Nucleic Acids Research, 1994, Vol. 22, No. 3 325–331

Spectinomycin interacts specifically with the residues G_{1064} and C_{1192} in 16S rRNA, thereby potentially freezing this molecule into an inactive conformation

Marcel F.Brink, Guido Brink, Martin Ph.Verbeet and Herman A.de Boer
Leiden Institute of Chemistry, Department of Biochemistry, Gorlaeus Laboratories, Leiden University, Einsteinweg 55, 2333CC Leiden, The Netherlands

Received October 22, 1993; Revised and Accepted January 4, 1994

ABSTRACT

The upper stem of helix 34, consisting of the basepaired sequences $C_{1063}G_{1064}U_{1065}$ and $A_{1191}C_{1192}G_{1193}$, is suggested to be involved in the binding of spectinomycin. In *E.coli* 16S rRNA, each of the three mutations at position C_{1192} confers resistance to spectinomycin. In chloroplast ribosomes from tobacco plants and algae, resistance is conferred by single mutations at positions 1064, 1191, and 1193 (*E.coli* numbering). Since each of these mutations disrupt any of the three basepairs in the upper stem of helix 34, it has been postulated that spectinomycin can bind to this region and inhibit protein synthesis, only if its nucleotides are basepaired. We have tested this hypothesis by introducing disruptive and compensatory mutations that alter the basepair G_{1064}-C_{1192}. Using the specialized ribosome system, the translational activity of such mutants was determined, in the absence and presence of spectinomycin. We show that any of the three disruptive mutations A_{1064}, C_{1064}, and U_{1064} confer resistance, in accordance with the model for spectinomycin binding. Compensatory mutations $A_{1064}U_{1192}$, $C_{1064}G_{1192}$, and $U_{1064}A_{1192}$, however, maintained the resistance. This indicates that a basepaired conformation as such is not sufficient for spectinomycin binding, but rather that a G-C pair at positions 1064 and 1192 is required. In addition, we find that the translational activity of specialized ribosomes containing the mutations $C_{1064}G_{1192}$ is 5-fold lower compared to that of ribosomes containing any of the other mutations introduced, regardless whether spectinomycin is present or not. Since the introduction of $C_{1064}G_{1192}$ is expected to increase the stability of the upper stem of helix 34, we suggest that these mutations impair ribosome function by preventing the (transient) disruption of the upper stem. By analogy, we speculate that spectinomycin blocks protein synthesis by stabilizing the upper stem. In both cases, the 30S subunit would be frozen into an inactive conformation.

INTRODUCTION

Spectinomycin (see Figure 1A) blocks the translocation of peptidyl-tRNAs from the A-site to the P-site (1) by inhibiting the binding of elongation factor G to the ribosome (2). In *E.coli*, resistance to this antibiotic can be conferred by substitutions at position C_{1192} of 16S rRNA (3,4). In the tobacco plant *Nicotiana* and the alga *Chlamydomonas*, resistance can be caused by the presence of mutations at positions 1064, 1191 and 1193 (*E.coli* numbering) in their chloroplast 16S rRNA (5).

In structural models of *E.coli* 16S rRNA, the resistance mutations are all located in helix 34 (6), which is formed by the basepairing between the regions 1046–1065 and 1191–1211 (see Figure 1B; 7). This helix consists of an upper and lower stem, separated from one another by an internal loop containing two U residues. Comparative sequence analyses of prokaryotic and chloroplast 16S rRNAs demonstrates that the consensus sequence of this upper stem is $C_{1063}G_{1064}U_{1065}$/ $A_{1191}C_{1192}G_{1193}$. *In vitro* footprinting on *E.coli* ribosomes indicated that spectinomycin protects only residues C_{1063} and G_{1064} from modification by dimethylsulphate (8). This suggests that the antibiotic interacts specifically with, at least, these two residues. Based on such footprinting data and the observation that every resistance mutation examined thus far causes disruption of a basepair in the upper stem of helix 34, Fromm *et al.* (5) postulated that spectinomycin interacts with the upper stem, provided that its nucleotides are basepaired. This hypothesis implies that resistance is conferred by mutations that impair the specific interaction with spectinomycin (i.e at positions C_{1063} and G_{1064}), and mutations that lead to disruption of the basepaired conformation (i.e. any mutation at position 1065 and 1191–1193). In other words, such mutations inhibit directly and indirectly the binding of the antibiotic.

The genetic evidence for this hypothesis is not conclusive, since only single mutations in the upper stem have been examined. Complementary mutations have not yet been introduced to substantiate the model. To further understand the mechanism of spectinomycin interaction with 16S rRNA, we have examined the effect of alterations at the G_{1064}-C_{1192} basepair. For that purpose, in 16S rRNA of *E.coli*, we have introduced any of the

