RNA (1999), *5*:585–595. Cambridge University Press. Printed in the USA.

UV-induced modifications in the peptidyl transferase loop of 23S rRNA dependent on binding of the streptogramin B antibiotic, pristinamycin IA

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ABSTRACT

The naturally occurring streptogramin B antibiotic, pristinamycin IA, which inhibits peptide elongation, can produce two modifications in 23S rRNA when bound to the *Escherichia coli* 70S ribosome and irradiated at 365 nm. Both drug-induced effects map to highly conserved nucleotides within the functionally important peptidyl transferase loop of 23S rRNA at positions m^2A2503/Ψ2504 and G2061/A2062. The modification yields are influenced strongly, and differentially, by P-site-bound tRNA and strongly by some of the peptidyl transferase antibiotics tested, with chloramphenicol producing a shift in the latter modification to A2062/C2063. Pristinamycin IA can also produce a modification on binding to deproteinized, mature 23S rRNA, at position U2500/C2501. The same modification occurs on an ~37-nt fragment, encompassing positions ~2496–2532 of the peptidyl transferase loop that was excised from the mature rRNA using RNAse H. In contrast, no antibiotic-induced effects were observed on in vitro T7 transcripts of full-length 23S rRNA, domain V, or on a fragment extending from positions ~2496–2566, which indicates that one or more posttranscriptional modifications within the sequence Cm-C-U-C-G-m^2A-Ψ-G_{2505} are important for pristinamycin IA binding and/or the antibiotic-dependent modification of 23S rRNA.

Keywords: 23S rRNA; peptidyl transferase; pristinamycin IA; RNAse H analysis; rRNA modifications; streptogramin B-ribosome interaction

INTRODUCTION

Antibiotics are important tools for the treatment of many serious human and animal infections. However, the increasing use and misuse of these drugs in both the health care and agricultural sectors have led to increasing problems with drug-resistant bacteria, with severe consequences for human health. One group of such therapeutically important antibiotics are the streptogramins, which contain A and B components and target the peptidyl transferase center of the large ribosomal subunit, where they inhibit peptide elongation. Resistance to both streptogramin components is widespread in diverse bacterial communities.

Streptogramins inhibit peptide elongation by a mechanism that is only partially understood. Moreover, the two components act synergistically such that although the individual A and B components are bacteriostatic, together they can be bacteriocidal (Gale et al., 1981; Di Giambattista et al., 1989). The observation that neither streptogramin component affects protein synthesis on polysomes suggests that they act during the initial rounds of protein synthesis in vivo (Contreras & Vázquez, 1977). In contrast to the A component, streptogramin B has no direct effect on peptide bond formation with puromycin, in vitro, a property which it shares with the smaller macrolides. Streptogramin B and these macrolides also share a similar resistance mechanism associated with either N-6 methylation (MLS_B phenotype), or mutation, at A2058 within the peptidyl transferase loop of 23S rRNA that is important for peptide bond formation (Cundliffe, 1990; Garrett & Rodriguez-Fonseca, 1995).

The binding sites of both streptogramin components have been assigned indirectly to nucleotides within the peptidyl transferase loop of 23S rRNA on the basis of

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chemical footprinting and mutational analyses (Vanuffel et al., 1992; Rodriguez-Fonseca et al., 1995; Porse & Garrett, 1999) and, for the B component, to the vicinity of ribosomal proteins L18 and L22 by affinity labeling (Di Giambattista et al., 1990). However, although several lines of evidence suggest that these and other peptidyl transferase drugs interact with rRNA, no direct binding to isolated 23S rRNA has been detected for any of them (reviewed by Kirillov et al., 1997).

In the present work, we provide evidence for both a direct interaction of the streptogramin B drug, pristinamycin IA (PIA; Fig. 1), with highly conserved nucleotides of the peptidyl transferase loop of 23S rRNA and for two PIA-dependent modifications in the same functional rRNA region. Moreover, we demonstrate that the presence of pristinamycin IIA (PIIA, a streptogramin A), chloramphenicol, carbomycin, tylosin, spiramycin and P-site-bound tRNA alter the PIA-induced effects. Finally, evidence is provided for the binding of PIA to protein-free mature rRNA, and to small rRNA fragments excised from the peptidyl transferase loop region, which is dependent on at least one posttranscriptional modification.

