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Antibiotic interactions at the GTPase-associated centre within *Escherichia coli* 23S rRNA

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A comprehensive range of chemical reagents and ribonucleases was employed to investigate the interaction of the antibiotics thiostrepton and micrococcin with the ribosomal protein L11-23S RNA complex and with the 50S subunit. Both antibiotics block processes associated with the ribosomal A-site but differ in their effects on GTP hydrolysis, which is inhibited by thiostrepton and stimulated by micrococcin. The interaction sites of both drugs were shown to occur within the nucleotide sequences A1067-A1098 within the protein L11 binding site on 23S RNA. This region of the ribosome structure is involved in elongation factor-G-dependent GTP hydrolysis and in the stringent response. No effects of drug binding were detected elsewhere in the 23S RNA. In general, the two drugs afforded 23S RNA similar protection from the chemical and nuclease probes in accord with their similar modes of action. One important exception, however, occurred at nucleotide A1067 within a terminal loop where thiostrepton protected the N-1 position while micrococcin rendered it more reactive. This difference correlates with the opposite effects of the two antibiotics on GTPase activity.

Key words: thiostrepton/micrococcin/ribosomal protein L11/antibiotic-RNA interaction/ribosomal A-site

Introduction

Current evidence strongly indicates that rRNA is an essential determinant of ribosomal function and, thus, the aim of present studies is to link rRNA structures with specific functional events in protein biosynthesis. To this end, antibiotics have proved helpful biochemical probes. Many antibiotics interact with a single binding site on the ribosome and inhibit a specific step, or related steps, of translation (Gale *et al.*, 1981). Data from modifications (Cundliffe, 1986) and mutations in rRNA (e.g. Sigmund *et al.*, 1984) that confer antibiotic resistance and from footprinting of antibiotics on rRNA (Moazed and Noller, 1987a,b) indicate that ribosome binding drugs interact directly with rRNA.

Thiostrepton and micrococcin are both modified peptide antibiotics of similar structure. Earlier data on the mutually competitive binding of the two antibiotics (reviewed by Cundliffe and Thompson, 1981) indicated that they either have the same or overlapping binding sites on the 50S subunit. This view was strengthened when *Bacillus megaterium* mutants that lack a protein homologous with *Escherichia coli* protein L11 were shown to be resistant to both drugs (Cundliffe and Thompson, 1981) although a further, unidentified protein, might also be involved in micrococcin resistance (Wienen *et al.*, 1979). Furthermore, the thiostrepton-producer *Streptomyces azureus* has a methylase specific for the 2'-O of A1067 (*E.coli* numbering) within the L11 binding site that conferred resistance to both antibiotics (Cundliffe and Thompson, 1981; Thompson *et al.*, 1982). Additional evidence for the binding site of thiostrepton lying within this region was attained in an earlier study when the drug was recovered bound to a complex of L11 and a fragment of 23S RNA (Thompson *et al.*, 1979). The 23S RNA binding site of L11 was subsequently shown to include A1067 (Schmidt *et al.*, 1981; Egebjerg *et al.*, 1989).

The physiological effects of both thiostrepton and micrococcin are linked to the ribosomal A-site (reviewed by Vázquez, 1979; Gale *et al.*, 1981), correlating well with their similarity in structure and ribosomal binding. There are, however, important differences in their effects on elongation factor-G (EF-G)-dependent hydrolysis of GTP which is inhibited by thiostrepton but stimulated by micrococcin (Cundliffe and Thompson, 1981). Additionally, methanogens are sensitive to thiostrepton but are unaffected by micrococcin (Beauclerk *et al.*, 1985).

In this study, we investigated the binding of thiostrepton and micrococcin to 23S RNA and established the effect of L11 upon the interactions. We reasoned that the different functional effects of the two drugs probably have a basis in their physical interaction with 23S RNA, most likely within the L11 binding region. To test this idea, we employed a range of chemical reagents and ribonucleases to probe the entire 23S RNA structure before and after binding the drugs to the L11-23S RNA complex and the 50S subunit.