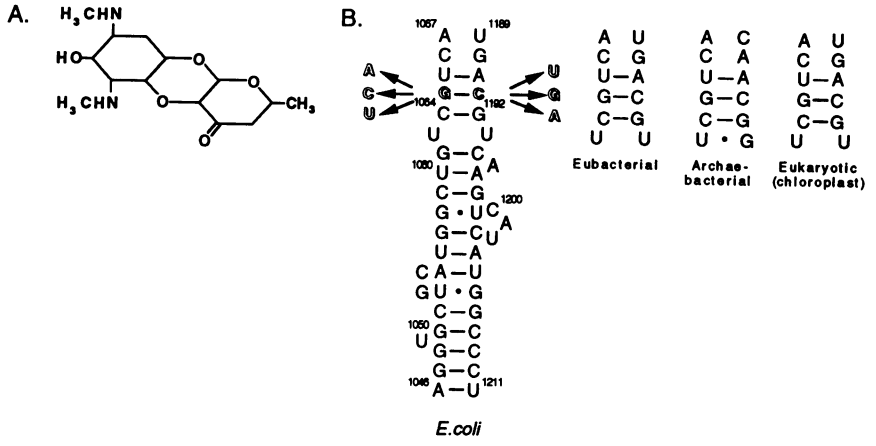

Figure 1. Structure of spectinomycin and its binding domain in 16S rRNA. (**A**) molecular structure of the aminoglycoside spectinomycin. (**B**) Proposed secondary structure of helix 34 and phylogenetic comparison of the upper stem. The sequence and secondary structure is according to Stern *et al.* (7). Based on phylogenetic evidence, the basepairs C_{1066}-G_{1190} and A_{1067}-U_{1189} are assumed not to be formed. The upper stem formed by the regions 1063–1065 and 1191–1193 is suggested to interact with spectinomycin (5). For each group indicated, the consensus sequence of this upper stem is shown. The G_{1064}-C_{1192} basepair, which is subjected to mutational analysis, is presented with open letters. The mutations introduced are indicated with arrows.

single mutation at position G_{1064}, and, in addition, constructed the compensatory mutants $A_{1064}U_{1192}$, $C_{1064}G_{1192}$, and $U_{1064}A_{1192}$.

To determine the effect of spectinomycin on the activity of such mutant ribosomes *in vivo*, the specialized ribosome system was used (9). This system offers the advantage that the translational activity of mutant ribosomes can be determined directly by measuring their translation of a single modified mRNA species, instead of determining ribosomal activity indirectly by monitoring the growth rate (10). Due to the altered anti-Shine–Dalgarno (ASD) sequence 5'CACAC3' near the 3'-end of 16S rRNA, specialized ribosomes are dedicated to the translation of a single, modified mRNA species having the complementary Shine–Dalgarno (SD) sequence 5'GUGUG3'. In the system used in our laboratory, this modified mRNA species encodes chloramphenicol acetyl transferase (CAT; 11,12). The wild-type ribosomes lacking the complementary ASD sequence, do not translate this CAT-mRNA. Therefore, the effect of rRNA mutations on the activity of the specialized ribosomes can be assessed by measuring the CAT-activity.

We show that spectinomycin does not block protein synthesis on specialized ribosomes containing any of the disruptive mutations at position 1064. In addition, the compensatory mutations at positions 1064 and 1192 maintain spectinomycin resistance. This means that the presence a G and a C residue at positions 1064 and 1192, respectively, is needed for binding of spectinomycin. None of the mutations affected the protein synthesis capacity significantly, except for the mutations $C_{1064}G_{1192}$. The activity of specialized ribosomes containing these latter mutations was 5-fold lower as compared to fully active specialized ribosomes, irrespective whether spectinomycin is present or not. Since the presence of a C_{1064}-G_{1192} would increase the stability of the upper stem, we infer that (transient) disruption of the helical structure may be a prerequisite for ribosome function. By analogy, we suggest that spectinomycin blocks protein synthesis in a similar way as mutations C_{1064} and G_{1192} do: i.e. by stabilizing the upper stem of helix 34. Both spectinomycin binding to normal specialized ribosomes, as well as the presence of the C-G basepair may freeze the upper stem in a basepaired conformation, thereby by locking the 30S subunit into a state incapable of beginning or completing translation.

MATERIALS AND METHODS

Strains, media and plasmids

The *E.coli* strains K5716 and K5637 used in these studies were described previously by Hui and de Boer (9). Cells were grown in LB medium containing 10 g/l of tryptone (DIFCO), 5 g/l of yeast extract (DIFCO) and 10 g/l of NaCl. Ampicillin (Sigma) was supplied at a final concentration of 100 mg/l. Plasmid pASDX-CATX encoding the specialized ribosome system, was derived from pASDX-PSDRX-hGH (9). The cat-gene is under the transcriptional control of a constitutive trp-promoter, whereas the 16S rRNA having the altered ASD sequence is driven by a thermo-inducible lambda P_L-promoter. Thus, induction of the synthesis of specialized ribosomes is achieved by changing the temperature from 30°C to 42°C.

Site-directed mutagenesis

Oligonucleotide-directed mutations were introduced using the PCR (13). Oligonucleotides were synthesized using the phosphoamidite method on a Cyclone-plus DNA synthesizer (Milligen/Biosearch). PCRs were carried out in 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2.5 mM $MgCl_2$ and 0.01% gelatin (Sigma). Fragments of *rrnB* were amplified from 5 ng plasmid DNA for 25 cycles using 25 pmoles of each primer and 2.5 units of Amplitac (Cetus). Plasmids used in PCRs encode the *rrnB* operon having either an A, C, G or U at 1192. The DNA fragment amplified by the primers 5'AGGAATTCACGGGGGCCCGCACAAGCG3' and 5'AACACGAGCTGA(A,G,T)GACAGCCATGCA3' spans the region 923–1076. It contains the mutation at position 1064 and an additional EcoRI site at the 5'-end. The DNA fragment amplified by 5'AGGAATTCACGGGGGCCCGCACAAGCG3' and 5'CCTCTAGACTCCCATGGTGTGACGG3' spans the region 923–1420. Depending on the plasmid DNA used in the PCR, it contains any of the four residues at position 1192. EcoRI–AluI and AluI–XbaI fragments containing the mutations at positions 1064 and 1192,

