Various effects of paromomycin on tmRNA-directed trans-translation

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SUMMARY

Trans-translation is an unusual translation in which tmRNA plays a dual function as a tRNA and an mRNA to relieve the stalled translation on the ribosome. In this study, we examined the effects of an aminoglycoside antibiotic, paromomycin, on several tmRNA-related events in vitro. The results of a chemical footprinting study indicated that paromomycin molecules bind tmRNA at G332/G333 in the tRNA domain and A316 in the middle of the long helix between tRNA and mRNA domains. Paromomycin bound at G332/G333 inhibited aminoacylation, and the inhibition was suppressed by the addition of SmpB, a tmRNA binding protein. It was also found that paromomycin causes a shift of the translation-resuming point on tmRNA by –1. The effect on initiation-shift was canceled by a mutation at the paromomycin binding site in 16S rRNA, but not by mutations in tmRNA. A high concentration of paromomycin inhibited trans-translation, whereas it enhanced the initiation-shifted trans-translation when SmpB was exogenously added or a mutation was introduced at 333. The effect of paromomycin on trans-translation differs substantially from that on canonical translation, in which it induces miscoding by modulating the A site of the decoding helix of the small subunit RNA of the ribosome.
INTRODUCTION

tmRNA (transfer-messenger RNA, also known as 10Sa RNA or SsrA RNA) is widely distributed among eubacteria and has also been found in some chloroplasts and possibly in some mitochondria (1-4). It is a novel molecule with both tRNA and mRNA properties. The tRNA mimicry of the upper-half structure of this molecule has been shown by comparative studies (5-9), chemical and enzymatic probing studies (8,10), the presence of tRNA-specific modified nucleosides in the putative TΨC-loop (11) and the capacity to accept alanine (5,6). The mRNA domain, encoding the last ten amino acids of the 11-amino-acid tag-peptide, is surrounded by four pseudoknot structures in the middle of this molecule. The tag-peptide was first found at the C-terminus of a fraction of mouse interleukin-6 expressed in *Escherichia coli* (12). Later, it was also found on other polypeptides when they are translated from artificial mRNAs lacking a termination codon (4) or possessing a cluster of rare codons (13) and from endogenous mRNAs in *E. coli* and *Bacillus subtilis* (14-16).

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 tmRNA. This would relieve stalled translation from mRNAs lacking a stop codon or possessing a cluster of rare codons with the addition of a specific tag-peptide as a degradation signal to the truncated C-termini of polypeptides decoded (4,7,17,18). This *trans*-translation model has also been supported by several other findings showing that the function as a tRNA is a prerequisite for the function as an mRNA *in vitro* (19), that an amino acid aminoacylated to tmRNA is actually incorporated into the tag-peptide (20) and that tmRNA binds predominantly to 70S ribosomes (4,21,22). A series of these processes may promote recycling of ribosomes and prevent accumulation of abortively synthesized polypeptides, providing some advantage to the cell for survival (16,23,24).
The mechanism by which tmRNA resumes translation from the first GCA codon for a tag-peptide is a central issue in the trans-translation. This resumption of translation is a mystery 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, but not the other three pseudoknots (PK2-PK4) downstream of the tag-encoding region, has been shown to be important for efficient tag-translation (25-27). It has also been shown both in vivo and in vitro that several nucleotides upstream of the tag-encoding region are involved in efficient tag-translation (28,29). Besides, some base substitutions around this region shift the tag-initiation point by –1 in vitro (29). Apart from the tag-encoding region, some nucleotides in the tRNA domain have been identified to be crucial for the mRNA function but not for the tRNA function (30). These sequences on tmRNA might have an intra- or intermolecular interaction to serve as a determinant for tag-translation. Several tmRNA-binding proteins such as EF-Tu (31-33), SmpB (34,35) and ribosomal protein S1 (36) have been identified, although how these proteins function in the ribosomal processes of trans-translation remains elusive.

In the present study, the effects of paromomycin, which belongs to a neomycin-class of aminoglycoside antibiotics, on several tmRNA-related events were examined in vitro. Paromomycin is known to interact with the A site of the decoding region of the 30S subunit of the ribosome to induce miscoding. In the present study, we found that paromomycin molecules bind tmRNA. We also found that it inhibits the aminoacylation of tmRNA and that it induces an initiation-shift of trans-translation. We identified which paromomycin molecule exerts each effect. The last effect was found to be caused not by paromomycins bound to tmRNA but by that bound to the decoding region of the ribosome. This effect differs substantially from that on canonical translation, in which paromomycin induces miscoding by modulating the decoding helix of the small subunit RNA of the ribosome.
EXPERIMENTAL PROCEDURES

