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Mutational analysis reveals two independent molecular requirements during transfer RNA selection on the ribosome

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Accurate discrimination between cognate and near-cognate aminoacyl-tRNAs during translation relies on the specific acceleration of forward rate constants for cognate tRNAs. Such specific rate enhancement correlates with conformational changes in the tRNA and small ribosomal subunit that depend on an RNA-specific type of interaction, the A-minor motif, between universally conserved 16S ribosomal RNA nucleotides and the cognate codon-anticodon helix. We show that perturbations of these two components of the A-minor motif, the conserved rRNA bases and the codon-anticodon helix, result in distinct outcomes. Although both cause decreases in the rates of tRNA selection that are rescued by aminoglycoside antibiotics, only disruption of the codon-anticodon helix is overcome by a miscoding tRNA variant. On this basis, we propose that two independent molecular requirements must be met to allow tRNAs to proceed through the selection pathway, providing a mechanism for exquisite control of fidelity during this step in gene expression.

During each cycle of protein chain elongation, the translation machinery must choose from the large cellular pool of aminoacyl-tRNAs (aa-tRNAs) the one that properly decodes the codon of the messenger RNA located in the A site of the ribosome. This tRNA-selection step occurs with high specificity *in vivo*, resulting in misincorporation frequencies of about 1 in 10^3–10^4 polymerized amino acids (reviewed in ref. 1). The current model that explains how such accuracy is achieved relies primarily on kinetic rather than thermodynamic discrimination[2–4].

Two strategies are used by the translation machinery to achieve high fidelity and speed during tRNA selection: kinetic proofreading and induced fit. Kinetic proofreading is made possible by the action of a GTPase elongation factor (EF-Tu in bacteria) that delivers aa-tRNAs to the ribosome in a ternary complex with GTP, allowing for two sequential opportunities to reject an incorrect aa-tRNA[5,6] (**Fig. 1a**). 'Induced fit' describes conformational changes that are thought to be responsible for accelerating GTPase activation and tRNA accommodation upon recognition of cognate tRNA species[4].

Important observations for understanding the physical basis of induced fit have come from high-resolution crystal structures that describe, in molecular detail, certain rearrangements of the small (30S) ribosomal subunit upon binding of cognate tRNA[7,8]. Specifically, A-minor interactions are established between the unpaired nucleotides A1492, A1493 and G530 of 16S rRNA and the minor groove of the first two base pairs of the codon-anticodon helix (**Fig. 1b**). To establish these contacts, A1492 and A1493 flip out from a bulge in helix 44 and G530 rotates from a *syn* to an *anti* conformation. These conformational changes are stabilized when the minor groove of the codon-anticodon helix has Watson-Crick geometry, and they in turn stabilize a 'closed' state of the small subunit. Aminoglycoside antibiotics such as paromomycin and streptomycin, known to stimulate miscoding, mimic some of the rearrangements caused by cognate aa-tRNA binding[9] (**Fig. 1b**) and accelerate the rate-limiting forward steps described above while reducing the rejection rates for near-cognate aa-tRNAs[10,11]. Thus, there is good correlation between observed kinetic effects and the conformational changes of the decoding site induced upon cognate codon-anticodon or aminoglycoside binding.

Despite this detailed understanding of some local and global conformational changes, insight into how these changes result in downstream events remains a challenge that is the focus of the work presented here. It has been proposed that communication from the small to the large subunit, to accelerate GTP hydrolysis and tRNA accommodation, can occur through the tRNA body[12] or through the intersubunit bridges[13], or both. Biochemical studies have begun to define the role of the intersubunit bridges[14,15] and the tRNA body[16,17] in this pathway. Such a role for the tRNA has been proposed to depend on the inherent flexibility between different domains of the tRNA body[18–21]. Furthermore, although both structural and biochemical studies[16,21] provide strong support for the idea that tRNA bending is important for tRNA selection, the specific mechanism by which conformational changes in the tRNA and the ribosome decoding

