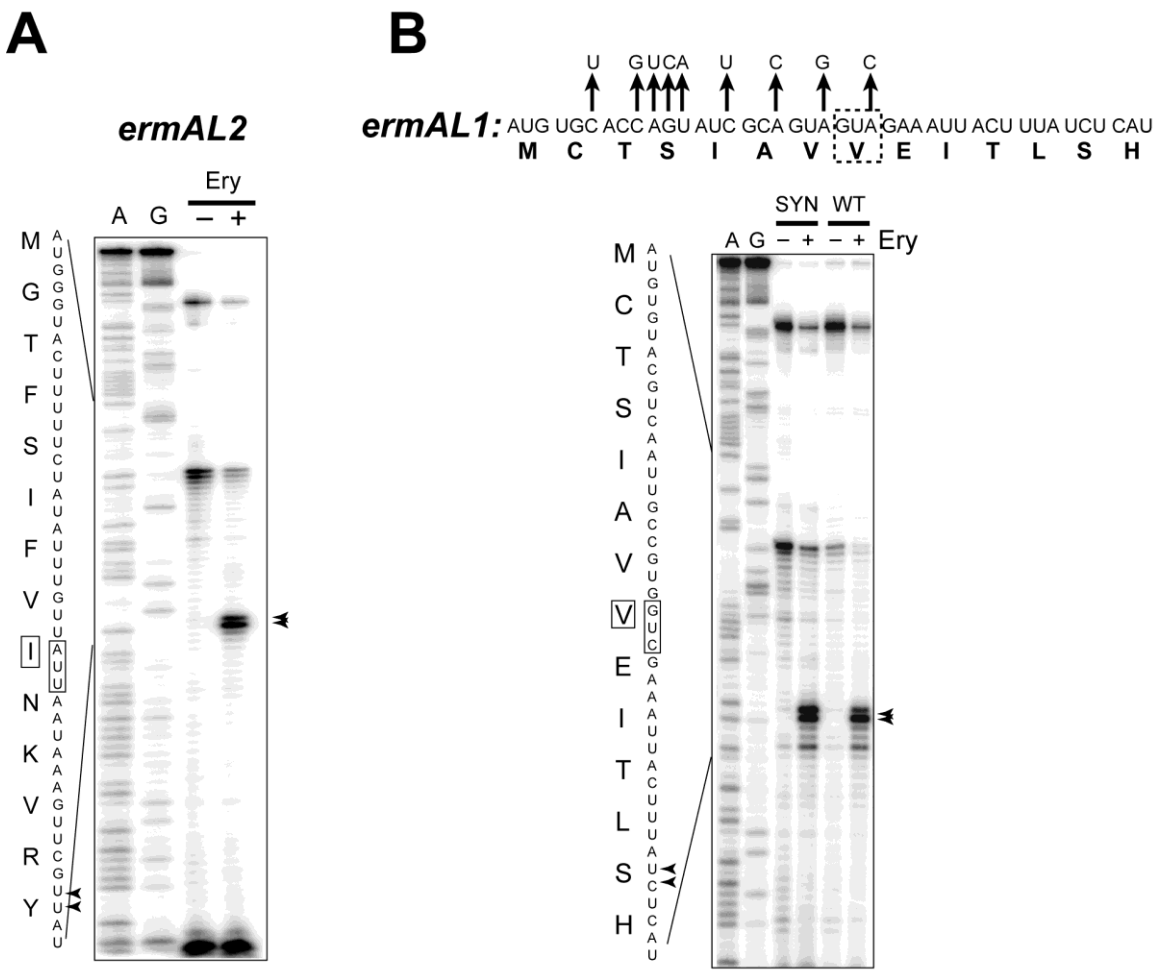


Figure S1, related to Figure 1. (A) Primer extension inhibition analysis of ribosome stalling at *ermAL2*. The *ermAL2* ORF was translated in the absence (-) or presence (+) of erythromycin (Ery). Primer extension revealed drug-dependent ribosome stalling at the codon 9 (Ile) of *ermAL2*, as indicated by arrowheads. (B) Effect of synonymous codon mutations on ribosome stalling at *ermAL1*. Top, Sequence of the *ermAL1* ORF and the encoded peptide. Arrows indicate the synonymous mutations that were introduced simultaneously at nine positions in *ermAL1*. Bottom, Primer extension inhibition analysis of erythromycin-dependent ribosome stalling at mutant *ermAL1* carrying synonymous mutations (SYN) or wild-type *ermAL1* (WT). Arrowheads indicate ribosome stalling sites. The codon located in the P-site of the ribosome stalled at *ermAL1* is boxed.

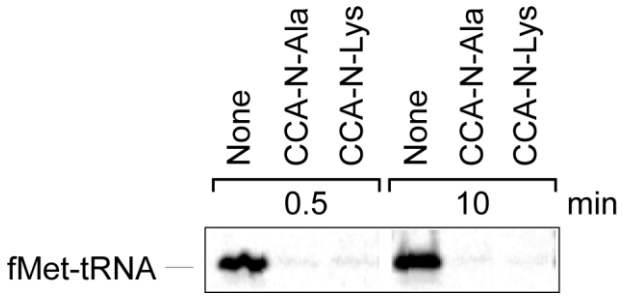


Figure S2, related to Figure 3A. Efficient transfer of formyl-methionine to aminoacyl-tRNA analogs CCA-N-Ala or CCA-N-Lys. 70S ribosomes (60 pmol) were preincubated for 5 min at 37°C with 280 pmol of *ermAL1* mRNA truncated after the 8th codon and 10 pmol of [^{35}S]-fMet-tRNA in PURE System buffer (Shimizu et al., 2001). Aminoacyl-tRNA analogs were added to a final concentration of 1mM, and incubation was continued for 30 sec or 10 min. The reactions were quenched by acetone precipitation, and remaining unreacted fMet-tRNA was resolved by electrophoresis in a 16.5% Bis-Tris gel (as described in http://openwetware.org/wiki/Sauer:bis-Tris_SDS-PAGE,_the_very_best based on US patent 6,162,338). The progression of the reaction was not affected by the presence of 50μM erythromycin (data not shown).