RESULTS

Identification of the pristinamycin IA-dependent modification sites on 23S rRNA

PIA was complexed to 70S ribosomes or 50S subunits from *Escherichia coli* and the complexes were irradiated with UV light at 365 nm. Total rRNA was isolated and 5S, 16S, and 23S RNAs were scanned for PIA-induced stops by primer extension using AMV reverse transcriptase. Only two sets of stops were observed in the 23S rRNA for drug complexes with either 70S ribosomes (Fig. 2) or 50S subunits (data not shown). They occurred at positions corresponding to m^2A2503 and Ψ2504 (Fig. 2A, compare lanes 1 and 2) and G2061 and A2062 (Fig. 2B, compare lanes 1 and 2) of the peptidyl transferase loop. The observation that the reverse transcriptase produced a weak stop at A2503, even in the absence of PIA, is likely to reflect the presence of the 2-methyl group at this position in *E. coli* 23S rRNA (Kowalak et al., 1995). Additional control experiments were performed to establish that the PIA-induced effects were dependent on UV irradiation (see Fig. 3A). The locations of the altered nucleotides within the peptidyl transferase loop of 23S rRNA are illustrated in Figure 2C.

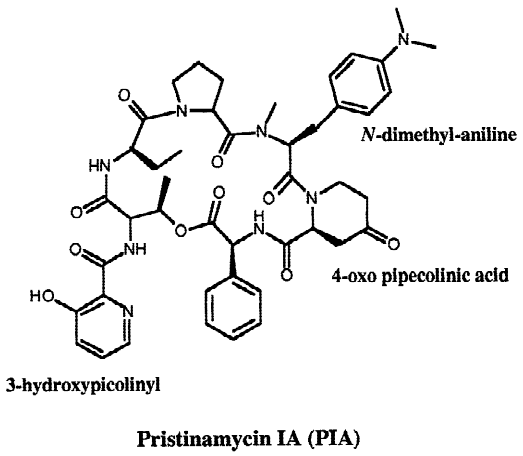

FIGURE 1. The chemical structure of PIA.

In principle, PIA-induced primer-extension stops could be caused by (1) direct crosslinking of the drug to 23S rRNA within the ribosome, (2) drug-induced cleavage of the RNA backbone, (3) a drug-induced protein–rRNA crosslink, or (4) a drug-induced rRNA–rRNA crosslink. To distinguish between these four possibilities, 70S ribosomes were irradiated with UV light at 365 nm in the presence and absence of PIA. The resulting complexes were extracted twice with phenol and small rRNA fragments encompassing sites m^2A2503/Ψ2504 (~124 nt, positions ~2443–2566) and G2061/A2062 (~190 nt, positions ~2048–2237) were excised from the 23S rRNA by oligonucleotide-directed RNAse H cleavage.

After purification on denaturating polyacrylamide gels, the RNAse H fragments were subjected to primer-extension analysis. The finding that the same PIA-dependent primer-extension stops occurred at m^2A2503/Ψ2504 on the isolated ~124-nt fragment (Fig. 2A, lanes 3 and 4) ruled out the possibilities that the stops were caused (2) by cleavage of the RNA backbone, (3) by a protein–rRNA crosslink, or (see (4)) that rRNA–rRNA crosslinks occurred with a sequence region outside of the ~124-nt fragment. Moreover, the finding that extension from primers located immediately 5′ to m^2A2503/Ψ2504 did not yield additional stops (data not shown), renders the possibility of an rRNA–rRNA crosslink occurring within the isolated fragment very unlikely. Thus, all the evidence strongly suggests, but does not prove, that PIA is crosslinked directly to nucleotides m^2A2503/Ψ2504, within the peptidyl transferase loop of 23S rRNA, in both 70S ribosomes and 50S subunits.