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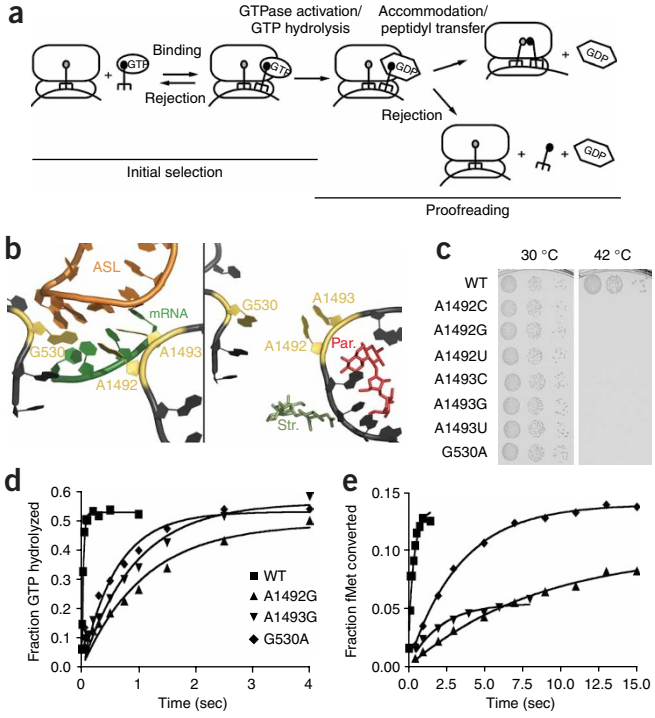

Figure 1 Role of conserved decoding-site nucleotides in cognate tRNA selection. (**a**) Simplified kinetic scheme showing the two stages of tRNA selection, with two chances for rejection and two crucial forward steps (GTPase activation and tRNA accommodation). Initial binding steps as well as additional conformational changes are not shown, for simplicity. (**b**) Left, structure of the decoding site in the small ribosomal subunit filled with a short mRNA and a cognate anticodon stem-loop (ASL), showing direct contacts between the codon-anticodon helix and 16S nucleotides A1492, A1493 and G530. Right, structure of an empty decoding site with the aminoglycoside antibiotics paromomycin (Par.) and streptomycin (Str.) bound. Figures were made from PDB entries 1IBM and 1FJG[7,9] using PyMOL (http://pymol.sourceforge.net). (**c**) Serial dilutions of strains carrying a plasmid that expresses either wild-type (WT) or mutant rRNA from a temperature-inducible promoter (see Methods). All tested substitutions cause dominant lethality when expressed (42 °C). (**d**) Representative time courses of GTP hydrolysis with 2.25 μM initiation complexes and substoichiometric ternary complex. (**e**) Representative time courses of dipeptide formation under same conditions as in **d**. Maximal expected endpoint is about 0.15 owing to the presence of excess ribosome complexes containing [^{35}S]fMet over ternary complex.

center are transmitted to more remote regions of the ribosome remains unclear.

The A-minor motif is a universal mode of helical packing for RNA molecules[22]. Recent detailed analysis of the 70S ribosome structure has led to the suggestion that ribosome dynamics might be facilitated in large part by the rearrangement of mutually exclusive A-minor interactions[23]. The energetic contribution of these interactions has been analyzed in the *Tetrahymena thermophila* group I intron by measuring the effect of introducing substitutions of either the unpaired adenosines or the receptor base pairs[22,24]. In both cases, the energetic penalties were large and indicated specific requirements for unpaired adenosines and for Watson-Crick base pair receptors. For the A-minor interactions in the decoding center, the effect of changing the receptor base pairs by introducing different codon-anticodon mismatches has been measured[25]. All non–Watson-Crick base pairs result in uniformly faster dissociation of the ternary complex from the ribosome as well as in slower, but more variable, rates of GTPase activation and tRNA accommodation.

In this work, we examine the contribution of universally conserved nucleotides in the decoding center of the ribosome to tRNA selection by introducing mutations in the three crucial 16S rRNA nucleotides A1492, A1493 and G530. Mutations in these three residues have previously been shown to cause dominant lethality and general translation defects in *Escherichia coli*[26–28], and the effects of substitutions at A1492 and A1493 on tRNA binding have been measured *in vitro*[27]. Here we have carried out a more detailed functional characterization of these variant *E. coli* ribosomes by measuring their effects on the crucial forward rates of GTPase activation and tRNA accommodation in tRNA selection. We have also addressed the question of how the decoding-center rearrangements act synergistically with those in the tRNA body by looking at the consequences of combining ribosomal mutations with two different miscoding reagents, the Hirsh suppressor tRNA and aminoglycoside antibiotics. Whereas aminoglycosides bind in the decoding center of the ribosome and stimulate miscoding both by decreasing tRNA rejection and increasing the rates of the forward reactions in tRNA selection[10,11], the Hirsh suppressor tRNA, which carries a single substitution in its D arm, acts simply by accelerating the forward steps in the process, with no discernible effect on tRNA dissociation[16]. This type of biochemical epistasis analysis has allowed us to determine whether the multiple molecular events crucial for tRNA selection might act in the same or in different paths. Notably, the stimulatory effects of the Hirsh suppressor tRNA are limited to disruptions of the codon-anticodon helix; the mutant tRNA has no compensating effect on disruptions of the A minor–type docking adenosine nucleotides. These observations suggest that the conformational changes that occur during decoding in the small subunit and in the tRNA are at least partially independent of one another. These ideas in turn lead to a model in which each of two irreversible chemical steps, GTP hydrolysis and peptidyl transfer, is limited by two distinct energetic barriers during the process of mRNA decoding.