Preparation of tmRNA and its mutants - Mutations were introduced into the *E. coli* tmRNA gene by primer-directed polymerase chain reaction, 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 ΔssrA, which lacks the tmRNA gene (6). tmRNA induced by the addition of 1.0 mM isopropyl-1-thio-β-D-galactopyranoside was purified as described previously (5). The nucleic acid fraction was extracted with phenol from mid-log-phase cells followed by 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 (Amersham Pharmacia Biotech). tmRNA was purified by electrophoresis on a 5% polyacrylamide gel containing 7M urea. Spectrophotometric measurements were made to determine the concentration of RNA. 2.0 A260 units of tmRNA are usually yielded from 1 liter of culture. After purification from the gel, each tmRNA variant was used with or without refolding procedure by heating at 75 °C for 3 min and cooling for 60 min at room temperature in a solution containing 10 mM HEPES-KOH (pH 7.5), 5 mM magnesium chloride and 20 mM ammonium chloride. And we confirmed that the refolding procedure does not affect the results.

Chemical footprinting - Chemical modifications and subsequent reverse transcription was carried out according to the method of Stern et al. (37) with a slight modification. For DMS modification, after 1 µM tmRNA had been incubated for 60 min at room temperature in a 50-µl reaction mixture of 80 mM K-HEPES (pH 7.0), 10 mM magnesium chloride, 150 mM potassium chloride, 6 mM 2-mercaptoethanol and varying concentrations of paromomycin, 5 µl of 16.6 % (v/v) DMS in 95% ethanol was added and the mixture was incubated for 60 min on ice. For KE modification, after 1 µM
tmRNA had been incubated for 60 min at room temperature in a 50-µl reaction mixture of 80 mM K-HEPES (pH 7.8), 10 mM magnesium chloride, 150 mM potassium chloride, 6 mM 2-mercaptoethanol and various concentrations of paromomycin, 5 µl of 37 mg/ml KE was added and the mixture was incubated for 15 min at 37°C. For CMCT modification, after 1 µM tmRNA had been incubated for 60 min at room temperature in a 50 µl reaction mixture of 80 mM K-borate (pH 8.0), 10 mM magnesium chloride, 150 mM potassium chloride, 6 mM 2-mercaptoethanol and varying concentrations of paromomycin, 5 µl of 42 mg/ml CMCT was added and the mixture was incubated for 15 min at 37°C. Reverse transcriptase reaction was carried out in a 20-µl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM potassium chloride, 3 mM magnesium chloride, 10 mM dithiothreitol, 1 mM each of dNTP, 2.6 pmol of modified tmRNA, 4 pmol of 5’ Texas Red labeled DNA primer complementary to a portion of tmRNA sequence, 10 units of ribonuclease inhibitor from human placenta (Takara) and 60 units of reverse transcriptase from Molony Murine Leukemia Virus (RNase H, Takara). After the addition of 3 µl of a stop solution containing 7 M urea and 0.5 % bromophenol blue, the positions of modifications were analyzed using a fluorescence DNA sequencer (Hitachi, SQ-5500).

Aminoacylation - Alanyl-tRNA synthetase was purified from an alanyl-tRNA synthetase-overproducing strain (6) with DEAE-Toyopearl 650 (Tosoh, Tokyo) and subsequent hydroxyapatite column chromatography (Gigapite, Seikagaku Corporation, Tokyo) (38). The final fraction of alanyl-tRNA synthetase had a specific activity of 16.78 units/mg, one unit of enzyme catalyzing the formation of 1 nmol of aminoacyl-tRNA per ten minutes under the reaction conditions described below. The aminoacylation reaction proceeded at 37°C in a 50-µl reaction mixture containing 60 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 30 mM potassium chloride, 5.0 mM dithiothreitol, 2.5 mM ATP and 25 µM L-[U-14C]alanine (6.5 GBq/mmol, NEN Life Science Products), 1.0 µM tmRNA variants (one A260 unit corresponding to 325
pmol), 8.5 x 10^{-3} units of alanyl-tRNA synthetase and varying amounts of paromomycin with or without 2 µM of C-terminally His-tagged SmpB (35). At the times specified, a 12-µl aliquot was withdrawn and spotted on Whatman 3MM filter paper, and radioactivity in the trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter.