In contrast, the PIA-dependent primer-extension stops at G2061/A2062 were not observed on the isolated ~190-nt fragment (Fig. 2B, lanes 3 and 4), suggesting that they derive from a protein–rRNA, or an rRNA–rRNA crosslink, formed in the presence of PIA. After treatment of the irradiated PIA–ribosome complexes with proteinase K, before isolation of the ~190-nt fragment, the primer-extension stops at G2061/A2062 were not seen either (data not shown), which supports the idea that PIA induces an rRNA–rRNA crosslink, although a residual crosslinked protein fragment could

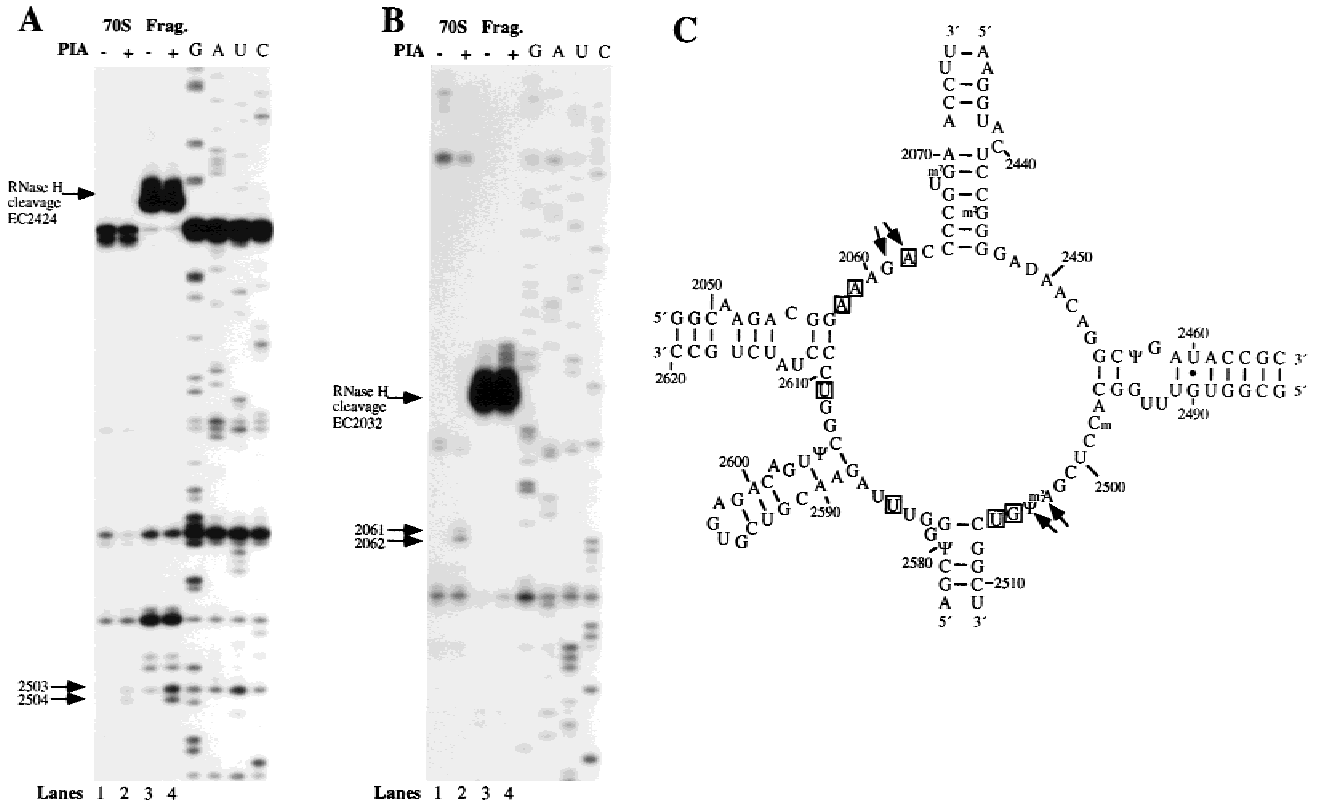

FIGURE 2. Characterisation of the PIA-dependent modifications of 23S rRNA. **A**: 70S ribosomes (0.15 μM) were incubated with (+) and without (−) PIA for 20 min at 37 °C, irradiated at 365 nm for 20 min, and rRNA was isolated as described in Materials and Methods. One half of each sample (70S) was analyzed directly by extension from primer EC2521. The other half of the sample was treated with RNAse H in the presence of oligonucleotides EC2424 and EC2563. The released fragment (~124 nt, Frag.) was gel-purified, and subjected to primer extension using primer EC2521. Bands seen at positions 2500/2501 (lanes 3 and 4) were produced by UV scanning of the gel containing RNAse H bands at 254 nm (see text). The rRNA modification-induced stop is displaced one base below the corresponding stop in the sequencing tracks (G, A, U, and C). **B**: The experiment was performed as in **A** except that RNAse H digestion was performed in the presence of oligonucleotides EC2032 and EC2234 resulting in a ~190-nt fragment (Frag.). The rRNA was analyzed by extension from primer EC2102. **C**: Secondary structure of the peptidyl transferase loop of 23S rRNA of *E. coli*. PIA-dependent modification sites are indicated by arrows and nucleotides that show altered chemical reactivities in the presence of the drug (Porse & Garrett, 1999) are boxed.