RESULTS

Decoding-site mutations impair cognate tRNA selection

Specific interactions of nucleotides A1492, A1493 and G530 with the codon-anticodon helix were disrupted by making single substitutions at each of these three positions (**Fig. 1b**). All mutations tested at these universally conserved residues resulted in dominant lethality (**Fig. 1c**), as previously observed[26,27] and consistent with their *in vivo* translation defects[28]. The growth defects associated with the decoding-site mutations required the use of our previously developed affinity-purification procedure to isolate homogeneous populations of mutant ribosomes from the wild-type, chromosomally-encoded background[29]. The mutant 30S particles were tagged by insertion of the MS2 RNA stem-loop into the backbone of the 16S rRNA in helix 6 (known as the spur) and affinity-purified through an MS2 coat protein fused to glutathione *S*-transferase (GST) on an FPLC glutathione column[30]. The biochemical activity of spur-tagged ribosomes was indistinguishable from that of untagged ribosomes in assays of both peptidyl transfer and EF-Tu–dependent GTP hydrolysis (data not shown).

Tagged variant ribosomes with mutations at positions A1492, A1493 and G530 were purified using this methodology[30], though the substantial growth defects associated with these mutations resulted in relatively low yields. For each position of interest, we focused on the effects on activity of the more conservative transition mutations

Table 1 GTPase and dipeptide formation rates of decoding-site variant ribosomes

	GTP hydrolysis rate (s^{-1})	Dipeptide formation rate (s^{-1})
WT	39.10 ± 2.90	3.00 ± 0.14
A1492G	0.90 ± 0.01	0.13 ± 0.01
A1493G	0.90 ± 0.30	0.57 ± 0.15
G530A	1.50 ± 0.10	0.29 ± 0.01

Rates of GTP hydrolysis and peptide bond formation for wild-type (WT) and mutant ribosomes with 2.25 μM initiation complexes and substoichiometric ternary complex. These rates are at saturation for peptide bond formation with all ribosomes, and for GTP hydrolysis with the mutant ribosomes. For GTP hydrolysis with wild-type ribosomes, the concentration of ribosomes used is close to the $K_{1/2}$.

(purine to purine). We first analyzed the activity of these variant ribosomes in two of the crucial steps during tRNA selection, GTPase activation and tRNA accommodation, while maintaining the integrity of the A-minor receptor base pair by using a cognate aa-tRNA–codon pair. These measurements were carried out under saturating, or nearly saturating, conditions using an *in vitro* system that recapitulates the high fidelity and speed of tRNA selection observed *in vivo*[3].

The rate of GTPase activation can be determined by measuring the rate of GTP hydrolysis, as this chemical step is limited by GTPase activation[31]. Time courses of GTP hydrolysis were obtained by mixing ribosome initiation complexes (carrying initiator fMet-tRNA^{fMet} bound to an AUG-programmed P site and the tryptophan codon UGG in the A site) with substoichiometric purified ternary complex composed of Trp-tRNA^{Trp}, EF-Tu and [γ-^{32}P]GTP. The single-turnover rates of GTP hydrolysis were obtained by fitting the data to single-exponential equations. Whereas wild-type ribosomes show the expected fast rate of GTPase activation when programmed with the cognate codon in the A site (~40 s^{-1}), all three mutants (A1492G, A1493G and G530A) tested have marked defects in this step, ranging from 30- to 60-fold in magnitude (**Fig. 1d** and **Table 1**). The concentration of initiation complexes (ribosomes) used for these assays (2.2 μM) was saturating for the three variants tested (**Supplementary Fig. 1** online). This is consistent with the prediction from the current kinetic framework that slow rates of GTPase activation result in low $K_{1/2}$ values because equilibration between the ribosome and ternary complex can be achieved. Also as predicted, wild-type ribosomes have a relatively high $K_{1/2}$ of ~2 μM for ternary complex because the fast rate of GTP hydrolysis precludes equilibration[3]. Because with wild-type ribosomes the measured rate of GTPase activation is not obtained at saturation, the observed rate defects are minimal estimates of actual differences in the values for wild-type and variant ribosomes. Nonetheless, these defects are reminiscent of what has been observed for GTPase activation of near-cognate ternary complexes on wild-type ribosomes (that is, complexes with mismatches in the codon-anticodon helix). With near-cognate aa-tRNAs, the rates are 50- to 200-fold slower than with the cognate aa-tRNA, depending on the nature of the mismatch supplied[4,16,25].