**Ribosomes having the 1408G mutation in 16S rRNA** - The 1408G mutation was introduced into the 16S rRNA gene on pKK3535 carrying the *E. coli* rrnB operon (39) using the primer-directed polymerase chain reaction. This plasmid was transformed into *E. coli* strain TA531 harboring two kinds of plasmids, pTRNA66 and pHK-rrnC^+ carrying the wild-type 16S rRNA gene (40), and then pHK-rrnC^+ was deleted by repeated selection on an ampicillin plate. The complete deletion of this plasmid was confirmed by polymerase chain reaction. The wild-type and mutant ribosomes were prepared from the S30 fractions from W3110 ΔssrA lacking the tmRNA gene and TA531 harboring pKK3535 having the 1408G mutation, respectively. The S30 fraction was separated into the ribosome and S100 fractions by centrifugation at 80,000 rpm for 30 min at 4°C using a Hitachi RP80TA rotor. The ribosome fraction was washed twice in 80 mM Tris-HCl (pH 7.8), 30 mM ammonium chloride, 10 mM magnesium chloride and 1 mM dithiothreitol and then stored at −80°C in 20 mM Tris-HCl (pH 7.8), 30 mM ammonium chloride, 10 mM magnesium chloride, 4 mM dithiothreitol and 10% (w/v) glycerol.

In vitro amino acid incorporations in the presence of poly (U) - The preincubated S30 fraction was prepared from middle-log-phase cells of *E. coli* strain W3110 (ΔssrA), as described previously (41). The reaction mixture (100 µl) contained 80 mM Tris-HCl (pH 7.8), 7 mM magnesium acetate, 150 mM ammonium chloride, 2.5 mM dithiothreitol, 5 mM phosphoenolpyruvate, 1 mM ATP, 0.2 mM GTP, 20 µM 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 (5.7 x 10^2 A_{260}, 2.5 x 10^2 A_{280}), and 250 µg of poly (U) (50 to 100 mer, Sigma). For the 1408G mutant ribosome, a total of 10 µl of the S100 fraction (3.2 x 10^2 A_{280}) and 10 µl of ribosomes (6.0 x 10^2 A_{260}) was used instead of 20 µl of the S30 fraction. 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 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

Paromomycin molecules specifically bind tmRNA - Neomycin-class aminoglycosides often bind various RNA species to inhibit their functions. We first found that paromomycin, a neomycin-class aminoglycoside antibiotic, significantly increased the melting temperature of *E. coli* tmRNA (Table 1). We then examined the binding of paromomycin to tmRNA using the chemical footprinting method. DMS, KE and CMCT methylate adenine/cytosine, guanine and uridine, respectively, at the Watson-Crick base-pair side, preferentially in the single strand region.

Various amounts of paromomycin were added to a reaction mixture of chemical modification. The chemically modified tmRNA was used as a template of reverse transcription. As shown in Figure 1, the site of methylation can be identified on the gel, since the methylation stops the chain elongation of reverse transcription. The band of DMS modification at A_{316} and the bands of KE modification at G_{332} and G_{333} disappeared with an increase of the paromomycin concentration (Fig. 1b). In contrast, the band intensity of DMS at A_{319} was increased (Fig. 1b). No other significant changes in the band intensity were observed over the whole tmRNA molecule. These results
indicate that paromomycin molecules bind tmRNA at A\textsubscript{316}, G\textsubscript{332} and G\textsubscript{333}, enhancing the accessibility of DMS to A\textsubscript{319}.

We then prepared a tmRNA mutant in which A\textsubscript{316} is substituted by C (316C). In this mutant, the KE modification at G\textsubscript{332} and G\textsubscript{333} still decreased, whereas the DMS modification at A\textsubscript{319} was not increased, with increase in the paromomycin concentration (Fig. 1b). Since DMS mildly modifies the C residues, a faint band was able to be found at 316 in the mutant. The intensity of this band did not change with increase in the paromomycin concentration. We also prepared a 333C mutant in which an A-to-C substitution had been made at position 333. In this mutant, neither the band intensity of DMS modification at 332 nor KE modification at 333 decreased with increase in the paromomycin concentration, whereas a decrease and increase in band intensity was still observed at 316 and 319, respectively (Fig. 1b). These results suggest that two different paromomycin molecules bind tmRNA in an independent manner, one to A\textsubscript{316} and the other to G\textsubscript{332}/G\textsubscript{333}, and that the binding of a paromomycin molecule at A\textsubscript{316} induces a local conformation change to enhance the accessibility of DMS to A\textsubscript{319}.

We next examined the effects of paromomycin on melting temperatures of 316C and 333C. As shown in Table 1, the melting temperatures of these tmRNA mutants were similar to that of wild-type tmRNA in the absence of paromomycin. The addition of 5.5 \mu M or 55 \mu M paromomycin increased the melting temperatures of these two mutants, although the melting temperatures of the mutants were significantly lower than that of wild-type. We also prepared a double mutant 316C333C, in which paromomycin did not significantly increase the melting temperature. These results support the above-stated idea that two paromomycin molecules bind wild-type tmRNA, while only one binds 316C or 333C mutant.

