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A minimum structure of aminoglycosides that causes an initiation shift of *trans*-translation

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ABSTRACT

***Trans*-translation is an unusual translation in which transfer-messenger RNA plays a dual function—as a tRNA and an mRNA—to relieve the stalled translation on the ribosome. It has been shown that paromomycin, a typical member of a 4,5-disubstituted class of aminoglycosides, causes a shift of the translation-resuming point on the tmRNA by −1 during *trans*-translation. To address the molecular basis of this novel effect, we examined the effects of various aminoglycosides that can bind around the A site of the small subunit of the ribosome on *trans*-translation *in vitro*. Tobramycin and gentamicin, belonging to the 4,6-disubstituted class of aminoglycosides having rings I and II similar to those in the 4,5-disubstituted class, possess similar effects. Neamine, which has only rings I and II, a common structure shared by 4,5- and 4,6-disubstituted classes of aminoglycosides, was sufficient to cause an initiation shift of *trans*-translation. In contrast, streptomycin or hygromycin B, lacking ring I, did not cause an initiation shift. The effect of each aminoglycoside on *trans*-translation coincides with that on conformational change in the A site of the small subunit of the ribosome revealed by recent structural studies: paromomycin, tobramycin and geneticin which is categorized into the gentamicin subclass, but not streptomycin and hygromycin B, flip out two conserved adenine bases at 1492 and 1493 from the A site helix. The pattern of initiation shifts by paromomycin fluctuates with variation of mutations introduced into a region upstream of the initiation point.**

INTRODUCTION

Transfer-messenger RNA or tmRNA (also known as 10Sa RNA or SsrA RNA), having both tRNA and mRNA properties, is widely distributed among eubacteria and has also been found in some chloroplasts and mitochondria (1–3). That the upper-half structure of this molecule mimicks tRNA has been shown by comparative, chemical and functional studies (4–8). The mRNA domain, encoding the last 10 amino acids of the 11-amino-acid tag-peptide fused at the truncated C-termini of various proteins expressed in eubacteria (9–13), is surrounded by four pseudoknot structures in the middle of this molecule. It has been proposed that molecular interplay between these two functions of this molecule facilitates an unusual translation reaction, *trans*-translation, in which a ribosome can switch from the translation of a truncated mRNA to the tag-encoded sequence of a tmRNA (6,10,14). This relieves stalled translation from those mRNAs lacking a stop codon or possessing a cluster of rare codons (15) with the addition of a tag-peptide as a degradation signal to the truncated C-termini of polypeptides decoded. These processes may promote recycling of ribosomes and the degradation of truncated mRNAs (16) and prevent the accumulation of abortively synthesized polypeptides involved in the normal cell growth (17,18) and other biological events (19–22).

Although several tmRNA-binding proteins including SmpB (23) have been identified, the mechanism by which tmRNA resumes translation from the first GCA codon for a tag-peptide is mysterious, since it involves no apparent codon–anticodon interaction before the first translocation event. The first pseudoknot (PK1) 12 nucleotides upstream of the tag-initiation point, and not the other three (PK2–PK4) downstream of the tag-encoding region, has been shown to be important for efficient tag-translation (24–26). It has also been shown that several nucleotides upstream of the tag-encoding region are involved in efficient tag-translation both *in vivo* and *in vitro* (27,28). Besides, some base substitutions around this region shift the tag-initiation point by −1 *in vitro*. These sequences on the tmRNA may have an intermolecular interaction with a ribosome directly or by means of another *trans*-acting factor to serve as a determinant for tag-translation.

Recent progress in the analysis of the tertiary structures of the ribosome and its complexes with various antibiotics has contributed greatly to an understanding of the molecular mechanism of the translation at the atomic level (29,30). Aminoglycosides are extensively studied antibiotics that bind around the A site on the small subunit to modulate coding accuracy and/or efficiency. In a previous study, we reported that paromomycin, which belongs to the 4,5-disubstituted class of aminoglycoside antibiotics, causes a shift in