To measure the effects of the decoding-site mutations on the proofreading stage of tRNA selection, we used a dipeptide-formation assay[3,4]. These experiments provide two pieces of information. First, the observed rate of peptide bond formation reports on the rate-limiting step of tRNA accommodation. Second, the reaction endpoint is a reflection of the rejection rate (the off rate) of tRNAs during the proofreading stage: a small fractional conversion of fMet into dipeptidyl product is an indication that aa-tRNAs are being irreversibly rejected after GTP hydrolysis. To measure the rate of peptide bond formation, ribosome initiation complexes (carrying initiator f[^{35}S]Met-tRNA^{fMet} bound to an AUG-encoded P site and the tryptophan codon UGG in the A site) were mixed with limiting amounts of cognate tRNA^{Trp} ternary complex, and f[^{35}S]Met-Trp formation was followed over time (**Fig. 1e** and **Table 1**). For wild-type ribosomes, the time course of dipeptide formation represents the rate of tRNA accommodation, because the rates of all preceding steps are considerably faster (including binding steps, GTPase activation and GTP hydrolysis) and accommodation is slower than the chemical step of peptidyl transfer[3]. In the case of the variant ribosomes, because the observed rates of dipeptide formation are very close to their respective rates of GTP hydrolysis, both steps are partially rate limiting and we cannot assign accurate rates of accommodation (in particular for A1493G, where the rates are very close). Nevertheless, it is clear that each of the variant ribosomes is somewhat slower at accommodation than the wild-type ribosome (**Table 1**).

The variant ribosomes with substitutions at 1492 and 1493 also have appreciably lower reaction endpoints than do wild-type ribosomes, indicating that they have increased rates of tRNA rejection during the proofreading stage (**Fig. 1e**). Notably, the G530A variant ribosomes seem to have no endpoint defect and thus no increased tRNA off rate at the proofreading stage. We established that the low endpoints of 1492 and 1493 mutants do not result from defects in the peptidyl-transfer center caused by, for example, improper subunit association or inadequate fMet-tRNA^{fMet} binding, by measuring the rates of peptide bond formation with the minimal A-site substrate puromycin. In these experiments, the mutant ribosomes have the same rate and endpoint for peptidyl transfer as their wild-type counterpart (data not shown). In addition, we have measured the rates of dipeptide formation using excess ternary complex relative to ribosomes (**Supplementary Fig. 2** online). In this case, even if irreversible rejection during proofreading occurs, there is extra ternary complex available for additional binding trials and ultimate peptidyl transfer. Under these conditions, we observed that the variant ribosomes reach the same reaction endpoint as wild-type ribosomes, thus establishing that they are not defective in other ribosomal functions, as mentioned above. These data further support the notion that the decreased endpoints observed when using substoichiometric ternary complex result from rejection after GTP hydrolysis.

The effects of the decoding-center mutations on tRNA selection are reminiscent of what has previously been observed with a variety of near-cognate pairing interactions in the codon-anticodon helix[25]: both GTPase activation and tRNA accommodation are similarly compromised in all cases. By looking at their combined effects, we examined whether the two classes of perturbation might act synergistically. Measurements with the variant ribosomes on a number of near-cognate codon-anticodon pairings yielded immeasurably low yields of dipeptide product, suggesting substantial defects in the rate of tRNA accommodation (data not shown). These data are consistent with the idea that these perturbations (in the rRNA and in the codon-anticodon helix) have independent effects that can synergistically contribute to the observed decreased rates during tRNA selection.