*Effects of paromomycin on aminoacylation of tmRNA in the presence or absence of SmpB* - We next examined the effects of paromomycin on aminoacylation of tmRNA
by alanyl-tRNA synthetase. Aminoacylation was significantly affected by the addition of paromomycin (Fig. 2a). The addition of 550 µM paromomycin decreased the aminoacylation efficiency by about 60%.

It has been shown that SmpB, a tmRNA-binding protein, is essential for trans-translation (34). It interacts with G19 and A334 or their surroundings in the lower portion of the tRNA domain of tmRNA to enhance aminoacylation efficiency, protect tmRNA from degradation in the cells and mediate ribosome binding to tmRNA (30,35). The binding sites of SmpB and paromomycin appear to be close each other. As shown earlier, aminoacylation was enhanced by about four fold by the addition of 2 µM SmpB (Fig. 2a). In the presence of 2 µM SmpB, more than 80% efficiency of aminoacylation remained even in the addition of 550 µM paromomycin. Apparently, SmpB suppressed the inhibition of aminoacylation by paromomycin.

Both the inhibition of aminoacylation and the suppression of the aminoacylation inhibition by SmpB were similarly observed when the 316C mutant was used instead of wild-type tmRNA (Fig. 2b). In contrast, inhibition of aminoacylation was hardly observed when the 333C mutant was used (Fig. 2c). The remaining aminoacylation efficiency after the addition of 550 µM paromomycin in the presence of SmpB (> 80%) was similar to that in the presence of SmpB.

These results indicate that the paromomycin molecule that bound at the lower portion of the tRNA domain around A334 inhibits the recognition by alanyl-tRNA synthetase and that the inhibition can be suppressed by SmpB.

*Paromomycin causes an initiation-shift in the poly(U)-dependent trans-translation in vitro - Himeno et al.* (19) have developed an *in vitro* system of poly(U)- and exogenous tmRNA-dependent tag-peptide synthesis via *trans*-translation using the S30 fraction extracted from tmRNA-depleted cells. Amino acids comprising the tag-peptide are incorporated in the *in vitro* poly (U)-dependent polypeptide synthesis
system. Using this system, we can evaluate not only the efficiency of *trans*-translation but also the shift of the initiation point of tag-translation (29). The tag-peptide from the normal initiation point (AANDENYALAA) contains five alanine residues. The tag-peptide from –1 (ARKRRKLFSSLITCLEPSLPSLRS) contains one alanine residue, five arginine residues, five serine residues and one threonine residue. The tag-peptide from +1 (AQTTKTTTL) contains one alanine and four threonine residues. In the present study, the effects of paromomycin on *trans*-translation were examined by measuring levels of alanine, arginine, glycine, serine and threonine incorporated in this system.

As shown earlier (19,29), amino acids were incorporated in a stoichiometrical fashion in the absence of paromomycin (Fig. 3a). In the presence of 5.5 µM paromomycin, the level of alanine incorporation was slightly decreased, and, instead, a detectable level of arginine incorporation was generated. In the presence of 55 µM paromomycin, the effect was most pronounced. The level of alanine incorporation was decreased more, and, instead, significant levels of incorporation of typical –1 frame amino acids, arginine and serine, were observed. Glycine, which does not appear in any of three frames, was not detected. Considering the amino acid compositions of the tag-peptides of three frames, about 60% of the total tag-peptides was tag-peptides from position –1 and the remainder were the tag-peptide from the normal initiation point. At 550 µM paromomycin, levels of incorporation of amino acids, were significantly lower than those at 55 µM paromomycin. Such an inhibitory effect at a high concentration of paromomycin was not observed in phenylalanine incorporation (Fig. 3a, right column) representing the canonical translation from poly (U), which is prerequisite for *trans*-translation.

*Exogenous addition of SmpB enhanced the initiation-shifted trans-translation at a high paromomycin concentration* - SmpB is essential for *trans*-translation, and its depletion affects the binding of tmRNA to the ribosomes (34,35). We therefore added 2...
µM SmpB into the in vitro trans-translation reaction mixture. No significant difference in alanine incorporation was observed in the absence of paromomycin (Fig. 3), indicating that the amount of endogenous SmpB in the reaction mixture was sufficient. With an increase in the concentration of paromomycin, alanine incorporation decreased less dramatically than that without exogenous addition of SmpB (Fig. 3b), suggesting that SmpB and paromomycin are competed with each other. On the other hand, the incorporations of −1 frame amino acids, arginine and serine, were significantly increased as compared to those without an exogenous addition of SmpB. The difference was most obvious at 550 µM paromomycin. Judging from the ratio of amino acids incorporated, more than 70% of the trans-translation products were estimated to be −1 frame. These results indicate that a sufficient, but not excess, amount of SmpB was contained in the in vitro trans-translation reaction mixture for paromomycin to elicit an inhibitory effect on trans-translation, especially at a high concentration, and that the addition of SmpB canceled it to enhance the initiation-shifted trans-translation. The results also suggested that the frequency of the initiation-shift itself becomes higher with an increase in the concentration of paromomycin.