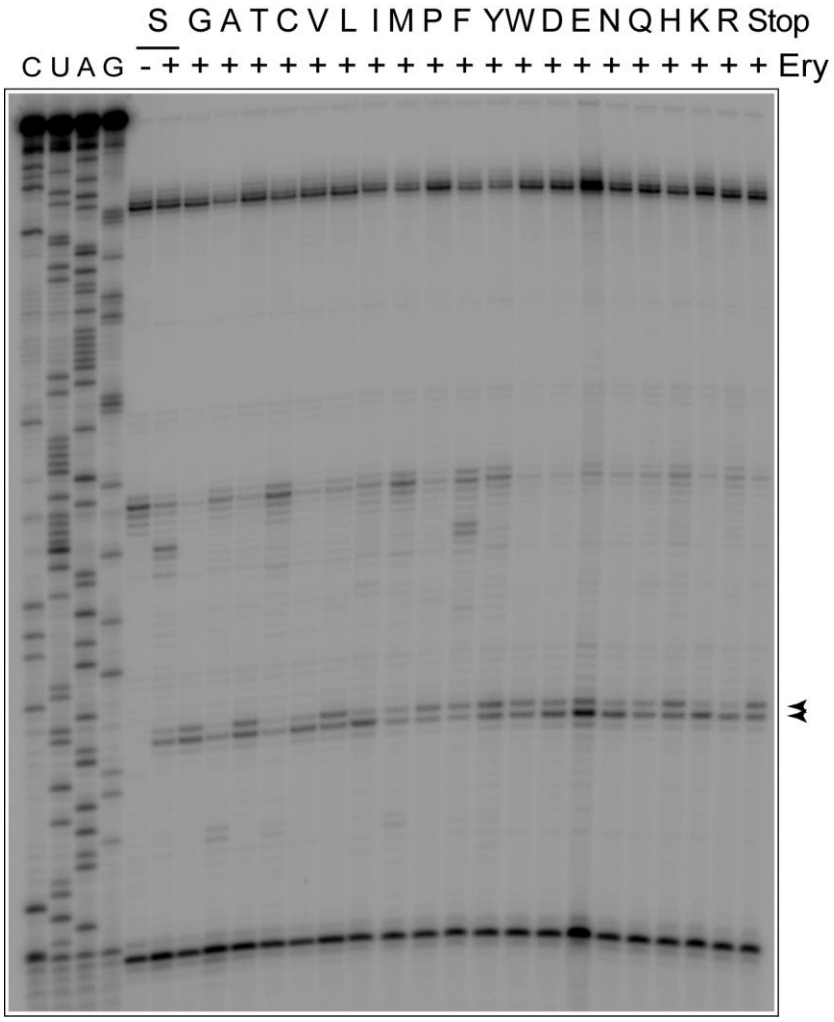
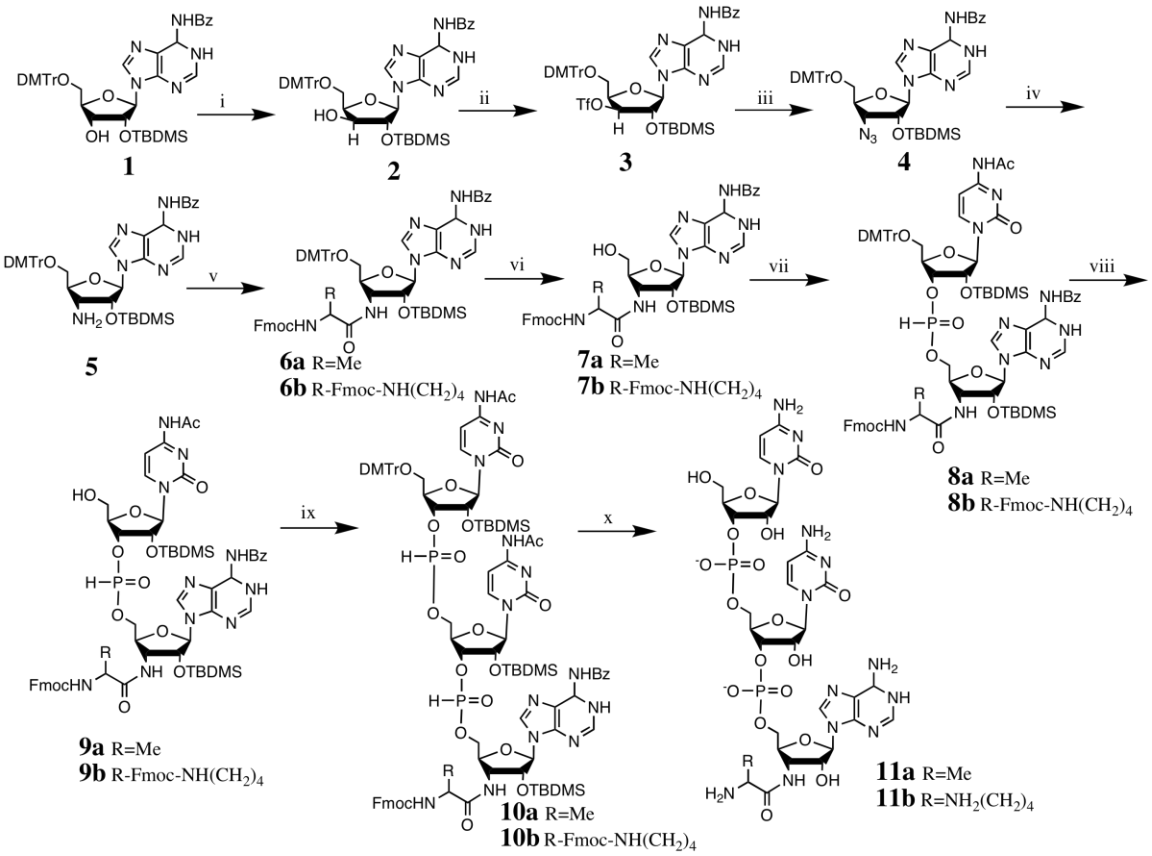


Figure S3, related to Figure 2B. Effect of mutations in the 10th *ermCL* codon on ribosome stalling. The primer extension bands corresponding to the stalled ribosome complex are marked by arrowheads.