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the translation-resuming point on tmRNA by −1 *in vitro* (31). Although paromomycin molecules also bind tmRNA at the tRNA domain to compete with SmpB and at the connector region between the tRNA and mRNA domains (31,32), the initiation shift of *trans*-translation is attributable to paromomycin bound at the major groove of the A site of the penultimate (44th) helix of the small subunit RNA of the ribosome. Thus, the initiation shift can be caused by the modulation of an interaction between tmRNA and the ribosome responsible for determination of the start point. The effect on *trans*-translation differs substantially from that on canonical translation in which paromomycin induces miscoding rather than a frameshift. In the present study, to address the molecular basis of this novel effect, we examined the effects of various aminoglycosides that can bind around the A site of the small subunit of the ribosome on *trans*-translation *in vitro*. It was found that the core structure, rings I and II, shared by the 4,5- and 4,6-disubstituted classes of aminoglycosides, is sufficient for this effect. We also found a relationship between the effects on the initiation shift of *trans*-translation and on the structure of the A site revealed by recent structural studies.

MATERIALS AND METHODS

Preparation of tmRNA and its mutant

Mutations were introduced into the tmRNA gene of *Escherichia coli* by primer-directed PCR, and the amplified DNA fragment was ligated under the T7 RNA polymerase promoter sequence of the plasmid pGEMEX-2. This plasmid was cotransformed with pACYC184 encoding the T7 RNA polymerase gene under the *lac*-promoter sequence into *E.coli* strain W3110 Δ*ssrA*, which lacks the tmRNA gene (6). The tmRNA was induced by the addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside tmRNA and purified as described previously (5). The nucleic acid fraction was extracted with phenol from mid-log-phase cells and then subjected to ethanol precipitation. After performing phenol extraction and ethanol precipitation, the resulting fraction was subjected to differential isopropylalcohol precipitations to roughly remove DNA, followed by incubation with RNase-free DNase I (Pharmacia). The tmRNA waspurified by electrophoresis on a 5% polyacrylamide gel containing 7 M urea and heated for 3 min at 75°C and slowly cooled down for 1 h at room temperature with refolding buffer (10 mM HEPES-KOH (pH 7.5), 5 mM magnesium chloride and 20 mM ammonium chloride). Spectrophotometric measurements were made to determine the concentration of RNA. Two A_{260} units of tmRNA are usually yielded by 1 liter of culture.

In vitro amino acid incorporation in the presence of poly(U)

The preincubated S30 fraction was prepared from mid-log-phase cells of *E.coli* strain W3110 (Δ*ssrA*) as described previously (33). The reaction mixture (100 µl) contained 80 mM Tris–HCl (pH 7.8), 7 mM magnesium acetate, 150 mM ammonium chloride, 2.5 mM DTT, 5 mM phosphoenolpyruvate, 1 mM ATP, 0.2 mM GTP, 20 µM of one of L-[U-^{14}C]alanine (6.7 GBq/mmol), L-[U-^{14}C]arginine (11.8 GBq/mmol), L-[U-^{14}C]serine (6.3 GBq/mmol), L-[U-^{14}C]threonine (7.7 GBq/mmol) or L-[U-^{14}C]phenylalanine (18.4 GBq/mmol) and 0.05 mM each of the remaining unlabeled 19 amino acids, 100 nM tmRNA, 20 µl of the S30 fraction, about 5.7×10^2 A_{260} and 2.5×10^2 A_{280}, and 250 µg of poly(U) (50–100mer; Sigma). The reaction mixture was incubated at 37°C. At each time point, a 23.5 µl aliquot was withdrawn from a 100 µl reaction mixture and spotted on a Whatman 3MM filter paper, and radioactivity in the hot trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter. The value of poly(U)-dependent amino acid incorporation was obtained by subtracting the value in the absence of poly(U) from that in the presence of poly(U).

RESULTS

Not only the 4,5-disubstituted class, but also the 4,6-disubstituted class of aminoglycosides caused an initiation shift of *trans*-translation

Amino acids comprising the tag-peptide are incorporated in a stoichiometrical fashion in the *in vitro* poly(U)-dependent polypeptide synthesis system using the S30 fraction extracted from tmRNA-depleted cells (33). Using this system, we can evaluate not only the efficiency of *trans*-translation but also the initiation point of tag-translation (28). The tag-peptide from the normal initiation point (AANDENYALAA) contains five alanine residues. The tag-peptide from −1 (ARKRRKLRFSSLITCLEPSLPSLRS) contains one alanine, five arginine, five serine and one threonine residues, and the tag-peptide from +1 (AQTTKTTL) contains one alanine and four threonine residues. In the present study, the effects of aminoglycosides on *trans*-translation were examined by measuring levels of alanine, arginine, glycine, serine and threonine incorporated in this system.