Effects of paromomycin on trans-translation directed by tmRNA mutants - We examined the effect of incorporations of amino acids in the in vitro system of trans-translation directed by a tmRNA mutant, 3A, having a G-to-A mutation at the third base pair position of the acceptor stem. This mutant has no alanine acceptor activity due to the lack of an identity determinant for recognition by alanyl-tRNA synthetase, thereby having no trans-translation activity (19). At any concentration of paromomycin, no detectable incorporations of amino acids other than phenylalanine were observed in the 3A mutant. This confirms that the observed paromomycin-induced −1 frame amino acid incorporations for wild-type tmRNA are the result of initiation-shifted trans-translation but not of aberrant translation from tmRNA or poly(U).
We next examined amino acid incorporations of 316C and 333C mutants to determine the effect of paromomycin binding to tmRNA on the initiation shift of trans-translation. As shown in Figure 4a, the 316C mutant had a pattern of amino acid incorporations quite similar to that for wild-type tmRNA at any concentration of paromomycin. The effect of a low concentration of paromomycin on 333C was also similar to that on wild-type tmRNA. These results indicate that the observed initiation shift of trans-translation was not caused by paromomycin binding to any of A316 and G333. Interestingly, at 550 µM paromomycin, the alanine incorporation of 333C was decreased more, and, in contrast, incorporations of -1 frame amino acids were significantly increased (Fig. 4b). Apparently, the 333C mutation canceled the inhibitory effect of paromomycin on trans-translation. The pattern of amino acid incorporations was similar to that using wild-type tmRNA with 2 µM of exogenously added SmpB. Judging from the ratio of amino acids incorporated, more than 80% of the trans-translation products were estimated to be -1 frame.

We also examined the effect of paromomycin on the deficient tag-translation directed by the 86C mutant having an A-to-C mutation four nucleotides upstream of the tag-initiation point. This mutant can associate with the ribosome as efficiently as wild-type tmRNA, although it has only a faint activity of trans-translation in vitro (29). Paromomycin did not induce any tag-related amino acid incorporations for the 86C mutant.

Lee et al. (29) have found several base-substitutions that cause a significant level of initiation shift of tag-translation by -1 in vitro. The 85A mutant having a U-to-A mutation five nucleotides upstream of the tag-initiation point is a typical initiation-shift mutant. About 45% of the total tag-peptides from this mutant tmRNA is estimated to be initiated from -1. We examined the effects of paromomycin on the tag-translation directed by this mutant (Fig. 4c). With increase in the paromomycin concentration, levels of amino acid incorporations became gradually decreased. However, the ratios of
incorporations of arginine, serine and threonine to that of alanine were almost constant at all concentrations of paromomycin, indicating that paromomycin induces no further shift in the initiation point for this initiation-shift mutant.

Initiation shift by paromomycin disappeared when a mutation was introduced in the decoding region of the ribosome - Neomycin-class aminoglycosides specifically interact with the decoding region, an internal bulge structure of the A site comprising three adenosine residues at 1408, 1492 and 1493 in the base of the penultimate helix of the 16S rRNA. It has been shown that a eukaryotic type of A-to-G mutation at 1408 in the decoding region of *E. coli* 16S rRNA decreased the sensitivity of aminoglycosides to bacteria as well as the binding capacity of aminoglycosides to the ribosome (42,43). We therefore made an *E. coli* strain in which all the ribosomes have a 1408G mutation according to Asai *et al.* (40), and we prepared 1408G mutant ribosomes to examine the effect of mutation on trans-translation. Cell-free trans-translation using the 1408G mutant ribosomes and the S100 fraction prepared from W3110ΔssrA cells was carried out.