also have produced an altered rRNA fragment mobility. No potential rRNA crosslinking partner was located in primer-extension analyses of 23S, 16S, and 5S rRNAs (data not shown).

Chemical characteristics of the PIA-dependent modification of m^{2}A2503/Ψ2504

To gain more insight into the chemistry of the rRNA modification at m^{2}A2503/Ψ2504, we tested the effects of time, drug concentration, and UV wavelength on the modification yield at m^{2}A2503/Ψ2504 (Fig. 3). It increased linearly, for at least 1 h of irradiation, suggesting that the UV doses employed neither reduced the capacity of ribosomes to bind PIA nor damaged the drug itself (Fig. 3A,B). When the modification yield at m^{2}A2503/Ψ2504 was monitored as a function of the concentration of PIA (Fig. 3C), a dissociation constant of approximately 0.1 μM was estimated (Fig. 3D), which agrees well with an earlier estimate for the closely related drug virginiamycin S (K_d = 0.26 μM, Vanuffel et al., 1992). Finally, we tested the effect of varying the UV wavelength. No modification was observed at 254 nm, whereas irradiation at 312 nm and 365 nm yielded weak and strong stops, respectively, at m^{2}A2503/Ψ2504 (Fig. 3E). The intensities of the latter stops were not affected by the presence of the filter that cuts off shorter wavelengths (<300 nm).

The effect of P/P′-site-bound tRNA

Next, we examined whether bound tRNA perturbed the PIA-dependent rRNA modifications. Deacylated tRNA was bound in the P/P′-site of 70S ribosomes in the presence of poly(U) and irradiated, with and without PIA. The results show that deacylated tRNA produced a threefold increase in the modification yield at m^{2}A2503/Ψ2504 (Fig. 4A, compare lanes 3 and 5),