Results

Drug binding to the L11-23S RNA complex and the 50S subunit

Accessibilities of nucleotides to the chemical and ribonuclease probes in the 1026-1128 region of 23S RNA were determined in the L11-23S RNA complex and in the 50S subunit and are shown for the latter in Figures 1 and 2A. The single-strand specific probes reacted mainly in the loops while cobra venom RNase (RNase CV) cuts primarily in double helical regions (Figure 2A), which correlates well with the specificities of the probes described in Materials and methods and with the putative secondary structure model for this region (Leffers *et al.*, 1987).

Nucleotide accessibilities were compared throughout the 23S RNA structure in both the L11-23S RNA complex and in the 50S subunit before and after antibiotic binding. Data are presented in Table I only for positions where the antibiotics induced altered nucleotide reactivities; these

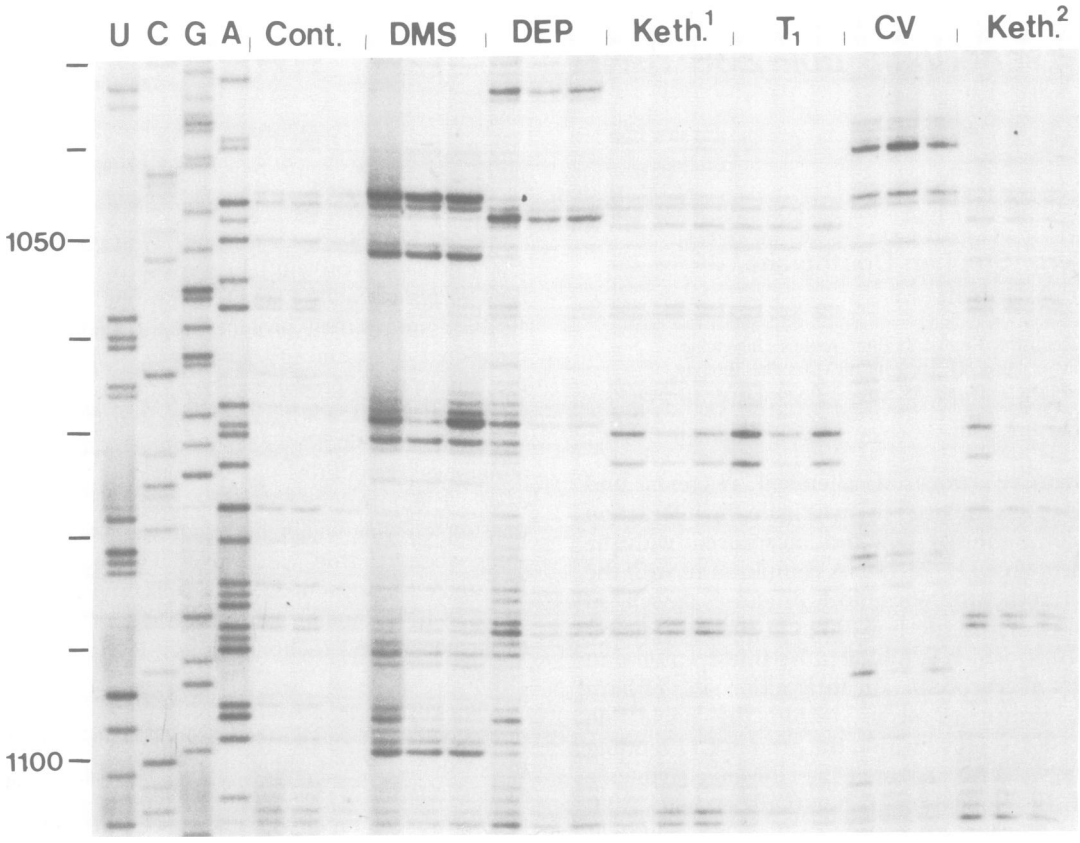

Fig. 1. Autoradiograph showing altered reactivities induced by thiostrepton and micrococcin binding to 50S subunits. The U, C, G and A tracks on the left were created by dideoxynucleotide sequencing on a 23S RNA template. Controls (Cont.), untreated samples; DMS (modified 25 min); DEP (60 min); Keth[1] and Keth[2] (20 μl Kethoxal, 60 min) RNase T1 (0.015 U, 30 min); RNase CV (0.16 U, 60 min). The order of the samples for each reagent is: first, no antibiotics; second, thiostrepton at a 5-fold molar excess relative to 23S RNA; third, micrococcin at a 10-fold molar excess. In Keth[2] micrococcin was added at a 25-fold molar excess.

included dimethylsulphate (DMS), diethylpyrocarbonate (DEP) and Kethoxal modifications, and RNase T1 and CV cutting, between nucleotides A1067 and A1098 (Figure 2B). No changes were observed in psoralen and CMCT reactivities or in RNase T2 cutting. Furthermore, no changes were detected in other parts of the 23S RNA molecule.