respectively, were jointly subcloned into pGEM-7Zf(+) (Promega) to allow for single stranded sequence analysis using the T_7-DNA-polymerase sequencing kit (Pharmacia). The ApaI−SmaI fragment (positions 932−1383) containing the desired mutation at position(s) 1064 and/or 1192 was cloned into the 16S rRNA gene containing the altered ASD sequence 5'CACAC3'.

Assessment of the translational activity; *in vivo* labeling of CAT and CAT-assays

Metabolic labeling of CAT with L-[S^{35}]-methionine (Amersham) was done as described by Hui and de Boer (9), except that M9 medium was supplied with all L-amino acids (Sigma) excluding L-methionine. CAT-assays were described by Brink *et al.* (11). In LB medium containing 100 mg/l of ampicillin, cells were grown for 1 h at 30°C. After induction at 42°C (t=0), samples of 1 ml were taken at t=0, 30, 60, 90 and 120 min. The CAT-activity was calculated as the amount of [H^3]-diacetyl-chloramphenicol (cpm) formed per optical cell density (OD_{650}) at the time of sampling.

Preparation of polysome profile and rRNA analysis

After 1 hour of induction, cells were harvested and polysome profiles were prepared as described by Brink *et al.* (11). rRNA was isolated from the 30S, 70S disome and trisome fractions and analyzed by the primer extension method (14, 15). The oligonucleotide complementary to the region 1194−1210 of 16S rRNA was extended by AMV reverse transcriptase (Promega) in the presence of ddGTP (Boehringer-Mannheim). Extension products were analyzed on 12.5% (w/v) polyacrylamide/urea gels. The amounts of specialized ribosomes and wild-type ribosomes present in the polysomal fractions were determined by direct measurement of the radioactivity present in the extension products using the Betascope 603 Blot Analyzer (Betagen).

RESULTS

Disruptive and compensatory mutations altering the G_{1064}-C_{1192} basepair confer spectinomycin resistance

The disruptive and compensatory mutations at positions G_{1064} and/or C_{1192} were introduced into 16S rRNA containing the altered ASD sequence 5'CACAC3'. The gene encoding this 'specialized' 16S rRNA is part of a plasmid-borne *rrnB* operon, which is controlled by the bacteriophage lambda P_L-promoter. In the *E.coli* strain K5637 encoding the temperature-sensitive repressor for this promoter, transcription of the *rrnB* operon is induced by changing the temperature from 30°C to 42°C, resulting in the synthesis of specialized ribosomes.

The effect of spectinomycin on the translational activity of these ribosomes was determined by metabolic labeling of CAT. After 2 hours of induction of specialized ribosome synthesis, spectinomycin was added to final concentration of 0.5 mg/ml; 15 minutes later, cells were labeled by adding L-[S^{35}]-methionine. Since spectinomycin inhibits protein synthesis by wild-type ribosomes and due to the altered SD sequence of the CAT-mRNA, labeling of CAT is exclusively dependent on the activity of the specialized ribosomes, as was demonstrated previously in cells harboring a deletion mutant that was not capable of forming specialized ribosomes (11). After labeling for 90 minutes, proteins were isolated and separated by SDS/PAGE followed by autoradiography (see Figure 2).

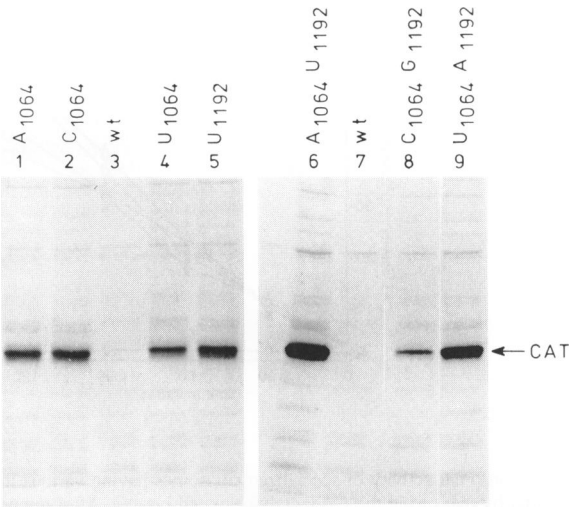

Figure 2. Effect of spectinomycin on the protein-synthesis-capacity of specialized ribosomes having various mutations in the upper stem of helix 34. Cells were grown in M9-medium at 30°C for three hours. At t=0, the synthesis of specialized ribosomes was induced at 42°C. At t=120 minutes, spectinomycin was added to a final concentration of 0.5 mg/ml. At t=135 minutes, *de novo* synthesized proteins were metabolically labeled by addition of L-[S^{35}]-methionine (final concentration 760 μCi/μmol). Proteins were separated by SDS/PAGE (12.5%). Lane 3 and 7 show the proteins synthesized by specialized ribosomes having the wild-type sequence. Lanes 1, 2, and 4 show the proteins synthesized by specialized ribosomes having the disruptive mutations A_{1064}, C_{1064}, and U_{1064}, respectively. Lane 5 shows proteins synthesized by the spectinomycin resistant control U_{1192}. Lanes 6, 8 and 9 show the proteins synthesized by specialized ribosomes having the compensatory mutations $A_{1064}U_{1192}$, $C_{1064}G_{1192}$, and $U_{1064}A_{1192}$, respectively. The position of CAT is indicated. Note that lanes 8 and 10 have been obtained from a separate SDS/PAGE gel. The single and double mutants were tested in separate experiments accounting for the difference in background labelling.