Aminoglycosides rescue the decoding-site mutants

Previous work has shown that addition of the aminoglycoside antibiotics paromomycin and streptomycin restores the otherwise slow rates of GTPase activation and accommodation that result from disruptions in the codon-anticodon helix (the A-minor receptor base pair)[10,11]. We examined whether defects caused by mutation of the decoding-center nucleotides (A1492, A1493 and G530) could similarly be rescued by the addition of these antibiotics by measuring the rates of GTP hydrolysis and peptide bond formation in the

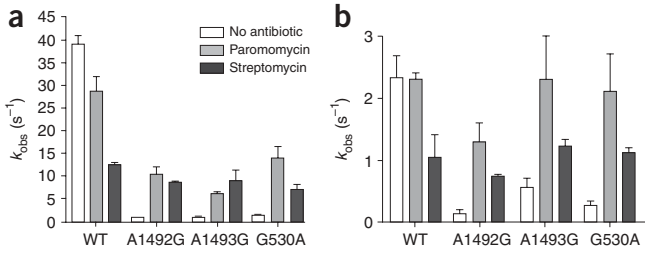

Figure 2 Effect of aminoglycosides on the decoding-site variant ribosomes. (**a**) Observed rates of GTP hydrolysis with 2.25 μM ribosome complex and substoichiometric ternary complex in the absence or presence of 5 μM paromomycin or 5 μM streptomycin. (**b**) Observed rates of peptide bond formation under same conditions as in **a**. WT, wild-type. Error bars represent s.d.

presence of saturating paromomycin (5 μM) and streptomycin (5 μM) (**Supplementary Fig. 3** online). As previously observed, paromomycin did not appreciably affect the rates of GTPase activation and accommodation of wild-type ribosomes carrying a cognate codon (**Fig. 2**)[11]. However, addition of paromomycin greatly increased the rates of GTPase activation for all three variant ribosomes (**Fig. 2a**). In the peptidyl-transfer assay in the presence of paromomycin, we observed both substantial increases in the rates of catalysis by the mutant ribosomes (**Fig. 2b**) and restoration of reaction endpoints to wild-type levels (data not shown). With the addition of streptomycin, we observed partial inhibition of both GTP hydrolysis and peptide bond formation rates for wild-type ribosomes carrying a cognate codon (**Fig. 2**), as previously reported[10]. In addition, as seen with paromomycin, streptomycin increases the rates of both reactions (GTP hydrolysis and peptidyl transfer) for the ribosome variants to wild-type, streptomycin-attenuated levels (**Fig. 2**) as well as increasing the peptidyl-transfer reaction endpoints (data not shown).

A miscoding tRNA does not rescue the decoding-site mutants

We have recently shown that the increased miscoding properties of a variant $tRNA^{Trp}$ carrying a G24A substitution in the D arm, the Hirsh suppressor[32], can be explained by its increased rates of GTPase activation and tRNA accommodation on a variety of near-cognate codons[16]. One of the key observations of this suppression analysis is that the Hirsh variant has quantitatively similar effects (five- to ten-fold stimulation) on the two distinct steps of GTPase activation and accommodation. These data reveal a crucial contribution of the tRNA body itself to high-fidelity decoding during tRNA selection. In the present study, we looked at the interplay between the tRNA body and the decoding-site nucleotides by examining whether the miscoding tRNA could similarly rescue the defects caused by mutation of the unpaired conserved nucleotides A1492, A1493 and G530. Notably, the rates of GTPase activation and peptidyl transfer by the variant ribosomes (A1492G, A1493G and G530A) were indistinguishable in the presence of either wild-type or miscoding G24A $tRNA^{Trp}$ (**Fig. 3**).

DISCUSSION

The analysis of variant ribosomes with single substitutions at three universally conserved nucleotides in the decoding site, A1492, A1493 and G530, has uncovered a number of interesting features of the tRNA selection process. First, we observed substantial defects caused by substitutions at each of these positions in both stages of decoding—initial selection and proofreading. All mutants have reduced rates of GTPase activation (at least 20- to 40-fold) and tRNA accommodation (from 5- to 20-fold) in the context of a cognate codon-anticodon interaction in the A site (**Table 1**). The concordance of the effects on GTPase activation and tRNA accommodation is consistent with earlier studies[11,16] and suggests that similar features of the translational apparatus are crucial for the two different stages of the tRNA selection pathway. In addition, substitutions at A1492 and A1493 cause an appreciable decrease in the stability of the cognate aa-tRNA on the ribosome, as reflected in the reduced fraction of dipeptide formed, which results from increased partitioning of aminoacyl-tRNA during the proofreading stage. Earlier studies of A1492 and A1493 variant ribosomes had similarly identified A-site binding defects in EF-Tu–independent binding experiments that probably report on this same off rate[27]. These data support previous evidence that the energy arising from A-minor interactions and small-subunit rearrangements is used both for increasing the stability of aa-tRNAs on the ribosome and for stimulating fast forward rates[3]. Accordingly, disruption of the A-minor interactions by substitution of the lone adenosines causes effects similar in magnitude and scope to those resulting from substitution of the receptor base pair[25]. These data complement analyses of other decoding-center A-minor contacts—the 2′-OH groups of the codon-anticodon helix[33–35]. The most quantitative of these studies[33] indicates that the contacts made by the 2′-OH groups in the minor groove make crucial contributions to the A-minor interaction, though the magnitudes of these contributions are generally smaller (about ten-fold effects or less) than what we observed here for the contribution of the unpaired docking nucleotide.