Several nucleotides that were accessible in this region of the 50S subunit were protected in the L11–23S RNA complex. These included the RNase T1 cuts at G1068 and G1071, Kethoxal reactivity at G1071, RNase CV cuts at A1080, U1081 and G1091 and the DEP modification of A1090 (Table I) and several RNase T2 cuts (data not shown). These constitute the most reactive RNA sites that were protected by L11 (Egebjerg *et al.*, 1989). However, the nucleotides that were reactive in the L11–23S RNA complex (Table I) were protected by the antibiotics to the same extent in both the complex and 50S subunits.

Thiostrepton and micrococcin binding sites on 23S RNA

Binding of thiostrepton to the 50S subunit resulted in the reduced reactivity of 13 nts between A1067 and A1098 (Figure 2B and Table I). Some nucleotides were shielded against more than one probe. For example, A1067 and A1095 were less reactive to both DMS and DEP which indicated that thiostrepton reduced the accessibility of both the N-1 and N-7 positions. Altered nucleotide reactivities induced by micrococcin binding were also limited to the A1067–A1098 region where there was a general protection against chemical and nuclease probes, with one notable exception. Nucleotide A1067 became more reactive to DMS which reflects an enhanced accessibility at the N-1 position; however, the N-7 position was protected against DEP modification (Figure 1).

Throughout the rest of the binding region, the protection patterns of the two antibiotics were the same. Only minor differences in the degree of protection were observed at some nucleotides. All the major effects listed in Table I were evident at the lower drug concentrations and became more pronounced at higher concentrations. This is illustrated in Figure 1 for micrococcin protection against Kethoxal.

Discussion

Evolutionary conservation of the antibiotic binding sites

The RNA region encompassing the antibiotic binding sites has been implicated in EF-G-dependent GTPase activity and in the stringent response. The secondary structure of this region, corresponding to helices 3 and 4 and loops B, C and D (Figure 2), is conserved in all the eubacterial, archaebacterial and eukaryotic cytoplasmic, chloroplast and mitochondrial 23S (and 23S-like) RNA sequences so far investigated (Leffers *et al.*, 1987). Such exceptional conservation may also extend to the highly abbreviated *Trypanosome*, *Crithidia* and *Leishmania* mitochondrial rRNA sequences which lack two-thirds of the total structure found in eubacterial 23S RNA (Gutell and Fox, 1988). Here,

Fig. 2. (**A**) Putative secondary structure model of the 1026–1128 region of 23S RNA (Leffers *et al.*, 1987) showing sites that are accessible in the 50S subunit to the chemical and nuclease probes. The positions of ribonuclease cuts are indicated by arrows, an open symbol denotes a weak cut, a filled symbol represents a medium cut and two or three symbols indicate strong or very strong cuts, respectively, for RNases T1 (⋆), T2 (■) and CV (•). DMS: weak (○), medium (•) and strong (••) modification; DEP: encircled nucleotides, weak (○), medium (●). Kethoxal: medium (★). The data are a summary of eight independent experiments. No differences were detected in the accessibilities of nucleotides on changing the $MgCl_2$ concentration from 10 to 20 mM or the KCl concentration from 100 to 300 mM. CMCT and the psoralen derivative did not react in this region of the 50S subunit, whereas a number of sites were reactive in the L11–23S RNA complex (Egebjerg *et al.*, 1989). (**B**) The common protection effects induced by micrococcin and thiostrepton on 23S RNA in the 50S subunit. The small and large symbols represent weak and strong protection effects, respectively (see Table I), and correspond to the same probes as in (A). Micrococcin enhanced the DMS modifications at A1067 (bold arrow), whereas this nucleotide was shielded by thiostrepton. The binding data for thiostrepton and micrococcin are summarized from six and four independent experiments, respectively.