As shown in a previous study (31), in the presence of paromomycin, a typical 4,5-disubstituted class of aminoglycosides having rings I, II, III and IV in which a five-membered ring III is linked to position 5 of ring II (4,5-2-deoxystreptamine ring) (Figure 1), the level of alanine incorporation was decreased and, instead, significant levels of incorporation of typical −1 frame amino acids, arginine and serine, were observed. Considering the amino acid compositions of the tag-peptides of three frames, ~60% of the total detected peptides were tag-peptides from position −1 and the rest were tag-peptide from the normal initiation point in 55 µM paromomycin. Since paromomycin binds to the A site of the decoding helix (44th helix) to flip out two adenine bases at the A site (34,35), the observed effect might be caused by the modulation of an interaction between tmRNA and the ribosome responsible for determination of the initiation point.

In the present study, we examined another class of aminoglycosides, 4,6-disubstituted class of aminoglycosides having rings I, II and III in which a six-membered ring III is linked to position 6 of ring II (Figure 1). The 4,6-disubstituted class of aminoglycosides can be further divided into two subclasses, the kanamycin subclass, including kanamycin, tobramycin and amikacin, and the gentamicin subclass, including gentamicin and geneticin (G418), based on their substitution patterns. Both subclasses bind to the major groove of the A site of the 44th helix to provide a conformational change similar