To further define the defects of the decoding-site variant ribosomes, we measured the effects of paromomycin and streptomycin on the kinetics of cognate aa-tRNA selection in wild-type and variant ribosomes. Titration experiments established that saturating concentrations of the aminoglycosides were used in each case (**Supplementary Fig. 3**), suggesting that binding of the aminoglycosides was not substantially disrupted by the various mutations in the context of the whole ribosome. Previous studies with model oligonucleotide RNAs had reported substantial binding losses associated with some of these same mutations[36]. Both paromomycin and streptomycin have previously been shown to both stabilize tRNA binding (decreasing off rates) and stimulate the forward rate constants for tRNA selection[10,11]. Here, both antibiotics substantially, though not completely, relieve the defects caused by mutations in each of the three conserved decoding nucleotides (**Fig. 2a**). Given the known role of these aminoglycoside antibiotics in inducing domain closure in the 30S subunit[9], these data suggest that the decoding-site mutants are defective in reaching the closed state, despite the presence of a cognate codon-anticodon interaction in the A site. The overall attenuated rates of GTPase

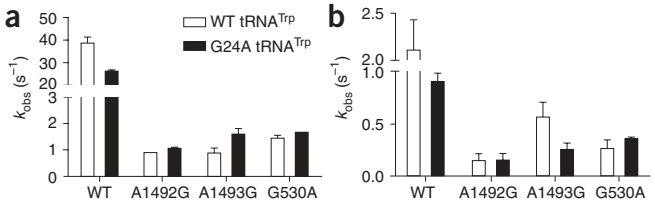

Figure 3 Effect of a miscoding tRNA variant on the decoding-site variant ribosomes. (**a**) Observed rates of GTP hydrolysis with ternary complex containing either the wild-type (WT) or the Hirsh suppressor (G24A) $tRNA^{Trp}$. (**b**) Observed rates of peptide bond formation under the same conditions as in **a**. Measurements were carried out under the same conditions as in **Figure 2**. Error bars represent s.d.

activation and accommodation observed in the presence of streptomycin in wild-type ribosomes and in the rescued variant ribosomes are also consistent with an earlier report indicating that streptomycin induces a discrete, but only partial, closure of the 30S subunit[9,10].

The most noteworthy aspect of the aminoglycoside suppression data is that defects associated with mutations at G530, A1492 and A1493 are all similarly compensated for by the two antibiotics paromomycin and streptomycin, though the two bind in distinct locations within the decoding region (**Fig. 1a**). The similarity of the effects suggests that these small molecules function independently of specific interactions with the nucleotide variants *per se*. Rather, they overcome general deficiencies in closure of the 30S subunit associated with defects either in the codon-anticodon helix or in the ribosomal RNA moieties that engage this ordered helix. Thus, despite the fact that G530 does not directly contact paromomycin, defects associated with variation at this position are compensated for by binding of the drug. These data argue that these functionally crucial nucleotides move cooperatively, so that addition of antibiotics stabilizes the closed structure independently of the perturbation. This conclusion further supports a recent model suggesting that the decoding center exists in two distinct functional states, 'accepting' and 'rejecting', that are key to specific recognition of cognate tRNAs during translation[25].

Our results also address the question of how structural changes in the decoding center are communicated to more remote regions of the ribosome (specifically the GTPase-activating center on the large subunit) and EF-Tu. Previous studies have established that a known miscoding tRNA (the Hirsh suppressor) functions by stimulating forward rate constants for GTPase activation and accommodation in the tRNA selection pathway and not by stabilizing the binding of the variant tRNA on the ribosome[16]. What is noteworthy in these studies is the ubiquity of the response of this variant tRNA to a variety of perturbations in the codon-anticodon helix (a number of first- and third-position mismatches). These data led to the suggestion that this tRNA mutation in the D arm functions to constitutively activate the pathway, independently of the signals normally required in the decoding center. Here we show that the same miscoding tRNA is unable to suppress defects in tRNA selection associated with mutation of the decoding-site nucleotides (**Fig. 3a**). These data indicate that disruption of the decoding-site interactions by substituting the unpaired adenosines is not equivalent to disruption by substituting the receptor base pair; in fact, each of these components might contribute to distinct events required for ultimate tRNA acceptance. This idea is further supported by two observations. First, the decoding-site variant ribosomes show even greater defects with a near-cognate codon-anticodon interaction (data not shown), consistent with synergistic contributions from these two independent events. Second, whereas the Hirsh suppressor has faster rates of dipeptide formation on near-cognate codons than the wild-type $tRNA^{Trp}$, addition of paromomycin further increases both the rates and end-points (data not shown).