Figure 2 shows the profile of proteins labeled by specialized ribosomes containing the various mutations at position 1064 and/or at position 1192. The background labeling of the endogenous proteins is predominantly caused by wild-type ribosomes, which, despite the presence of spectinomycin, have retained low-level activity. Lanes 1, 2, and 4 show that the amount of CAT labeled by the mutants A_{1064}, C_{1064} and U_{1064} is similar to the amount labeled by ribosomes containing the 'classical' resistance mutation U_{1192}; spectinomycin does not significantly impair the ribosomal activity of these mutants. Similar amounts of CAT are labeled also by ribosomes containing the compensatory mutations $U_{1064}A_{1192}$ and $A_{1064}U_{1192}$ (lanes 6 and 9, respectively; Note that the stronger background of endogenous proteins indicates that the overall labeling capacity of the cells containing the double mutants was higher than that of cells containing the single mutants. Thus, the apparent higher amount of CAT labeled by the double mutants should not be considered significant). Only in the case of $C_{1064}G_{1192}$ (lane 8), a significantly lower amount is labeled; i.e. merely 20−25% of the amount synthesized by any of the other mutants. These results demonstrate that each of these mutant ribosomes is capable of synthesizing CAT in the presence of spectinomycin, whereas this is not the case for ribosomes containing the wild-type residues $G_{1064}C_{1192}$ (lanes 3 and 7). We conclude that not only mutations at position 1192 confer resistance to spectinomycin (3, 4) but also those introduced at position 1064. Compensatory mutations at these positions maintain resistance, indicating that basepairing

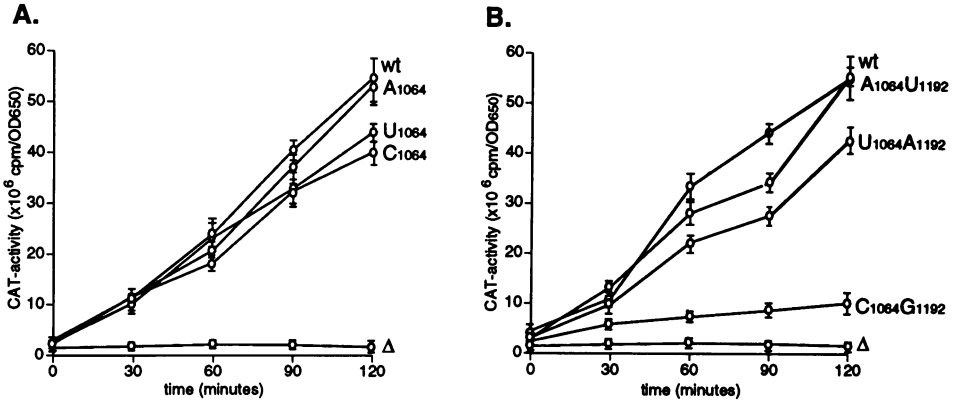

Figure 3. Ribosomal activity of specialized ribosomes having various mutations in the upper stem of helix 34. The ribosomal activity of the specialized ribosomes was assessed by measuring the CAT-activity in cell lysates. After temperature induction of the synthesis of specialized ribosomes (t=0), samples were taken at 30 min intervals. The CAT-activity was determined by measurement of the amount of [^{3}H]di-acetyl-chloramphenicol formed. The CAT-activity in cells harboring the various mutants is compared with the activity in cells harboring specialized ribosomes containing the wild-type spectinomycin binding domain (wt), or negative control cells lacking specialized ribosomes (Δ). Errors bars are indicated. **(A)** the disruptive mutants A_{1064}, C_{1064} or U_{1064}, **(B)** the compensatory mutants $A_{1064}U_{1192}$, $C_{1064}G_{1192}$ or $U_{1064}A_{1192}$.

between the residues at positions 1064 and 1192, as such, is not sufficient for spectinomycin binding. This suggests that the G-residue at 1064 and the C-residue at 1192 are both crucial for the interaction with spectinomycin.

The presence of a C_{1064}-G_{1192} basepair is detrimental to the ribosome

As demonstrated by Figure 2, in the presence of spectinomycin, specialized ribosomes containing $C_{1064}G_{1192}$ synthesize a lower amount of CAT as compared to specialized ribosomes having any of the other mutations introduced. This may mean that ribosomes containing $C_{1064}G_{1192}$ are, to some extent, sensitive to spectinomycin at the concentration used (0.5 mg/ml), or that these mutations reduce the translational activity of the ribosome, irrespective whether spectinomycin is present or absent.