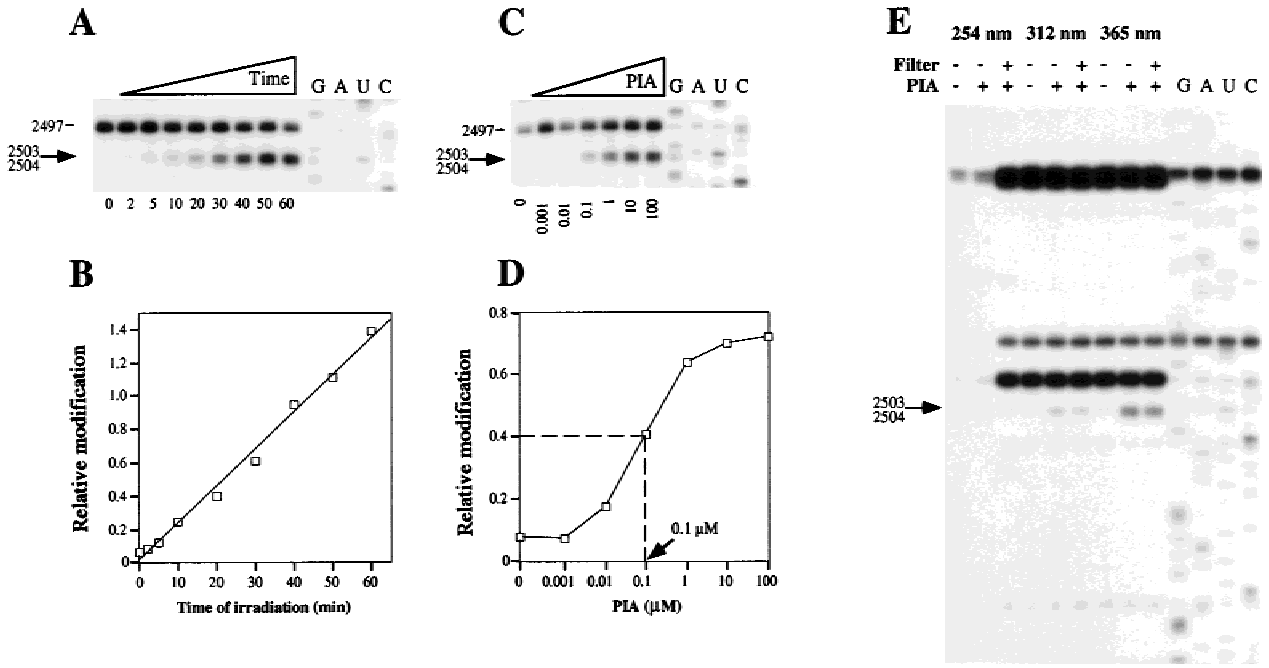

FIGURE 3. The dependence of the yield of the PIA-dependent rRNA modification on time of irradiation, PIA concentration, and wavelength of the UV light. **A,B**: Time dependence: Complexes of 70S (0.15 μM) ribosomes and PIA (10 μM) were irradiated at 365 nm for the time (minutes) indicated below the gel. rRNA was isolated as described in Materials and Methods and analyzed by extension from primer EC2621. The yield of PIA-dependent modification at m^2A2503/Ψ2504 was quantified relative to the natural stop at A2497 using an Instant Imager and plotted as a function of time. **C,D**: PIA concentration dependence: Complexes of 50S subunits (50 nM) and increasing amounts of PIA were irradiated at 365 nm for 20 min and treated further as described above. The concentration of PIA (in μM) is indicated below the gel. The yield of modification at m^2A2503/Ψ2504 was quantified relative to the natural stop at A2497 using an Instant Imager and plotted as a function of the concentration of PIA. **E**: Wavelength dependence: 50S subunits (0.15 μM) were irradiated for 20 min in the presence (+) and absence (−) of PIA (10 μM) at 254 nm, 312 nm, or 365 nm. When indicated (+), a petri dish was used as a filter (<300 nm). G, A, U, and C represent nucleotide sequencing tracks.

whereas no change was observed in the yield at G2061/A2062 (Fig. 4B, compare lanes 3 and 5). Similar results were found when N-Ac-Phe-tRNA was bound (data not shown). Control experiments performed in the presence of tRNA established that the primer-extension stops observed at these positions were entirely dependent on PIA (data not shown; these data were provided to the editor). Since these experiments were performed at saturating concentrations of PIA, the stimulatory effect of the tRNA was not because of increased binding of PIA. Therefore, we infer that the tRNA renders the stereochemistry of the drug-23S rRNA interaction more favorable for modification at m^2A2503/Ψ2504.