Table I. Nucleotide reactivities in the L11–23S RNA complex and in the 50S subunit before and after binding of antibiotics

Nucleotide	Nuclease chemical	23S– L11	23S–L11 + thiostrepton	23S–L11 + micrococcin	50S	50S + thiostrepton	50S + micrococcin
A 1067	DMS	++	+	+++	++	+	+++
	DEP	+	(+)	(+)	+	(+)	(+)
G 1068	T1	–			++	–	(+)
	K	(+)	–	–	+	–	–
A 1069	DEP	+	–	–	+	–	–
A 1070	DEP	+	–	–	+	–	–
G 1071	T1	–			+	–	–
	K	–			+	–	–
A 1073	DMS	(+)		–	(+)		–
A 1080	CV	–			+	(+)	(+)
U 1081	CV	–			+	(+)	(+)
A 1089	DEP	(+)	–	–	+	–	–
A 1090	DEP	–			+	–	–
G 1091	CV	–			+	–	(+)
A 1095	DMS	++	(+)	–	++	(+)	–
	DEP	+	–	–	+	–	–
A 1098	DEP	+	(+)	–	+	(+)	–

The reactivities of the nucleotides to chemical reagents and ribonucleases are the visual estimations of autoradiograph band intensities from several experiments (see legend to Figure 2). +++, very strong band; ++, strong band: +, medium band; (+), weak band. '–', indicates complete protection against the probes. The blank spaces indicate that there was no change in band intensity upon drug binding. These data correspond to binding at the highest antibiotic concentrations (Materials and methods). Abbreviations are given in Materials and methods, except for 'K' (Kethoxal).

the evolutionary conservation of this putative GTPase site is comparable with the conservation (and, presumably, the functional importance) of the peptidyl transferase loop in domain V of 23S RNA (Noller, 1984).

Nucleotide A1067 is of pivotal importance. It is conserved in eubacterial, archaebacterial and chloroplast 23S RNAs although a guanosine occurs at the equivalent position in most eukaryotic cytoplasmic ribosomes. *S.azureus* has a 2′-O-

methylation at the position equivalent to A1067 that affords resistance to both thiostrepton and micrococcin (Cundliffe and Thompson, 1981; Thompson *et al.*, 1982). Moreover, mutagenesis of this nucleotide gives thiostrepton resistance in halobacteria *in vivo* (Hummel and Böck, 1987) and in *E.coli* ribosomes *in vitro* (Thompson *et al.*, 1988).

Antibiotic–RNA interactions

The results demonstrate that both thiostrepton and micrococcin interact with the L11–23S RNA complex and the 50S subunit within the region between A1067 and A1098. The antibiotics induced protection at 13 nucleotides, that are concentrated in loops B and D and helix 4 (Figure 2B). The results are compatible with a tertiary structural model in which loops B and D are folded close together (Egebjerg *et al.*, 1989) enabling the drugs to contact both of these regions simultaneously. Since no effects were detected elsewhere in the 23S RNA, we infer that this region constitutes the sole site of drug–RNA interaction, which correlates with the finding that thiostrepton can be recovered together with L11 bound to a fragment of 23S RNA after RNase T1 digestion (Thompson *et al.*, 1979). However, an additional interaction between the drugs and protein L11 cannot be excluded. The observation that protein L11 greatly enhances the binding constant of thiostrepton (Cundliffe, 1986) could reflect either a protein–drug interaction or a protein-induced change in the RNA structure with an accompanying increase in the affinity of the drug for the RNA. The absence of change in the nucleotide reactivities when the drugs were incubated with free 23S RNA does not resolve this ambiguity.

The interactions of a wide range of antibiotics with RNA have been mapped by Moazed and Noller (1987a,b). For example, the binding sites of chloramphenicol and erythromycin were located on 23S RNA, and that of hygromycin was placed on 16S RNA. Each site involved two to five nucleotides within a discrete region of secondary structure. The interactions of vernamycin B with 23S RNA and other aminoglycosides with 16S RNA were more extensive and spanned two or more rRNA domains although it is unlikely that they protected all of the implicated nucleotides. The same possibility exists for thiostrepton and micrococcin, despite their large structures. They may interact directly in one region of the RNA and indirectly lower the RNA reactivity either by inducing a tightening of the RNA structure or by strengthening the L11–23S RNA interaction, as discussed below.

Are there functional conformers in the L11 site?