Several differences were observed in the patterns of alanine and phenylalanine incorporations, compared with those using wild-type ribosomes (Fig. 5). The ratio of alanine incorporation to phenylalanine incorporation was slightly lower in the absence of paromomycin. The addition of 5.5 µM paromomycin did not change, or even slightly enhanced, the alanine incorporation as well as the phenylalanine incorporation. Alanine incorporation decreased with an increase in paromomycin concentration, as was the case using wild-type ribosomes. Interestingly, basal levels of incorporations of −1 frame-tag amino acids, serine or arginine were detected at any concentration of paromomycin. The pattern of −1 frame-tag amino acid incorporations differed from that using wild-type ribosomes, the difference most apparent at 55 µM paromomycin (see Fig. 3a). These results indicate that the observed difference in the −1 frame-tag amino
acid incorporations is due to the difference in the ribosomes used and strongly suggest that the initiation shift in trans-translation is caused by modulation of the decoding region by binding of a paromomycin molecule.

DISCUSSION

Aminoglycosides often bind various RNA species, such as the decoding region of 16S rRNA, group I intron (44), hammerhead ribozyme (45), hepatitis delta virus ribozyme (46), RNase P RNA (47) and tRNA (48), to inhibit or modulate their functions. The present study demonstrated that paromomycin, a neomycin-class aminoglycoside, exerts various effects on tmRNA-related events. It binds tmRNA, inhibits aminoacylation and shifts the resuming point of trans-translation.

The results of the present study indicated that paromomycin molecules bind tmRNA at A316 and G332/G333. A316 and G315 are opposed to A38 and A39, respectively, so that they interrupt the long helix connecting the two separated functional domains. Similar adenine-rich non-Watson-Crick base pairs in the middle of the helix can be observed in another aminoglycoside-binding RNA, the A site of the decoding region of 16S rRNA. A316 is close to the region of binding to the GDP form of EF-Tu (33). Whether this unusual mode of binding to EF-Tu is affected by paromomycin has not yet been determined. Even if it is true, it would not be relevant to the initiation shift in trans-translation in consideration of the present results for mutations at 316 of tmRNA and 1408 of 16S rRNA. A316 itself or its surrounding adenine-rich non-Watson-Crick base pairs is not so highly conserved. In contrast, G332 and G333 are almost completely conserved. It has been proposed that G333 together with A334, G19 and A20 can form a sheared GA:GA base pair, a characteristic RNA structure (30,49). An alternative tertiary structure model has also been proposed (50). Further NMR and/or crystallographic studies will clarify the mode of binding of paromomycin molecules to tmRNA.
The present study showed that paromomycin molecule bound at 332/333 inhibits aminoacylation, although base substitutions at G332 or G333 cause no significant decrease in aminoacylation (35). Very recently, Corvaisier et al. have also reported the inhibition of aminoacylation of tmRNA or its tRNA-like fragment by aminoglycosides (51). Inhibition of aminoacylation has also been observed in yeast tRNA<sup>Phe</sup>, in which an aminoglycoside binds the region connecting the anticodon stem with the D- and T-arms (G20, A23, A44 and G45) (49).

The present results demonstrate that the binding of paromomycin to this region may also affect the binding of SmpB, which is consistent with an earlier finding that G332 and G333 are just at or close to the site of binding to SmpB (35,52). The 333C mutant should be free of this effect, probably leading to the pronounced initiation-shift at 550 µM paromomycin. The inhibitory effect of a high concentration of paromomycin seems specific to trans-translation rather than general to the translation, and either the 333C mutation or the addition of SmpB canceled this effect. This finding is consistent with the fact that SmpB serves as a trans-translation-specific factor, although it is not required for canonical translation (35).

The shift in the resuming point of trans-translation seems to be a novel effect of paromomycin. The results of the present study revealed that it was caused not by binding of paromomycin to tmRNA but by modulation of the decoding region of the ribosome by paromomycin. Neomycin-class aminoglycosides typically induce miscoding of the translation via binding to the decoding region of the ribosomes (53). They also induce a readthrough at the termination codon (54), inhibit the assembly of the 30S ribosomal subunit (55), and inhibit the RRF-dependent disassembly of the post termination complex (56). The readthrough at the termination codon often produces ribosomes stalled at the 3’ end of mRNA, which would subsequently be released by tmRNA-mediated trans-translation (57). It has recently been shown that gentamycin or paromomycin causes only a very small increase in the level of the intrinsic
frameshifting upon translation of lacZ gene (58). To the best of our knowledge, the present study is the first study to show that an aminoglycoside significantly shifts the frame or initiation point of translation. The event of determination of the resuming point in trans-translation would be more fragile than the codon-anticodon interaction in canonical translation. Another ribosome-binding reagent, polyamine, sometimes induces translational frameshifting (59), although the molecular mechanism of this event is not clear.