The effect of PIIA

The effect on the rRNA modifications of PIIA, which acts synergistically with PIA, was also tested. While PIIA alone does not produce primer-extension stops at either m^2A2503/Ψ2504 or G2061/A2062, it strongly reduced the PIA-induced stops at both sites (Fig. 4A,B, compare lanes 3 and 4). These effects cannot be solely due to drug competition because studies of the synergistic behavior of the two drugs (Di Giambattista et al., 1989), and rRNA footprinting results (Porse & Garrett, 1999), clearly demonstrate that the drugs can bind simultaneously to the ribosome. Hence, it appears that PIIA perturbs the interaction between PIA and 23S rRNA via either alteration of the rRNA structure or drug–drug contact, such that the interacting chemical groups are no longer oriented favorably for rRNA modification.

Interference by other peptidyl transferase antibiotics

We tested the capacity of several other peptidyl transferase inhibitors to influence the PIA-dependent rRNA modification on 70S ribosomes (Fig. 5). Of the drugs tested, virginiamycin M_1 (Vm, a streptogramin A), chloramphenicol, and the macrolides carbomycin, tylosin, and spiramycin all interfered with the modifications at both rRNA sites, whereas sparsomycin, gougerotin, lincomycin, and clindamycin had no effect on either. Moreover, the same results were obtained irrespective of the order of addition of PIA and the second drug (data not shown).

Virginiamycin M_1 (Fig. 5A,B), like PIIA (Fig. 4A,B), strongly inhibited modifications at both sites (see above).

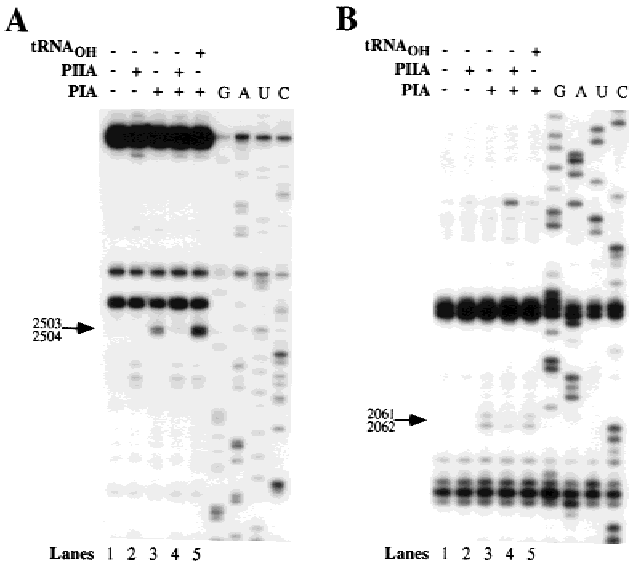

FIGURE 4. The effect of deacylated tRNA, and PIIA, on the PIA-dependent modification of 23S rRNA. **A**: Complexes were formed between PIA or PIIA (10 μM) and 70S ribosomes (0.15 μM) as indicated above the numbered gel lanes. Deacylated tRNA (0.18 μM) was included (+) in some samples. After irradiation at 365 nm for 20 min, the rRNA was isolated and analyzed by extension from primer EC2621. **B**: As in **A** except that primer extension was performed using primer EC2102. G, A, U, and C represent nucleotide sequencing tracks.