Several nucleotides that were protected in the L11–23S RNA complex were reactive in the 50S subunit (Table I). The possibility that there was a low stoichiometry of L11 in the subunits could be ruled out on the basis of the drug protection data. The complete protection of eight nucleotides (for example the strong RNase T1 cut at G1068 which was rendered unreactive by thiostrepton) indicated stoichiometric binding of the drugs which, in turn, was dependent on stoichiometric binding of L11. Therefore it is likely that L11 is present in all subunits and exhibits a spatial arrangement on the 23S RNA that differs partly or wholly from that in the L11–23S RNA complex. Such a conformational change could occur in the L11 or in the 23S RNA and be stabilized by the other 50S subunit components. Drug binding to the 50S subunits could then induce a change in the L11 binding such that it resembles that in the isolated L11–23S RNA complex. However, we cannot eliminate the possibility that the antibiotics bind to the 50S subunit in a manner that mimics the protection effects produced by L11 in the L11–23S RNA complex, although, as discussed in the previous section, this explanation is unlikely.

Cundliffe (1986) has suggested that the RNA binding region for L11 has multiple functional conformations and that it may be locked into one of them by thiostrepton. The occurrence of RNA conformational heterogeneity receives support from DMS modification at A1088 and A1090. In the L11–23S RNA complex, A1088 was accessible and A1090 was resistant (Egebjerg *et al.*, 1989); in 50S subunits, both were accessible (Figure 2); and in poly U-bound 70S ribosomes with $tRNA^{Phe}$ at the A- and P-sites, A1088 was inaccessible but A1090 was accessible (J.Egebjerg, unpublished data). The latter result is in accordance with the phylogenetically supported model (Figure 2). It is feasible that the function of L11 is to facilitate such changes within its RNA binding region.

A functional rRNA site

Translational steps shown to be inhibited by thiostrepton involve the binding of ribosomal factors, including initiation factor-2 and EF-Tu and -G, which are accompanied by GTP hydrolysis, (p)ppGpp formation and peptide chain termination (reviewed by Vázquez, 1979 and Gale *et al.*, 1981). Micrococcin also inhibits several A-site-associated steps, primarily blocking EF-Tu-dependent aminoacyl-tRNA binding (reviewed by Vázquez, 1979 and Cundliffe and Thompson, 1981). This correlates well with the results of the present study, which show that both antibiotics interact with the same site in 23S RNA.

An essential question to be addressed here is: Can one explain on the basis of the limited binding site of the antibiotics their wide-ranging effects on protein synthesis? The antibiotic site has been linked with most of the above processes and it undoubtedly constitutes part of the ribosomal A-site. The interaction of EF-G at this site has been directly established by RNA cross-linking studies (Sköld, 1983) and by chemical footprinting (Moazed *et al.*, 1988). The RNA region has been implicated in (p)ppGpp production on the basis of studies of *relC* (L11) strains (Cundliffe, 1986). (p)ppGpp production requires an uncharged tRNA in the A-site (Block and Heseltine, 1974) and it would thus be inhibited by either A-site-bound antibiotic. The other inhibitory effects of the antibiotics necessitate that the ribosomal factors have overlapping binding sites and various lines of evidence support this for EF-G and EF-Tu (Liljas, 1982; Moazed *et al.*, 1988).

Despite the similarities between thiostrepton and micrococcin, they exhibit a striking difference in their effects on EF-G-dependent GTP hydrolysis. EF-G binds to the ribosome in a complex with GTP and promotes translocation and subsequent GTP hydrolysis, which is followed by dissociation of the EF-G·GDP complex from the ribosome (Spirin, 1986). Presumably, the drugs bind to the 50S subunit after this latter stage. At the next cycle of elongation, EF-G·GTP binding is prevented by thiostrepton, thus inhibiting GTP hydrolysis (Gale *et al.*, 1981). Micrococcin, however, stimulates EF-G-dependent GTPase (Cundliffe and Thomp-

son, 1981) and presumably allows, or even aids, EF-G·GTP binding. The N-1 position of A1067 is strongly shielded from DMS modification by ribosome-bound EF-G (Moazed *et al.*, 1988). This observation correlates with the differences we observed at the N-1 of A1067, which was less reactive to DMS after thiostrepton binding but more reactive after micrococcin binding. We infer therefore that a conformational change is induced by micrococcin at this nucleotide that results in a higher association constant for the EF-G·GTP complex.