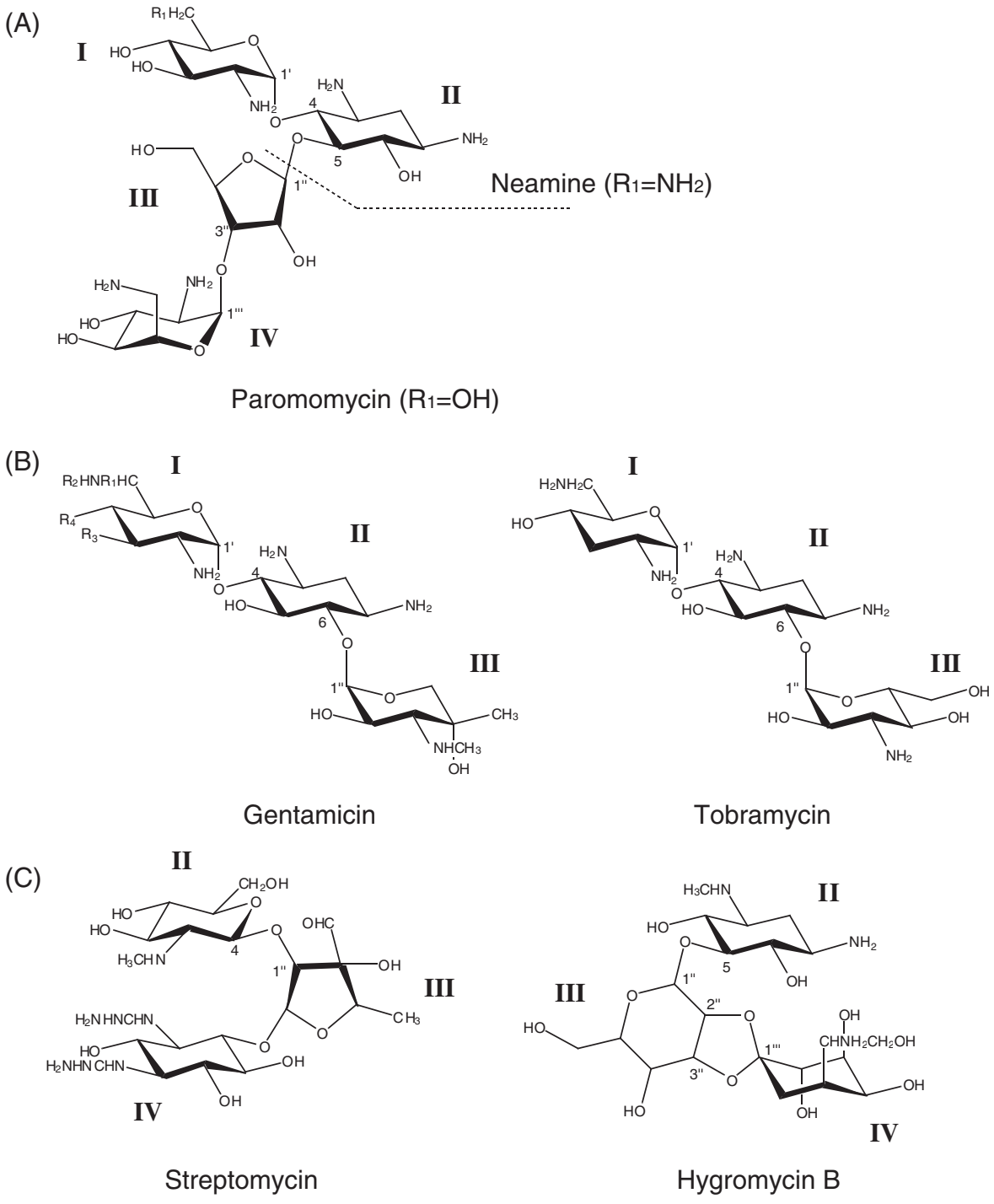

Figure 1. Chemical structures of aminoglycosides used in this study. (**A**) The 4,5-disubstituted class of aminoglycosides, paromomycin and neamine. (**B**) The 4,6-disubstituted class of aminoglycosides, gentamicin and tobramycin. Gentamicin is a mixture of gentamicin C1a (R_1=H, R_2=H, R_3=H and R_4=H), gentamicin C1 (R_1=CH_3, R_2=CH_3, R_3=H and R_4=H) and gentamicin C2 (R_1=H, R_2= CH_3, R_3=H and R_4=H). Geneticin (R_1=CH_3, R_2=OH, R_3=OH and R_4=OH) is also categorized into the gentamicin subclass. (**C**) Structurally dissimilar aminoglycosides, streptomycin and hygromycin B.

to that of the 4,5-disubstituted class (36–39). Varying concentrations of tobramycin or gentamicin were added to the reaction mixture for poly(U)-dependent tag-translation. As in the case of paromomycin, tobramycin reduced the level of alanine incorporation and, instead, increased the levels of incorporation of typical −1 frame amino acids, arginine and serine (Figure 2A). A similar effect was observed when gentamicin was added to the reaction mixture (Figure 2B). The effect was most pronounced at 55 μM tobramycin or gentamicin, in which ∼70% of tag-peptides was estimated to be from −1.