The conclusions presented above can be summarized in a model where there are two different requirements for tRNA selection on the ribosome, one of which is likely to be related to the rearrangements in the decoding site and the small subunit, and the other to conformational changes in the ternary complex. Our initial analyses of decoding led to models involving a single energy barrier that has to be crossed twice during tRNA selection, first for GTPase activation and then for tRNA accommodation. In such models, near-cognate codon-anticodon pairs raise the energy barrier, thus decreasing both forward rate constants, whereas miscoding agents such as the aminoglycosides and the Hirsh suppressor lower the barrier and thus increase near-cognate misincorporation. The present data showing the inability of the Hirsh variant tRNA to suppress decoding-site mutants is inconsistent with such a single-barrier model. The simplest model that reconciles these data involves two roughly equivalent energetic barriers that must be overcome during each stage (initial selection and proofreading) in the tRNA selection process (**Fig. 4**). Our data suggest that introducing a mismatch in the codon-anticodon helix predominantly raises one of the barriers, thus slowing the rates of GTPase activation and tRNA accommodation. The variant miscoding tRNA counteracts this effect by specifically lowering this activation barrier. By contrast, mutations in the decoding site itself (at A1492, A1493 or G530) raise the other barrier (or potentially both) and thus also cause a decrease in both forward rate constants (GTPase activation and accommodation). The mutant tRNA, though capable of lowering one of the energy barriers, does not affect the other and thus does not rescue the rate defects of the variant ribosomes. We propose that paromomycin is able to lower both energy barriers, thus accelerating GTPase activation and tRNA accommodation on the variant ribosomes as well as with the codon-anticodon mismatches. It is important to note that the energy diagrams shown in **Figure 4** depict the potential consequences of independent molecular recognition events during tRNA selection but give no indication of a temporal sequence. Our data do not allow us to evaluate whether these molecular conditions must be met simultaneously or sequentially.

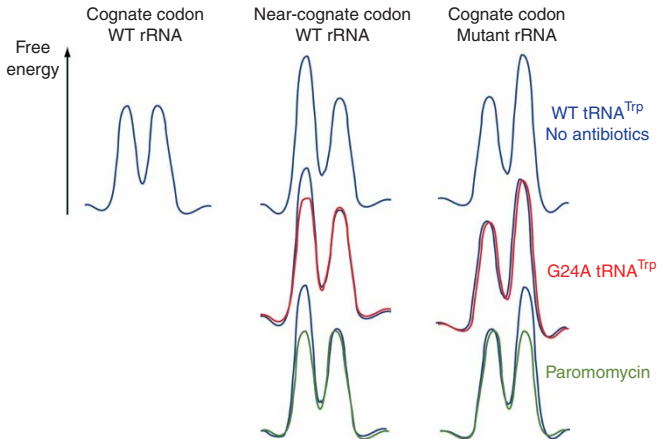

Figure 4 Scheme for a two-barrier model for tRNA selection. Two energy barriers would have to be crossed for both GTPase activation and tRNA accommodation (left). One of these barriers can be affected by disrupting the decoding-site interactions by introducing a mismatch in the codon-anticodon helix (center) or 16S rRNA mutations (right). The proposed effects of the variant G24A $tRNA^{Trp}$ and paromomycin are shown in red and green respectively (see text for description). WT, wild-type.

The barriers that we describe limit the progression of the tRNA through the selection process and are thus at the heart of the communication between the small-subunit decoding center and remote regions of the ribosome involved in GTPase activation and tRNA accommodation. In addition, although there has been much discussion of the possibility of multiple paths for communication during tRNA selection[2], these data provide concrete evidence that more than one molecular recognition event must contribute. It seems reasonable to suggest that one barrier is related to the molecular properties of the tRNA and the consequences of its deformability[21], whereas the other is related to molecular rearrangements in the

decoding center and the downstream consequences of these[7]. These two events probably converge to achieve the global rearrangements that are thought to be fundamental to the process of tRNA selection[8]. The molecular contributions that we identify here may ultimately be correlated with intermediate states in tRNA selection visualized by earlier fluorescence and FRET-based approaches[12,37]. A notable consequence of such a mechanism dependent on two independent molecular requirements for ultimate tRNA acceptance is that, through its redundancy, tighter control on the specificity of this crucial step of gene expression can be imposed.