Paromomycin is the best-characterized neomycin-class aminoglycoside. The mode of binding to the decoding region of the ribosome has been revealed by NMR (60,61) and X-ray crystallographic studies (62). It binds the internal bulge of the A site of the decoding helix comprising three adenosine residues at 1408, 1492 and 1493 of 16S rRNA. The results of a crystallographic study using the small subunit of the Thermus thermophilus ribosome and a short RNA helix mimicking the anticodon stem-loop has suggested that the adenine bases at 1492 and 1493 were flipped out from the helix in the presence, but not in the absence, of normal codon-anticodon interaction (63). In the presence of paromomycin, this phenomenon occurs regardless of the presence or absence of the codon and anticodon interaction, apparently leading to the failure of proofreading.

The 1408G mutation in E. coli 16S rRNA has been shown to decrease the affinity of aminoglycosides to ribosomes and confer resistance to aminoglycosides upon cells (43,64). In the present study, the 1408G mutation conferred higher fidelity of the trans-translation initiation with a milder effect on efficiency of trans-translation in the presence of a low concentration of paromomycin (5.5 µM). At higher concentrations of paromomycin, a significant level of inhibition of trans-translation was still observed as in the case using wild-type ribosomes.

The mechanism by which tmRNA resumes translation from the first GCA codon for a tag-peptide remains unknown. It might be determined by the interaction between
tmRNA and ribosome either directly or via a specific factor. It has been shown that the sequence at –5 to –2 upstream of the translation resuming point on E. coli tmRNA as well as the start nucleotide serve as a cis-element to determine the initiation point (28,29). How does paromomycin induce the -1 initiation shift in tmRNA translation? Paromomycin induces distortion of the decoding region of the A site to modulate the normal interaction between tmRNA and ribosome. The -1 initiation shift has also been found in the in vitro trans-translation directed by some tmRNA mutants having a single point or multiple mutation around the cis-element upstream of the translation resuming point. In this case, the -1 initiation shift is thought to be caused by an aberrant interaction between the altered cis-element and ribosome. Local conformation changes in the decoding region caused by paromomycin and a mutation of the cis-element on tmRNA produce apparently similar effects. If these two effects are independent of each other, a combination of two events would produce an additive effect. The present study showed that paromomycin has neither a synergistic, additive nor complementary effect on the initiation-shift for a typical initiation-shift mutant, 85A. It has been shown that the ribosomal protein S1 interacts with tmRNA (36,65). Since it can crosslink with the uridine residue 5 nucleotides upstream of the tag-initiation point as well as PK2 and PK3, it is a candidate for recognizing the cis-element, although it is not universal among the eubacterial kingdom (66). SmpB, another tmRNA-binding protein, would not involve the event around the decoding region, considering that it can still efficiently bind a trans-translation-deficient mutant, 86C, having a mutation in the center of the cis-sequence for tag-initiation (35).

A toe printing study has shown that aminoglycosides increase the span of mRNA downstream of the first nucleotide of translation covered by the E. coli ribosome from 16 to 17 nucleotides, suggesting a global conformational change in the translating ribosome (67). Such a conformation change in the ribosome would also affect the interaction between tmRNA and the ribosome. It is conceivable that it causes
an unexpected interaction between the ribosome and H4 helix 18 nt downstream of the start nucleotide G, which would affect the normal selection of the initiation point, although it has been shown that some single-nucleotide additions in the downstream region on tmRNA do not change the normal initiation point of the *trans*-translation (29).

The results of this study have revealed various effects of paromomycin on *trans*-translation. It has recently been shown that the lack of tmRNA increases sensitivity to some antibiotics in a cyanobacterium (68) and in *E. coli* (56). Although the relevance between these *in vivo* effects and the *in vitro* effects revealed by the present study remains unknown, the results of further studies along these lines should lead to clarification of the molecular mechanism of *trans*-translation mediated by tmRNA.
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FOOTNOTES

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The abbreviations used are: DMS, Dimethyl sulfate; KE, kethoxal; CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate; EF-Tu, elongation factor Tu

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Figure legends

Figure 1. Chemical modifications for *E. coli* tmRNA in the presence of paromomycin. (a) The bases of which the level of modification was decreased and increased with increase in paromomycin concentration are indicated by black and white arrowheads, respectively, on the secondary structure model of tmRNA. The tag-encoded sequence is highlighted by white with a black background. Non-Watson-Crick base pairs are shown by open circles. The dotted lines indicate the conserved GA/GA quartet that can take a characteristic sheared base pair (30, 49). This RNA has two tRNA specific modified nucleotides, 5-methyl U and pseudouridine (11), designated as T and Ψ, respectively. (b) The gel electrophoresis patterns of primer extension for tmRNA variants modified by DMS, KE and CMCT in the presence of varying amounts (0, 5.5, 55 and 550 µM) of paromomycin. The area of A<sub>316</sub> to A<sub>334</sub> is shown. The band appears at the site of interruption of reverse transcription just before the base of modification. The bands of interest are indicated by arrowheads. The lanes of DNA sequencing of pGEMEX-2 carrying the tmRNA gene with the same primer are designated by T, G, C and A. The lane without any treatment of chemical modification is indicated by “n”.