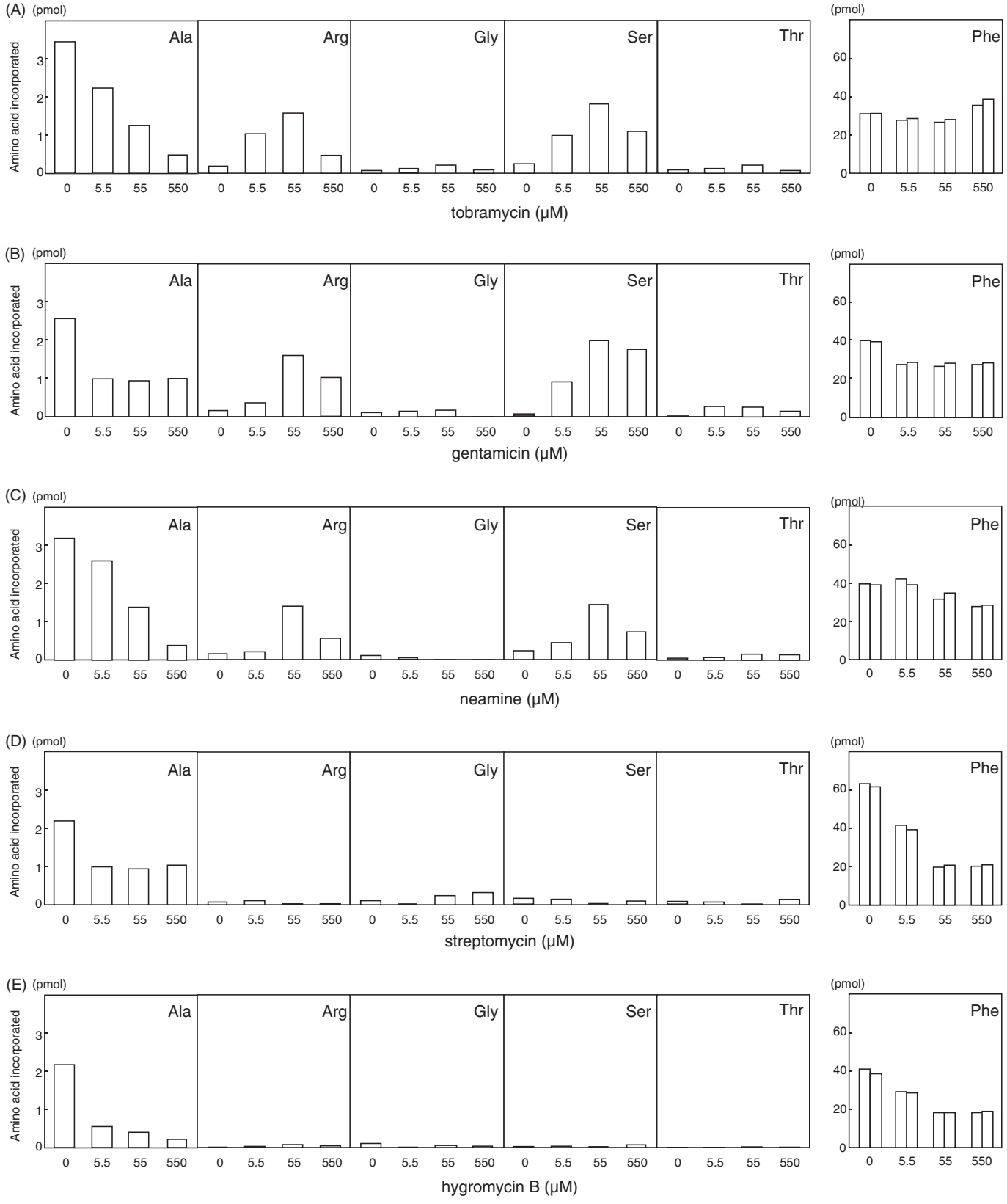

Figure 2. Effects of aminoglycoside on the poly(U)-dependent incorporations of amino acids directed by *E.coli* tmRNA in the presence of various aminoglycosides. The levels of incorporation of alanine, arginine, glycine, serine, threonine and phenylalanine were measured in the presence of varying concentrations of: (**A**) tobramycin, (**B**) gentamicin, (**C**) neamine, (**D**) streptomycin and (**E**) hygromycin B. The value of poly(U)-dependent incorporation of each of the amino acids except phenylalanine was obtained by subtracting the value in the absence of tmRNA from that in the presence of tmRNA. For phenylalanine incorporation, both the values in the presence (left) and absence (right) of tmRNA are shown.

Neamine, a minimum structure of 4,5- and 4,6-disubstituted classes of aminoglycosides, also caused an initiation shift of *trans*-translation

Neamine, which has only rings I and II, a common structure to both 4,5- and 4,6-disubstituted classes of aminoglycosides (Figure 1), also binds the major groove of the 44th helix (40). Ring II is identical to that of paromomycin, and the 6-OH group of ring I of paromomycin is substituted by the 6-NH_2 group. This simple structure also reduced the level of alanine incorporation and increased the levels of incorporation of arginine and serine (Figure 2C). The effect was most pronounced at 55 μM neamine, in which ~50% of tag-peptides was estimated to be from −1.

Streptomycin or hygromycin B did not cause an initiation shift of *trans*-translation

We also examined other types of aminoglycosides, streptomycin and hygromycin. Streptomycin comprises rings II, III and IV, in which five-membered ring III is linked to position 4 of ring II (Figure 1). Hygromycin B also comprises rings II, III and IV, in which six-membered ring III is linked to position 5 of ring II. Like 4,5- and 4,6-disubstituted classes of aminoglycosides, both streptomycin and hygromycin B bind around the A site of the 44th helix of 16S rRNA (29,30). Both aminoglycosides reduced the incorporation of alanine, whereas none of them induced incorporation of any of arginine and serine (Figure 2D and E).

Effects of aminoglycosides on the mutant tmRNA-directed *trans*-translation

Lee *et al*. (28) found several base-substitutions upstream of the tag-encoding region that caused a significant level of initiation shift of tag-translation by −1 *in vitro*. It is possible that this upstream region undergoes an interaction with the ribosome to determine the tag-initiation point and that the observed initiation shift is due to the perturbation of this interaction by base substitution. If this is the case, such an intermolecular interaction around the A site might also be perturbed by aminoglycosides.