Supplemental Experimental Procedures

Preparation of 5'-CCA-N-Lys and 5'-CCA-N-Ala.

5'-CCA-N-Ala (**11a**) and 5'-CCA-N-Lys (**11b**) were synthesized according to Scheme 1.



Scheme 1, Synthesis of 5'-CCA-N-Ala and 5'-CCA-N-Lys. i. (a) CrO_3/Py/Ac_2O in CH_2Cl_2; (b) $NaBH_3CN$ in EtOH, 30% yield for two steps. (ii). CF_3SO_2-Cl/DMAP/CH_2Cl_2, 75%. (iii). NaN_3/DMF, 88%. (iv) Ph_3P/THF/H_2O, 78%. (v). HOBt, *i*-PrN=C=NPr-*i*, THF, Fmoc-Ala for **6a** (75%) and di-Fmoc-Lys for **6b** (72%). (vi) 3% TCA, for **7a** (86%) and for **7b** (88%). (vii). **12**, trimethylacetyl chloride/2,6-lutidine/CH_3CN, for **8a** (72%) and for **8b** (68%). (viii) 3% TCA, for **9a** (82%) and for **9b** (85%). (iv). **12**, trimethylacetyl chloride/2,6-lutidine/CH_3CN, for **10a** (64%) and for **8b** (59%). (x). (a) I2/THF/py/H2O; (b) 3% TCA; (c) NH_4OH/EtOH/55°C/2h; (d) NH_4F/MeOH; (e) HPLC.

The synthesis started from commercially available compound **1** from Chemgene.Oxidation of the 3′-hydroxyl group of **1** with CrO_3/Py/Ac_2O in CH_2Cl_2 afforded the corresponding ketone intermediate, which was reduced by $NaBH_3CN$ stereoselectively to generate the 3′-β-isomer **2**. Intermediate **2** was converted to the triflate **3** by treatment with trifluoromethanesulfonyl chloride in the presence of DMAP. The SN_2 replacement reaction of **3** with sodium azide in DMF at room temperature gave 3′-α-azido nucleoside **4**. The azide **4** was converted into amine **5** by treatment **4** with

triphenyl phosphine and then coupled amine **5** with protected amino acids to give **6a** or **6b**. After removal of 5′-DMTr by trichloracetic acid, intermediate **7a** or **7b** were obtained, respectively. Using compound **7a** as a model compound, we optimized the conditions to remove the protecting groups. Fmoc and benzoyl groups were removed by treatment with 3:1 NH_4OH:EtOH at 55 °C for 2 h. The ^1H NMR indicated that both the benzoyl group and the Fmoc group were removed completely.

Cytidine 3′-H-phosphonate **12** was then coupled with **7a** in the presence of 2,6-lutidine with trimethylacetyl chloride as coupling reagent and the reaction was monitored by TLC. About 10 equivalents of trimethylacetyl chloride was required to push the reaction into near completion. TCA treatment removed the 5′-DMTr group of **8a**, and the coupling product **8a** was purified by column chromatography. The ^{31}P NMR spectrum of **8a** shows two peaks at 10.2 and 9.6 ppm--- the typical region of H-phosphonate. Compound **8b** was prepared similarly. The 5′-DMTr group of **8a** and **8b** was then removed by treatment of TCA to give **9a** and **9b**, respectively. The 5′-hydorxyl group of **9a** or **9b** was coupled with **12** again under the same coupling condition to give **10a** and **10b**, respectively. Without purifying **10a** and **10b**, the crude **10a** or **10b** was treated with iodine followed by removal of 5′-DMTr group by treatment of TCA. The resulting intermediates were fully deprotected by ammonia treatment followed by fluoride treatment to give the final products **11a** and **11b**, which were further purified by HPLC. The MALDI-MS confirmed their structures. With **11a**, $[MH]^+$=948, with **11b**, $[MH]^+$=1005.