Figure 2. Effect of paromomycin on aminoacylation of tmRNA. Top: 0 µM (white), 55 µM (hatched) and 550 µM (black) of paromomycin for (a) wild-type tmRNA, (b) 316C and (c) 333C in the presence (circles) or absence (squares) of 2 µM SmpB were used. Bottom: the aminoacylation activity relative to that in the absence of paromomycin is plotted against the paromomycin concentration. The values of 12 min in each top figure were used.

Figure 3. Effects of paromomycin on the poly(U)-dependent incorporations of amino acids directed by wild-type *E. coli* tmRNA. The incorporations of alanine, arginine, glycine, serine, threonine and phenylalanine were measured (a) in the absence
or (b) presence of 2 μM exogenous SmpB. 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 the phenylalanine incorporation, both the values in the presence (black) and absence (white) of tmRNA are shown.

Figure 4. Effects of paromomycin on the poly(U)-dependent amino acid incorporations directed by tmRNA variants. The poly(U)-dependent incorporations of alanine, glycine, arginine, serine, threonine and phenylalanine directed by (a) 316C, (b) 333C and (c) 85A mutants were measured in the presence of varying concentrations of paromomycin. 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 the phenylalanine incorporation, both the values in the presence (black) and absence (white) of tmRNA are shown.

Figure 5. Effects of paromomycin on the poly(U)-dependent incorporations of amino acids using the 1408G mutant ribosomes. The value of poly (U)-dependent incorporation of each of alanine, glycine, arginine, serine and threonine was obtained by subtracting the value in the absence of tmRNA from that in the presence of tmRNA. For the phenylalanine incorporation, both the values in the presence (black) and absence (white) of tmRNA are shown.
Table I

*Melting temperatures of tmRNA variants*

Melting temperatures were measured in a solution containing 10 mM sodium cacodylate (pH 6.8), 0.1 mM EDTA, 50 mM NaCl, 10 mM magnesium chloride, 162 nM tmRNA variant and varying amounts of paromomycin. Parentheses indicate the difference from the melting temperature in the absence of paromomycin.

<table>
<thead>
<tr>
<th>paromomycin (µM)</th>
<th>0</th>
<th>5.5 (+1.5)</th>
<th>55 (+8.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>71.1</td>
<td>72.6</td>
<td>79.9</td>
</tr>
<tr>
<td>316C</td>
<td>70.3</td>
<td>70.6 (+0.3)</td>
<td>76.2 (+5.9)</td>
</tr>
<tr>
<td>333C</td>
<td>71.4</td>
<td>71.6 (+0.2)</td>
<td>76.3 (+4.9)</td>
</tr>
<tr>
<td>316C333C</td>
<td>71.3</td>
<td>71.5 (+0.2)</td>
<td>72.6 (+1.3)</td>
</tr>
</tbody>
</table>
Figure 2

(a) Alanine aminoacylated (pmol) over time (min).

(b) Alanine aminoacylated (pmol) over time (min).

(c) Alanine aminoacylated (pmol) over time (min).

Relative activity (%) vs. paromomycin (μM):

- Graph (a) shows relative activity (%) decreasing with increasing paromomycin concentration.
- Graph (b) shows relative activity (%) decreasing with increasing paromomycin concentration.
- Graph (c) shows relative activity (%) decreasing with increasing paromomycin concentration.
Figure 3

(a) Paromomycin (µM) vs. Amino acid incorporated (p mol)

(b) Paromomycin (µM) vs. Amino acid incorporated (p mol)
Figure 4

(a) Amino acid incorporated (pmol) vs. Paromomycin (µM) for Ala, Arg, Gly, Ser, and Phe.

(b) Amino acid incorporated (pmol) vs. Paromomycin (µM) for Ala, Arg, Gly, Ser, and Phe.

(c) Amino acid incorporated (pmol) vs. Paromomycin (µM) for Ala, Arg, Gly, Ser, and Thr.
Figure 5

Amino acid incorporated (pmol) vs. Paromomycin (µM) for Ala, Arg, Gly, Ser, and Thr. The graph shows the incorporation of amino acids in response to varying concentrations of Paromomycin. The x-axis represents Paromomycin concentration in µM, and the y-axis represents the amount of amino acid incorporated in pmol. The data points are indicated with error bars to show variability.
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