The 23S RNA region that interacts with thiostrepton and micrococcin is clearly of functional importance. It should also be borne in mind, however, that EF-G·GTP binding also involves domain VI of 23S RNA (Moazed *et al.*, 1988; Leffers *et al.*, 1988). Moreover site-directed mutagenesis studies of A1067 have shown that an adenine at this position is not essential for GTPase activity (Thompson *et al.*, 1988). Thus, the picture presently emerging of EF-G binding and subsequent GTP hydrolysis is that domains II and VI of 23S RNA and one or more ribosomal proteins are involved. The latter either participate directly, or indirectly by stabilizing functional RNA conformers as is suggested here for L11.

Materials and methods

Binding of antibiotics to ribonucleoprotein complexes

50S subunits and 23S RNA were prepared from *E.coli* strain MRE 600 as described by Garrett *et al.* (1984). Protein L11 was prepared on CM-Sephadex (Pettersson *et al.*, 1976) and stored in 5 mM Tris–Cl, pH 7.0, 300 mM NH_4Cl, 5 mM dithiothreitol at −80°C. The complex was formed by incubating 8 pmol renatured 23S RNA with a 3-fold molar excess of L11 in 200 μl of 50 mM Hepes–OH, pH 7.6, 20 mM $MgCl_2$, 300 mM KCl ($H_{50}M_{20}K_{300}D_1$) at 37°C for 1 h followed by slow cooling. The antibiotics thiostrepton and micrococcin were generous gifts from Eric Cundliffe. Prior to binding of the antibiotics, 50S subunits were preincubated for 15 min at 30°C in 50 mM Hepes–OH, pH 7.6, 10 mM $MgCl_2$, 100 mM KCl and 1 mM dithiothreitol; duplicate samples were preincubated in $H_{50}M_{20}K_{300}D_1$. Antibiotics, dissolved in DMSO, were then added at a 3- to 25-fold molar excess (thiostrepton) or a 5- to 100-fold molar excess (micrococcin) relative to 23S RNA. The mixtures were incubated for a further 20 min at 30°C, slowly cooled and placed on ice prior to probing. Control samples contained an equal amount of DMSO, which never exceeded 1% of the final volume.

Chemical and ribonuclease probing

Chemical reagents and nucleases were chosen according to their specificities to facilitate probing of all nucleotides (Egebjerg *et al.*, 1987; Christiansen, 1988). Most of the chemical reagents are single-strand specific: dimethylsulphate (DMS, Merck) modifies adenosine (N-1) and cytidine (N-3) more slowly; diethylpyrocarbonate (DEP, Eastman Kodak) reacts with adenosine (N-7); CMCT (1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methyl-*p*-toluene-sulphonate, Sigma) modifies uridine (N-3) and guanosine (N-1) more slowly; Kethoxal modifies guanosine (N-1 and N-2). The psoralen derivative, 8-{[3-(4-methyl-1-piperazinyl)propyl]oxy} psoralen (a gift from Ole Buchardt), modifies pyrimidines, preferentially uridines, that are located at the ends of helices or in irregular double helical regions; DMS also reacts at guanosine (N-7) within regular double helical regions. The RNases T1 (guanosine) and T2 (preference for adenosine) are single-strand specific and RNase CV is specific for double-stranded structures.

Antiobiotic-ribonucleoprotein complexes, and control samples without antibiotics, were subjected to limited modification with chemical reagents or digestion with ribonucleases at 0°C in the buffers described above. Probing with DMS, DEP, Kethoxal, CMCT and RNases T1, T2 and CV were otherwise carried out according to Egebjerg *et al.* (1987), and the psoralen derivative was used as described by Christiansen (1988).

Reverse transcriptase analyses of modified and digested sites in 23S RNA

Nucleotides that were reactive to the various probes were identified by primer extension using reverse transcriptase and by sequencing gel electrophoresis (Moazed *et al.*, 1988); Christiansen *et al.*, 1987). Deoxyoligonucleotides complementary to the following sequences were used as primers to screen the entire 23S RNA (Noller, 1984): 417–433; 617–635; 888–905; 1157–1174; 1309–1324; 1687–1703; 1906–1922; 2234–2251; 2607–2634; 2888–2904.

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