In this study, we examined the effect of paromomycin on tag-translation mediated by the 84UG mutant having an AU-to-UG mutation six to five nucleotides upstream of the tag-initiation point (Figure 3). In the absence of aminoglycosides, ~60% of the total tag-peptides from this mutant tmRNA was estimated to be initiated from −1 (Figure 4B). In the presence of 55 μM paromomycin, the ratios of incorporation of alanine, arginine, serine and threonine was similar to that in the absence of paromomycin, although their levels were lower (Figure 4B). This result indicates that paromomycin induced no further shift in the initiation point for this initiation-shift mutant.

We also examined the effect of paromomycin on incorporation of some upstream mutants, 84CC, 86GA and 88AG mutants (Figure 3), in which almost none or only a faint level of incorporation of any among arginine, serine and threonine was detected in the absence of paromomycin (Figure 4C–E). In the presence of 55 μM paromomycin, these frame-shifted amino acids were obviously incorporated for these three mutants. The ratio of −1 frame amino acid, serine or arginine, to alanine fluctuated with the variation of

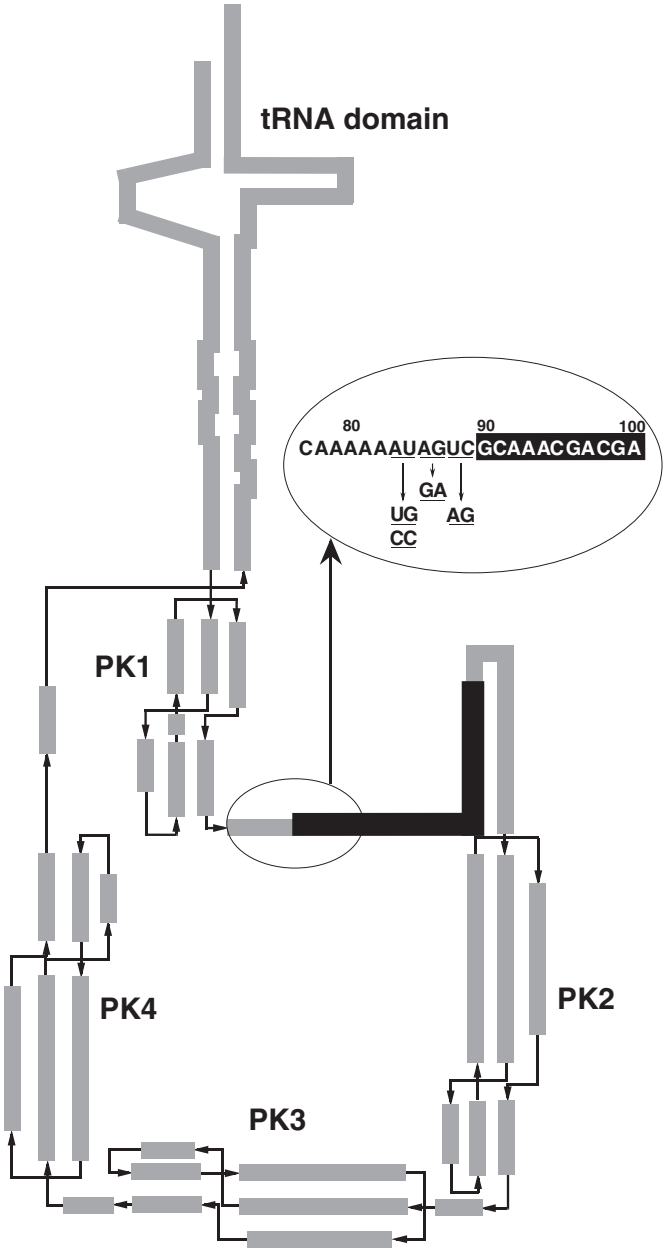

Figure 3. Secondary structure of *E.coli* tmRNA. The region around the tag-initiation point is highlighted in the oval, in which the mutations used in this study are shown. The tag-encoding region is designated by black colour.

mutations. The −1 frame amino acids were incorporated more than alanine in the 84CC mutant. Not only −1 frame amino acids but also a typical +1 frame amino acid, threonine, was incorporated in the 86GA mutant.