The p-ACCA-N-Lys or p-ACCA-N-Ala conjugates that were also tested in the peptidyl transfer experiments but are not reported in the paper were prepared as described by Moroder et al., (2009).

Table S1. List of primers used in this work

Primer Name	Primer Sequence (5' to 3')
Universal primers for generating templates for cell-free transcription-translation [a)]	
NV1	GGTTATAATGAATTTTGCTTATTAAC
T7fwd	TAATACGACTCACTATAGGG
Oligonucleotides used for generating wild-type *ermAL1* template	
ermAfwd	TACATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGTGCACCAGTATCGCAGTAG
ermArev	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTCTACTACTGCGATACTGGTG
Oligonucleotides used for generating synonymous mutations in *ermAL1*	
ermAL1syn-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGTGTACGTCAATTGCCGTGG
ermAL1syn-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTCGACCACGGCAATTGACGTA
Oligonucleotides used for generating mutant *ermAL1* templates for alanine scanning	
ermA-A2-F (used with ermArev)	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGCCACCAGTATCGCAGTAG
ermA-A3-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGTGCGCCAGTATCGCAGTAG
ermA-A3-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTCTACTACTGCGATACTGGCG
ermA-A4-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGTGCACCGCAATCGCAGTAG
ermA-A4-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTCTACTACTGCGATTGCGGTG
ermA-A5-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGTGCACCAGTGCAGCAGTAG
ermA-A5-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTCTACTACTGCTGCACTGGTG
ermA-G6-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGTGCACCAGTATCGGAGTAG
ermA-G6-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTCTACTACTCCGATACTGGTG
ermA-A7-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGTGCACCAGTATCGCAGCAG
ermA-A7-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTCTACTGCTGCGATACTGGTG
ermA-A8-R (used with ermAfwd)	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTCTGCTACTGCGATACTGGTG
ermA-A9-R (used with ermAfwd)	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTGCTACTACTGCGATACTGGTG
ermA-A10-R (used with ermAfwd)	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTTGCTTCTACTACTGCGATACTGGTG
Oligonucleotides used for generating mutant *ermAL1* templates with codon 9 substitutions [b)]	
ermA-E10F-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATAAATACTACTGCGATACTGGTG
ermA-E9Q-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTGTACTACTGCGATACTGGTG
ermA-E9K-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATTTTTACTACTGCGATACTGGTG
ermA-E9P-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATGAGATAAAGTAATAGGTACTACTGCGATACTGGTG