METHODS

Buffers. Buffer A for ribosome preparation contained 20 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA and 6 mM β-mercaptoethanol (BME). Sucrose cushions contained 1.1 M sucrose, 20 mM Tris-HCl (pH 7.5), 500 mM NH_4Cl, 10 mM $MgCl_2$ and 0.5 mM EDTA. MS2 binding buffer was composed of 20 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl and 10 mM $MgCl_2$, and the elution buffer was the same with 10 mM reduced glutathione added. Reaction buffer was HiFi unless otherwise indicated; HiFi contains 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 3.5 mM $MgCl_2$, 0.5 mM spermidine, 8 mM putrescine and 2 mM DTT. Buffers for tRNA preparation were as described[16].

Expression and purification of MS2-tagged ribosomes. Strain DH5α (Invitrogen) was transformed with pLK35-derived plasmids that carry a copy of the *rrnB* operon under control of a λ promoter[38]. These plasmids had an MS2 tag inserted in the spur region of 16S rRNA[30] and either the wild-type decoding-site sequence or the indicated substitutions (made by QuikChange, Invitrogen). A plasmid containing a temperature-sensitive allele of the cI repressor was cotransformed to allow for expression repression. Cultures were grown to saturation at 30 °C, diluted 1:35 into LB medium with 100 μg ml^{-1} ampicillin and grown for 2–2.5 h at 42 °C to an A_{600} of 0.6–0.7. Cells were pelleted and resuspended in buffer A and cracked using a French press, and the lysates were clarified by centrifugation. The clarified lysates were pelleted over sucrose cushions in a Beckmann Ti45 rotor for 16 h at 37,000 r.p.m. (107,000*g*). Ribosome pellets were resuspended in buffer A and purified over an FPLC GST-Trap column (Pharmacia) to which a GST-MS2 coat protein fusion had been prebound. Elution was carried out with glutathione and eluted ribosomes were concentrated over Amicon Ultra filters (MWCO 100,000, Millipore). Purified ribosomes were depleted of 50S subunits, so purified MRE600 50S subunits were added back for the formation of initiation complexes.

Purification of MRE600 50S subunits. *E. coli* MRE600 (ATCC 29417) was grown in LB to an A_{600} of 0.6–0.8, and crude 70S ribosomes were prepared as described above. 10,000–15,000 A_{260} units of 70S ribosomes were dialyzed overnight against the same buffer, but with 1 mM $MgCl_2$ to promote subunit dissociation. These samples were loaded onto a 10%–30% (w/v) sucrose gradient in buffer A with 1.5 mM $MgCl_2$ and separated by zonal centrifugation in a Ti15 rotor at 26,000 r.p.m. (36,600*g*) for 20 h. RNA was extracted from the collected fractions and analyzed on 4% (w/v) polyacrylamide gels run in 1× TBE with 7 M urea. Fractions containing only 23S RNA were pooled, $MgCl_2$ was added to 10 mM and ribosomes were pelleted in a Ti45 rotor at 37,000 r.p.m. (107,000*g*) for 18 h. Pellets were resuspended in HiFi buffer and stored in aliquots at –80 °C. We tested 50S subunit preparations for contamination with 30S subunits by carrying out a dipeptide-formation assay in the absence of added 30S subunits.

Expression and purification of wild-type and G24A $tRNA^{Trp}$. tRNAs were overexpressed and purified from strain MY87 as described[16].

Kinetic assays. Initiation complexes containing wild-type or mutant ribosomes, f[^{35}S]Met-$tRNA^{fMet}$ and mRNA having AUG and UGG codons in the P and A sites, respectively, were formed and purified as described[3,16]. Ternary complexes with wild-type or G24A $tRNA^{Trp}$, EF-Tu and either unlabeled GTP or [γ-^{32}P]GTP were also formed and purified as described[3,16]. Complexes were mixed in a rapid-quench apparatus, and the reaction was stopped at the desired time point with 0.5 M KOH for the peptide bond formation assay or with 40% (v/v) formic acid for the GTPase assay. The products of the peptide bond formation reaction were resolved by electrophoresis on cellulose thin-layer chromatography plates as described[3,16]. The GTPase products were analyzed by PEI cellulose thin-layer chromatography in 0.5 M KH_2PO_4 at pH 3.5.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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