To distinguish between these two alternatives, the synthesis of CAT was measured without adding this antibiotic. In the absence of spectinomycin, however, the translational activity of the specialized ribosomes can not be determined properly by metabolic labelling of CAT, since wild-type ribosomes generate a strong background of labeled endogenous proteins. Therefore, the amount of CAT synthesized by each of the mutants was quantified by measuring the CAT-activity. Upon induction of the synthesis of specialized ribosomes (t=0), samples were taken at 30 minute intervals and the CAT-activity in cell lysates was determined by measuring the amount of [H^3]-diacetyl-chloramphenicol formed (cpm/OD_{650}). In cells transformed with a plasmid in which part of the *rrnB* operon was deleted, the background level of CAT synthesized by wild-type ribosomes was assessed. The deletion in plasmid pASDΔ(SmaI−SstI)CATX spans the region from position 1383 in 16S rRNA to position 365 in 23S rRNA. In control cells harboring this plasmid, specialized ribosomes are not formed (11).

Figure 3A shows that, upon induction of the synthesis of specialized ribosomes having the wild-type G_{1064}-C_{1192} basepair, a large increase in CAT-activity is measured. In cells that do not harbor specialized ribosomes, the CAT-activity remains at a low level. For the disruptive mutants A_{1064}, C_{1064}, and U_{1064} a similar increase in the CAT-activity is measured as the wild-type control, although the activity of the latter two mutants appears to be slightly lower. Nevertheless, these results indicate that, in the absence of spectinomycin, the activity of specialized ribosomes having such disruptive mutations is nearly identical to that of ribosomes having the wild-type G_{1064}-C_{1192} basepair. Figure 3B demonstrates that the translational activity of ribosomes containing the compensatory mutations $A_{1064}U_{1192}$ and $U_{1064}A_{1192}$ is also similar to that of ribosomes having the wild-type basepair. In contrast, in cells harboring the mutant $C_{1064}G_{1192}$, however, at 2 hours after induction, the CAT-activity is merely at 20% of that of the wild-type level. The CAT-activity of this mutant corresponds with the amount of CAT labeled with L-[S^{35}]-methionine; both being a fifth of that of the wild-type level (Figure 2). This means that the low amount of CAT synthesized in the presence of spectinomycin reflects the overall ribosomal activity of the specialized ribosomes, and is not due to inhibition by this antibiotic. Therefore, the mutations $C_{1064}G_{1192}$ confer a similar (high) level of resistance to spectinomycin as any of the other mutations introduced, although the presence of this basepair as such already impairs the ribosomal activity.

30S subunits containing $C_{1064}G_{1192}$ can not form 70S and polysomal complexes

The low amount of CAT synthesized in cells harboring the mutant $C_{1064}G_{1192}$ indicates, that these mutations either interfere with the assembly of the 16S rRNA into ribosomal particles, or that the activity of such particles is severely impaired. To determine whether the formation of 30S, 70S, or polysomal complexes is affected, these ribosomal fractions were isolated and analyzed for the presence of the mutant 16S rRNA. For this purpose, in the absence of spectinomycin, cells were induced at 42°C for 1 hour, before polysome profiles were prepared. From the 30S, 70S, disome, and trisome fractions the rRNA was isolated, and the relative level of mutant 16S rRNA present in each fraction was determined by primer extension analysis (14).

Since this technique exploits a mutation at position 1192 to distinguish between plasmid-encoded specialized and chromosome-encoded wild-type 16S rRNA, specialized