ermA-E9D-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATATCTACTACTGCGATACTGGTG
ermA-E9stop-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATTTATACTACTGCGATACTGGTG
ermA-E9L-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATTAATACTACTGCGATACTGGTG
ermA-E9I-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATGATTACTACTGCGATACTGGTG
ermA-E9M-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATCATTACTACTGCGATACTGGTG
ermA-E9V-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATGACTACTACTGCGATACTGGTG
ermA-E9T-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATTGTTACTACTGCGATACTGGTG
ermA-E9Y-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATGTATACTACTGCGATACTGGTG
ermA-E9H-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATGTGTACTACTGCGATACTGGTG
ermA-E9N-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATGTTTACTACTGCGATACTGGTG
ermA-E9C-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATGCATACTACTGCGATACTGGTG
ermA-E9R-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATACGTACTACTGCGATACTGGTG
ermA-E9G-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATACCTACTACTGCGATACTGGTG
ermA-E9W-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATCCATACTACTGCGATACTGGTG
ermA-E9S-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATTGATACTACTGCGATACTGGTG
ermA-E9Q2-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATCTGTACTACTGCGATACTGGTG
ermA-E9S2-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATACTTACTACTGCGATACTGGTG
ermA-E9L2-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATGAGTACTACTGCGATACTGGTG
ermA-E9R2-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATGAATG AGATAAAGTAATCCGTACTACTGCGATACTGGTG
Oligonucleotides used for site-directed mutagenesis of cloned *ermAL1*	
ermAL-shift1	TCGCAGTAGTAGAAATTACTATCTCATTCATAAGTGATAG
ermAL-shift2	CTATCACTTATGAATGAGATAGTAATTTCTACTACTGCGA
permAL.FS-E9F-F	GCACCAGTATCGCAGTAGTATTCATTACTATCTCATTCATAAG
Oligonucleotides used for generating *ermCL-ermAL1 hybrids* [c)]	
ermC-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGGC ATTTTTAGTATTTTTGTAATC
ermC-rev	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATGAAC TGTGCTGATTACAAAAATACTAAAAATGCC
ermC-S10E-R (used with ermC-fwd)	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATGAAC TGTTTCGATTACAAAAATACTAAAAATGCC
ermALshort-rev (used with ermAfwd)[d]	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATAAAGT AATTTCTACTACTGCGATACTGGTG
ermALshort-E9S-R (used with ermAfwd)	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTATAAAGT AATTGATACTACTGCGATACTGGTG
ermCLshort-F7A-I9V-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGGC ATTTTTAGTATTGCAGTAGTA

ermCLshort-F7A-I9V-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATGAAC TGTGCTTACTACTGCAATACTAAAAATGCC
ermCLshort-F7A-I9V-S10E-R (used with ermCLshort-F7A-I9V-F)	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATGAAC TGTTTCTACTACTGCAATACTAAAAATGCC
ermCLshort-I9V-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGGC ATTTTTAGTATTTTTGTAGTA
ermCLshort-I9V-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATGAAC TGTGCTTACTACAAAAATACTAAAAATGCC
ermCLshort-I9V-S10E-R (used with ermCLshort-I9V-F)	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATGAAC TGTTTCTACTACAAAAATACTAAAAATGCC
ermCLshort-F7A-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGGC ATTTTTAGTATTGCTGTAATC
ermCLshort-F7A-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATGAAC TGTGCTGATTACAGCAATACTAAAAATGCC
ermCLshort-F7A+S10E-R (used with ermCLshort-F7A-F)	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATGAAC TGTTTCGATTACAGCAATACTAAAAATGCC
Oligonucleotides used for generating *ermAL2* template	
ermAL2-F	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGGT ACTTTTTCTATATTTGTTATTAATAAAGTTCG
ermAL2-R	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAATTTTG ATTTGGTTGATAACGAACTTTATTAATAACAAA
Oligonucleotide probes for Northern hybridization	
$tRNA^{Val}$ (UAC)	TGGGTGATGACGGGATCGAACCGCCGACCCCCTCCTTGTAAGGGAGGTGC TCTCCCAGCTGAGCTAATCACCC
$tRNA^{Glu}$ (UUC)	CGTCCCCTAGGGGATTCGAACCCCTGTTACCGCCGTGAAAGGGCGGTGTC CTGGGCCTCTAGACGAAGGGGAC

[a)] For generating temples for cell-free transcription-translation, each primer pair was used in combination with T7fwd and NV1.

[b)] For *ermAL1* codon 9 mutations, the forward primer was ermAfwd in all cases.

[c)] The *ermCL* ORF was truncated to 13 codons.

Supplemental references

Moroder, H., Steger, J., Graber, D., Fauster, K., Trappl, K., Marquez, V., Polacek, N., Wilson, D.N., and Micura, R. (2009). Non-hydrolyzable RNA-peptide conjugates: a powerful advance in the synthesis of mimics for 3'-peptidyl tRNA termini. Angew. Chem. Int. Ed. Engl. *48*, 4056-4060.