DISCUSSION

The initiation point of tag-translation is precisely determined by an as yet unidentified mechanism to ensure the degradation of the *trans*-translation products. This mechanism can be perturbed when mutation is introduced into the upstream region of the tag-initiation point, bearing −1 initiation-shifted

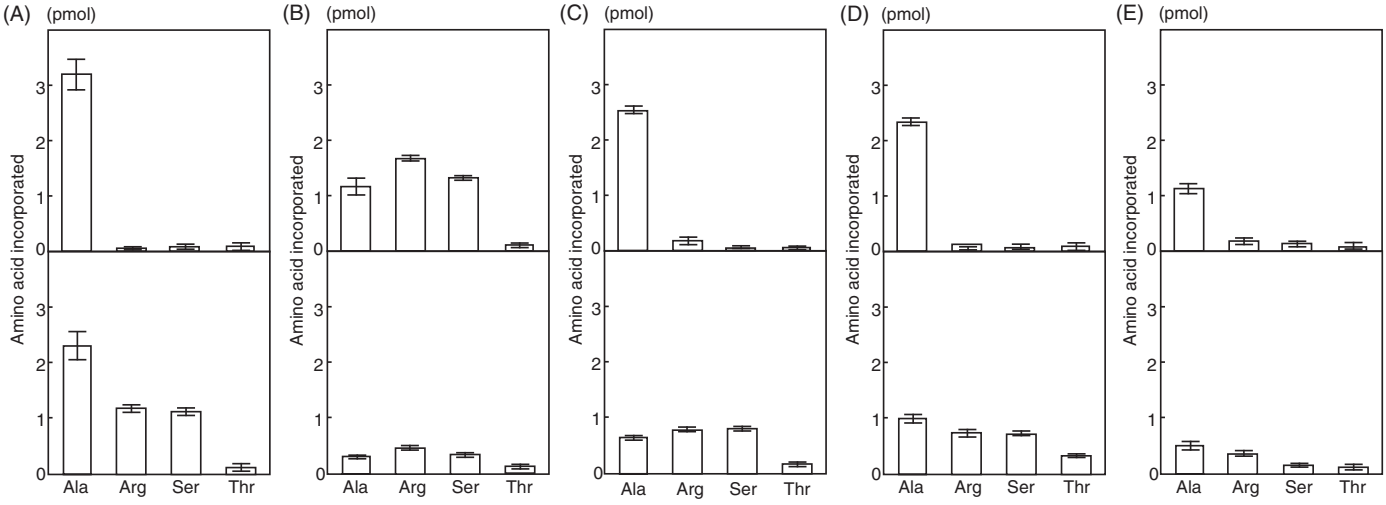

Figure 4. Effects of paromomycin on the poly(U)-dependent amino acid incorporations directed by *E.coli* tmRNA mutants. The levels of poly(U)-dependent incorporation of alanine, arginine, serine and threonine directed by: (**A**) wild-type tmRNA (31) and (**B**) 84UG, (**C**) 84CC, (**D**) 86GA and (**E**) 88AG mutants were measured in the presence (lower) or absence (upper) of 55 μM paromomycin. The value of poly(U)-dependent incorporation of each of the amino acids was obtained by subtracting the value in the absence of tmRNA from that in the presence of tmRNA.

trans-translation products (28). In the present study, we found that both 4,5- and 4,6-disubstituted classes of aminoglycosides also caused an initiation shift of *trans*-translation by −1. Both classes of aminoglycosides share a common 4,5-2-deoxystreptamine ring (ring II) of which position 4 is linked to ring I. Neamine, a minimum structure of these aminoglycosides comprising only rings I and II, was sufficient to cause initiation shift of *trans*-translation. Thus, it was concluded that rings I and II play an essential role in this event. Although with variation of the substitution pattern, both classes of aminoglycosides including neamine bind to the major groove of the A site of the penultimate helix to flip out two adenine bases at 1492 and 1493 from the helix (30,34,35,40). A recent structural study has indicated that the binding of paromomycin slightly reduces the distance between helix 44 and the shoulder (S12) even in the absence of tRNA to fix the closed conformation of the small subunit from the open conformation (41). This may reduce the energy cost of tRNA binding, leading to miscoding by stabilizing the conformation of the A site, which normally occurs only when a cognate codon–anticodon interaction takes place (42).