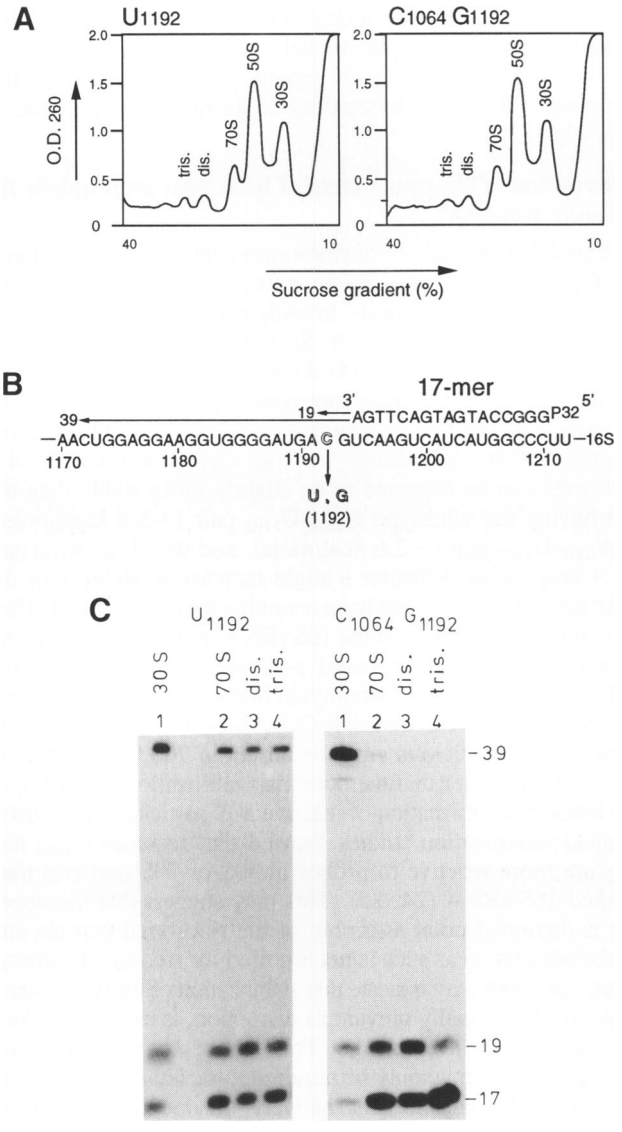

Figure 4. Primer extension analysis of 16S rRNA present in the 30S, 70S, disome, and trisome fractions. (**A**) Polysome profiles of cells harboring specialized ribosomes with mutations in the upper stem helix 34. Cells were grown in the absence of spectinomycin, and harvested 1 h after induction of the synthesis of specialized ribosomes. Polysome profiles were prepared from cell lysates using 10–40% sucrose gradients (see Material and Methods). The profile of cells containing the positive control U_{1192} and the mutant $C_{1064}G_{1192}$ are compared. The 30S, 50S, 70S, disome, and trisome fraction are indicated. (**B**) Primer sequence and lengths of expected extension products. A [^{32}P]-end-labeled-oligonucleotide complementary to the region 1194–1210 was extended by reverse transcriptase. The residues at position 1192, which distinguishes wild-type from specialized 16S rRNA, is presented with an open letter. In the presence of ddGTP, extension of the oligonucleotide 1194–1210 on wild-type and specialized 16S rRNA results in the synthesis of an 19- and 39-mer, respectively. (**C**) Primer extension product synthesized on rRNA isolated from 30S, 70S, disome, and trisome fractions. For both panels, i.e., U_{1192} (left) and $C_{1064}G_{1192}$ (right), in lanes 1 to 4 rRNA was used from 30S, 70S, disome, trisome fractions, respectively. The lengths of the unextended (17-mer) and extended primer (19- and 39-mer) are shown.

ribosomes containing the wild-type $G_{1064}C_{1192}$ basepair can not be used as a positive control to be compared with the mutant $C_{1064}G_{1192}$. Instead, cells harboring the fully active specialized ribosomes containing U_{1192} were used as a positive control.

Table I. Percentage of specialized ribosomes present in the 30S, 70S, disome and trisome fractions

mutant	fraction 30S	70S	disome	trisome
U_{1192}	59	36	34	38
$C_{1064}G_{1192}$	65	<10	<10	<10

Relative levels were determined by measuring the radioactivity (cpm) present in the 19- and 39-mers, as shown in Figure 4B. Relative levels of specialized ribosomes (%) were calculated as: [(cpm in 39-mer) /(cpm in 19-mer + cpm in 39-mer)]×100%.

Figure 4A shows the polysome profiles of cells harboring the U_{1192} control (left panel) and the mutant $C_{1064}G_{1192}$ (right panel). These profiles display a similar distribution of 30S, 50S, 70S and polysome fractions. In both profiles, large amounts of free 30S and 50S subunits are present, because the majority of specialized ribosomes can not initiate translation due to the limiting amount of CAT-mRNA in the cell (Brink, Verbeet and de Boer, unpublished results). The absence of shoulders or peaks containing particles smaller than 30S indicates that 16S rRNA having $C_{1064}G_{1192}$ does not lead to the formation of particles that are grossly different in size.

The rRNA was isolated from each fraction and the presence of the specialized 16S rRNA was assessed by extension of a [^{32}P]-end-labeled oligonucleotide complementary to the region 1194–1210, in the presence of ddGTP. When this 17-mer is annealed to chromosome-encoded wild-type 16S rRNA (see Figure 4B), termination occurs at position C_{1192}, resulting in a 19-mer. Upon annealing to plasmid-encoded specialized 16S rRNA, termination will not occur at position 1192 due to the presence of a G or U-residue, and a 39-mer is made. Since the 17-mer anneals to the identical sequence in both wild-type and specialized 16S rRNA, the relative intensities —i.e. amount of radioactivity— of the end-labeled 18-mer and 38-mer reflect the relative levels of wild-type and specialized ribosomes present in each fraction.

Figure 4C shows the result of this primer extension experiment for the U_{1192} control (left panel) and the $C_{1064}G_{1192}$ mutant (right panel). In both panels, lanes 1 to 4 show the extension products synthesized on rRNA isolated from the 30S, 70S, disome, and trisome fraction, respectively. In lanes 1 to 4 of the left panel, the presence of the 39-mer indicates that each of the ribosomal fractions contain specialized 16S rRNA having U_{1192}. In the right panel, however, a large amount of the 39-mer is only synthesized on rRNA from the 30S fraction. When rRNA is used from either the 70S or the polysomal fractions, the 39-mer is barely detectable. These results indicate that 16S rRNA containing $C_{1064}G_{1192}$ is assembled into 30S particles, while such particles can not form 70S and polysome complexes.

Based on the radioactivity present in the 19- and 39-mers, we calculated the relative levels of the specialized 16S rRNA present in the various fractions (Table I). The Table shows that 16S rRNA containing either U_{1192} or $C_{1064}G_{1192}$ is abundant in 30S particles, forming close to 60% of the 30S fraction. In the 70S and polysome fractions, 36% on average consists of 16S rRNA containing U_{1192}. The relative level of specialized ribosomes present in these fractions is significantly lower as compared to that in the 30S fraction, because the majority of the specialized ribosomes can not initiate translation and form 70S or polysomal complexes, due to the limited amount of CAT-mRNA in the cell. In the 70S and polysome fractions, the 16S rRNA containing

$C_{1064}G_{1192}$ is even less abundant as compared to 16S rRNA having U_{1192}; being less than 10% in each fraction. From these results, we infer that the 30S particles having a C_{1064}-G_{1192} can not form 70S and, consequently, polysomal complexes.

DISCUSSION

Spectinomycin interacts with the residues G_{1064} and C_{1192}

Because each of the spectinomycin resistance mutations located at positions 1064, 1191, 1192 and 1193 in 16S rRNA (3, 4, 5), lead to disruption of the three basepairs forming the upper stem of helix 34, Fromm *et al.* (5) postulated that spectinomycin can bind to this region, only if it is in a basepaired conformation. To determine whether basepairing between the nucleotides at positions 1064 and 1192 is sufficient for spectinomycin binding, or whether this should be a G-C pair, accordingly, we have introduced disruptive and compensatory mutations that alter the G_{1064}-C_{1192} basepair. We have shown that any nucleotide other than G_{1064} confers spectinomycin resistance. All these substitutions make basepairing with C_{1192} impossible, and, therefore, this result does not contradict the model proposed by Fromm *et al.*. Our observation that complementary mutations at position 1064 and 1192 also caused resistance, suggests that spectinomycin can interact directly with at least the residues G_{1064} and C_{1192}. This would be in agreement with a previous footprinting study showing that the residues G_{1064} is protected from chemical modification by dimethylsulphate, when spectinomycin is bound to 70S ribosomes *in vitro* (8).

Spectinomycin is a small molecule, which, in diameter, is of similar size as two basepaired residues. It contains only four functional groups, i.e. one hydroxyl, one keto, and two secondary amino-groups, which could form hydrogen bonds (see Figure 1A). Therefore, this antibiotic should be capable of interacting only with nucleotides that are in the immediate vicinity of one another; e.g. G_{1064} and C_{1192}. The lack of complexity in the molecular structure of this antibiotic appears to be in conflict with the observation that spectinomycin binds exclusively to the upper stem of helix 34 and not to other regions in 16S rRNA. An explanation may be, that the upper stem of helix 34 is one of the few regions in 30S or 70S particles that is accessible for spectinomycin, while other potential binding sites are 'shielded' by r-proteins. The involvement of r-proteins in spectinomycin binding is illustrated by the observation that amino acid substitutions at positions 20−22 of S5 confer spectinomycin resistance (16). These substitutions cause a structural change in a part of S5, which is located in the vicinity of the upper stem of helix 34 (17). This structural change in S5 may render the upper stem inaccessible for spectinomycin, suggesting that this antibiotic does not bind to S5 directly.

Antibiotics that are structurally related to spectinomycin, e.g. streptomycin and neomycin-like aminoglycosides, have also been suggested to bind to specific helices in 16S rRNA. For example, streptomycin may interact with nucleotides in the helix formed by the regions 12−16 and 911−915 (18). Footprinting experiments had already shown that streptomycin protects the residues 911−915 (8). Similar to our results, mutations disrupting the interaction between residues U_{13} and A_{914} conferred streptomycin resistance, while complementary substitutions at these positions also resulted in a resistant phenotype (19). Neomycin-related antibiotics were shown, by chemical footprinting, to bind in the vicinity of the residues A_{1408} and G_{1494} (8), which are both located at the decoding site (20, 21). Resistance to such antibiotics was conferred by mutations disrupting the last basepair of helix 44: i.e. C_{1409}-G_{1491} (22). This indicates that binding of neomycin-like antibiotics to this region depends on the basepaired conformation at the base of helix 44.

Is disruption of the upper stem of helix 34 a prerequisite for ribosome function?

We found that the activity of ribosomes containing the mutations $C_{1064}G_{1192}$ is 5-fold lower as compared to ribosomes harboring any of the other mutations investigated, regardless whether spectinomycin is present or not. Since the single mutations C_{1064} (our own results) and G_{1192} (4) do not affect ribosomal activity, only the combination of these mutations appears to be deleterious. Calculation of the free energy ΔG^0 at 42°C (23) indicates that an upper stem containing a C_{1064}-G_{1192} basepair (−4.4 kcal/mole) can be expected to be slightly more stable than the stem having the wild-type G_{1064}-C_{1192} pair (−3.8 kcal/mole), the A_{1064}-U_{1192} pair (−2.6 kcal/mole), and the U_{1064}-A_{1192} pair (−2.4 kcal/mole). Whether a slight increase in stability of the upper stem of helix 34 can have a significant (deleterious) effect on the functional activity of the 16S rRNA molecule is still highly speculative, however, it could potentially shed light on the mechanism by which spectinomycin impairs ribosome function.