We also found that other classes of aminoglycosides, streptomycin and hygromycin B, both lacking ring I, caused no detectable level of initiation shift of *trans*-translation, supporting the above conclusion that rings I and II play an essential part. Hygromycin B also binds to the major groove of the A site of helix 44, and the binding site is very close to and partially overlaps the site to which the 4,5- or 4,6-disubstituted class of aminoglycosides bind. Streptomycin binds to the region between the A site of helix 44 and S12 to cause miscoding by modulating both initial selection of cognate tRNA at the A site and subsequent GTP hydrolysis by EF-Tu (43). As in the case of paromomycin, streptomycin also reduces the distance between helix 44 and the shoulder, although in a way slightly different from that in the presence of paromomycin or cognate tRNA (41). A conformational change in the ribosome has also been detected by a toe printing study, in which the conformational change was found to be induced not only by the 4,5- or 4,6-disubstituted class of aminoglycosides but also by streptomycin or hygromycin (44). More importantly, neither streptomycin nor hygromycin B flips out A_{1492}/A_{1493} (29,30). Thus, we postulate that the observed initiation shift of *trans*-translation by the 4,5- or 4,6-disubstituted class of aminoglycosides is due to the flipping out of A_{1492}/A_{1493} from the decoding helix.

It has been shown that some base substitutions upstream the tag-encoding region reduce the efficiency of *trans*-translation, while other base substitutions, such as U_{-5}-to-A_{-5} mutant (85A) and $A_{-6}U_{-5}$-to-$U_{-6}G_{-5}$ mutant (84UG), cause a shift of the initiation point of *trans*-translation (28). Although the molecular basis for this shift has not been rationalized, the vast majority is a shift by −1. Interestingly, the initiation shift by aminoglycosides is also a shift by −1. It is possible that base substitutions around the initiation point or A_{1492}/A_{1493} untimely flipped out from helix 44 perturb some intermolecular interactions involving the upstream sequence which is important for determination of the initiation point of tag-translation. Alternatively, they may produce an unexpected interaction with the upstream sequence to modulate the normal determination of the initiation point. If the effects of base substitutions in the upstream region and of aminoglycosides are independent of each other, a combination of them would produce an additive effect. However, paromomycin induced no further shift of *trans*-translation directed by a typical initiation shift mutant, 85A, in a previous study (31). In the present study, this was also the case for another initiation shift mutant, 84UG (Figure 4B).

The present study also showed that the initiation point of tag-translation in the presence of paromomycin fluctuated with variation of the nucleotide sequence upstream of the tag-encoding region (Figure 4C–E). This result also indicates that the effect of base substitutions in the upstream region has some connection with that of aminoglycosides, supporting the idea that some of these upstream nucleotides have

important interaction with A_{1492}/A_{1493} that has flipped out from the decoding helix, which can be disturbed either by base substitution or by paromomycin. Alternatively, A_{1492}/A_{1493} that has flipped out unexpectedly from the decoding helix by the addition of aminoglycosides might undergo an unexpected interaction with the upstream bases prior to the first tRNA selection, leading to the –1 initiation shift of *trans*-translation, and that substitutions of these bases affect the interaction, leading to the fluctuation of the initiation point. At which step the upstream region on tmRNA accesses the A site and how it does so have yet to be determined.

Several tmRNA-binding proteins such as SmpB, which is involved in binding to ribosome and the enhancement of aminoacylation with alanine (23,45–47), ribosomal protein S1 (45,48) and EF-Tu (49–52) have been identified. Some of them can interact with the upstream region on tmRNA in any step of *trans*-translation to determine the initiation point. Since ribosomal protein S1 can cross link with U_{85} which is 5 nt upstream of the tag-initiation point as well as PK2 and PK3 (48), it is a candidate for the recognition of the upstream sequence, although it is not universal among the eubacterial kingdom (53). SmpB, another tmRNA-binding protein, binds to the tRNA domain rather than the mRNA domain (47,54,55). A recent cryo-EM study has revealed a tertiary structure of a complex of ribosome, EF-Tu(GDP), SmpB and kirromycin, which can represent a *trans*-translation step just prior to dissociation of the GDP form of EF-Tu (56). In this complex, the coding region as well as the upstream region is distant from the decoding region, and S1 is missing around the upstream region on tmRNA and the decoding region on the ribosome. It is possible that the proposed interaction between the upstream region and the flipped-out adenine bases occurs at a later step.

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