Since 16S rRNA containing $C_{1064}G_{1192}$ was present in 30S subunits, whereas it was virtually absent in 70S and polysomal complexes, we infer that the potential stabilization of the upper stem leads to the formation of inactive 30S particles. Previously, chemical modification studies showed that residues G_{1064} and C_{1192} are more reactive to probes in 30S or 70S particles than in naked 16S rRNA (24, 25). This may suggest that the upper stem is disrupted upon assembly of the ribosomal particle and that the upper stem as such is not required for ribosomal activity. In fact, our result may indicate that stabilization of the upper stem, which could potentially prevent its disruption, is even deleterious to the activity of the ribosome. Thus, the temporary formation of the upper stem may only be required to facilitate the assembly of the ribosomal particle. Alternatively, the opening and closing of this helical structure may define two different functional states of the ribosome separated by a small energy barrier. In this light, the small increase in free energy of the upper stem, caused by the presence of a C_{1064}-G_{1192} basepair, may stabilize the upper stem sufficiently to hamper the disruption of this helical structure. As a result the ribosome would be frozen into one particular conformation, hence being incapable of beginning or completing the elongation cycle. By analogy, due to its interaction with residues G_{1064} and C_{1192}, spectinomycin may also stabilize the basepaired conformation of the upper stem, thereby freezing the ribosomal particle in one particular (translational) state.

Our model describing the mechanism by which spectinomycin inhibits ribosomal activity may also apply to antibiotics that are structurally related to spectinomycin, e.g. streptomycin and neomycin-like aminoglycosides. As already discussed above, the binding of these antibiotics depends on the presence of helical structures in specific regions of 16S rRNA. Mutations that disrupt the basepaired conformation of such regions confer resistance to the individual antibiotics (19, 22). Since each of the helices are located in regions of the 16S rRNA that have been suggested to be able to adopt alternative conformations (18, 21), these antibiotics may freeze, like spectinomycin, such regions in one particular (helical) conformation, thereby blocking the protein synthesis capacity of the ribosome.

ACKNOWLEDGEMENTS

We are grateful to Dr Albert E.Dahlberg for generously giving us plasmids pKK3535A_{1192} and pKK3535G_{1192}.

REFERENCES

1. Burns, D.J.W., and Cundliffe, E. (1973) *Eur. J. Biochem.*, **37**, 570–574.
2. Bilgin, N., Richter, A.A., Ehrenberg, M., Dahlberg, A.E., and Kurland, C.G. (1990), *EMBO J.* **9**, 735–739.
3. Sigmund, C.D., Ettayebi, M., and Morgan, A. (1984) *Nucleic Acids Res.*, **12**, 4653–4663.
4. Makosky, P.C., and Dahlberg, A.E. (1987) *Biochimie*, **69**, 885–889.
5. Fromm, H., Edelman, M., Aviv, D., and Galun, E. (1987) *EMBO J.*, **6**, 3233–3237.
6. Brimacombe, R., Atmadja, J., Stiege, W., and Schüler, D. (1988). *J.Mol. Biol.*, **199**, 115–136.
7. Stern, S., Weiser, B., and Noller, H.F. (1988) *J. Mol. Biol.*, **204**, 447–481.
8. Moazed, D., and Noller H.F. (1987) *Nature*, **327**, 389–394.
9. Hui, A.S., and de Boer, H.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4762–4766.
10. Gourse, R.L., Stark, M.J., and Dahlberg, A.E. (1982) *J. Mol. Biol.*, **159**, 397–416.
11. Brink, M.F., Verbeet, M.Ph., and de Boer, H.A. (1993) *EMBO J.*, **12**, 3987–3996.
12. Brink, M.F., Nels, N.M., Verbeet, M.Ph. and de Boer, H. A. (1994) J. Mol. Biol., *in press*.
13. Saiki, R.S. (1989) in *PCR Technology*, H.A. Erlich (ed.) pp. 7–16, Stockton Press, New York.
14. Sigmund, C.D., Ettayebi, M., Borden, A., and Morgan, E.A. (1988) *Methods Enzymol.*, **164**, 673–690.
15. Triman, K., Becker, E., Dammel, C., Katz, J., Mori, H., Douthwaite, S., Yapijakis, C., Yoast, S., and Noller, H.F. (1989) *J. Mol. Biol.*, **209**, 645–653.
16. Bollen, A., Davies, J., Ozaki, M., and Mizushima, S. (1969) *Science*, **165**, 85–86.
17. Ramakrishnan, V., and White, S.W. (1992) *Nature*, **358**, 768–771.
18. LeClerc, D., and Brakier-Gingras, L. (1991) *FEBS Lett.*, **279**, 171–174.
19. Pinard, R., Payant, C., Melançon, P., and Brakier-Gingras, L. (1993) *FASEB J.*, **7**, 173–176.
20. Prince, J.B., Taylor, B.H., Thurlow, D.L., Ofengand, J., and Zimmerman, R.A..(1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5450–5454.
21. Noller, H.F., Asire, M., Barta, A., Douthwaite, S., Goldstein T., Gutell, R.R., Moazed, D., Normanly, J.,Prince, J.B., Stern,. S., Triman, K., Turner, S., Van Stolk, B., Wheaton, V., Weiser, B., and Woese, C.R. (1986) in *Structure, Function and Genetics of Ribosomes* (B. Hardesty and G.Kramer, eds.) Springer Verlag, NY, 143–163.
22. De Stasio,E.A., Moazed,D., Noller,H.F., and Dahlberg,A.E. (1989) *EMBO J.*, **8**, 1213–1216.
23. Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T., and Turner, H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9373–9377.
24. Baudin, F., Ehresmann, C., Romby, P., Mougel, M., Colin, J., Lempereur, L., Bachellerie, J.P., Ebel, J.P., and Ehresmann, B. (1987) *Biochimie*, **69**, 1081–1096.
25. Laughrea, M., and Tam, J. (1992) *Biochemistry*, **31**, 12035–12041.