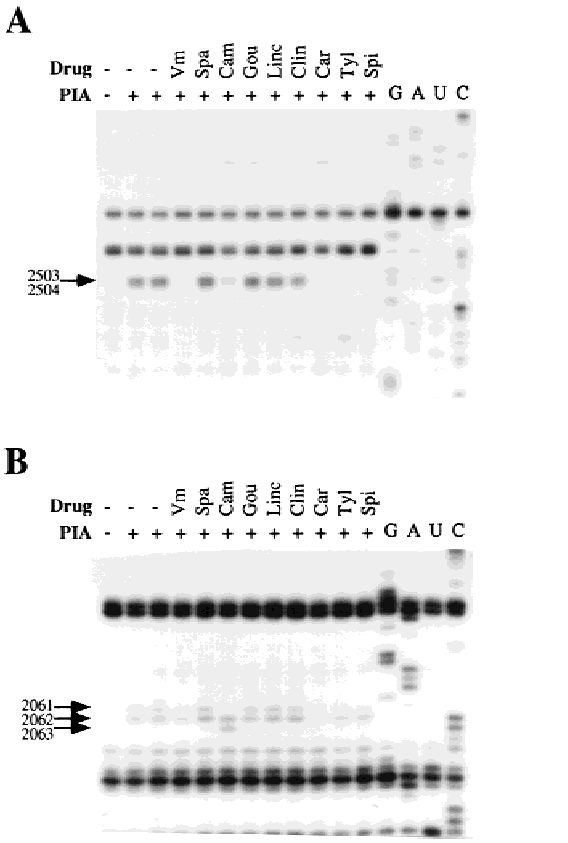

FIGURE 5. Interference of peptidyl transferase antibiotics with the PIA-dependent modifications at m^{2}A2503/Ψ2504 (**A**) and with the modification at G2061/A2062 (**B**). Complexes of 70S ribosomes (0.15 μM) and PIA (10 μM) were formed by incubating at 37 °C for 10 min, antibiotics were then added, and incubation was continued for another 10 min before UV irradiating (20 min at 365 nm). Drug concentrations were: virginiamycin M$_1$ (Vm, 200 μM), sparsomycin (Spa, 100 μM), chloramphenicol (Cam, 1.2 mM), gougerotin (Gou, 200 μM), lincomycin (Linc, 200 μM), clindamycin (Clin, 200 μM), carbomycin (Car, 200 μM), tylosin (Tyl, 200 μM), and spiramycin (Spi, 200 μM). G, A, U, and C represent nucleotide sequencing tracks.

Inhibition by the three macrolides (Fig. 5A,B) probably results from direct competition because they are likely to occupy physically overlapping binding sites (Di Giambattista et al., 1987). Unlike the effects observed for the other drugs, the presence of the stops at A2062, for samples containing tylosin and spiramycin (Fig. 5B), was independent of the presence of PIA (data not shown) and we are currently investigating the nature of these effects. Chloramphenicol produced the most dramatic effects by blocking the modification at m^{2}A2503/Ψ2504 (Fig. 5A) and producing a shift in the primer-extension stops from G2061/A2062 to A2062/C2063 (Fig. 5B). Since chloramphenicol, like the streptogramin A drugs, binds to the ribosome in the presence of PIA (de Bethune & Nierhaus, 1978; Di Giambattista et al., 1989), the results strongly suggest that it also alters the conformation of the peptidyl transferase loop, which is consistent with the complex rRNA footprints produced by these drugs (Fig. 2C; Moazed & Noller, 1987; Rodriguez-Fonseca et al., 1995; Porse & Garrett, 1999).

Modification of protein-free mature 23S rRNA

Since the PIA-dependent modifications occurred at an rRNA level, we tested whether they occurred on deproteinized 23S rRNA. 50S subunits were treated for a total of four rounds with phenol (+0.1% SDS) followed by proteinase K (+0.5% SDS), which completely eliminated peptidyl transferase activity (data not shown) and the resulting protein-free 23S rRNA was complexed with PIA and irradiated at 365 nm. Two strong primer-extension stops appeared after UV irradiation, corresponding to nt U2500 and C2501, exclusively in the presence of PIA (Fig. 6A,D). Primer-extension analysis of the entire 23S rRNA molecule revealed no additional PIA-induced effects (data not shown).

To rule out the possibility that PIA had induced either cleavage of the RNA backbone or formation of a long-range rRNA–rRNA crosslink, an ~83-nt RNAse H fragment that encompasses the U2500/C2501 site (positions ~2443–2566) was isolated as described above, and analyzed by primer extension (Fig. 6B). The presence of PIA-dependent stops at U2500/C2501, within the isolated RNAse H fragment, indicated that PIA binds directly to protein-free 23S rRNA.

The wavelength dependence of this PIA-dependent modification was also tested by irradiating PIA-23S

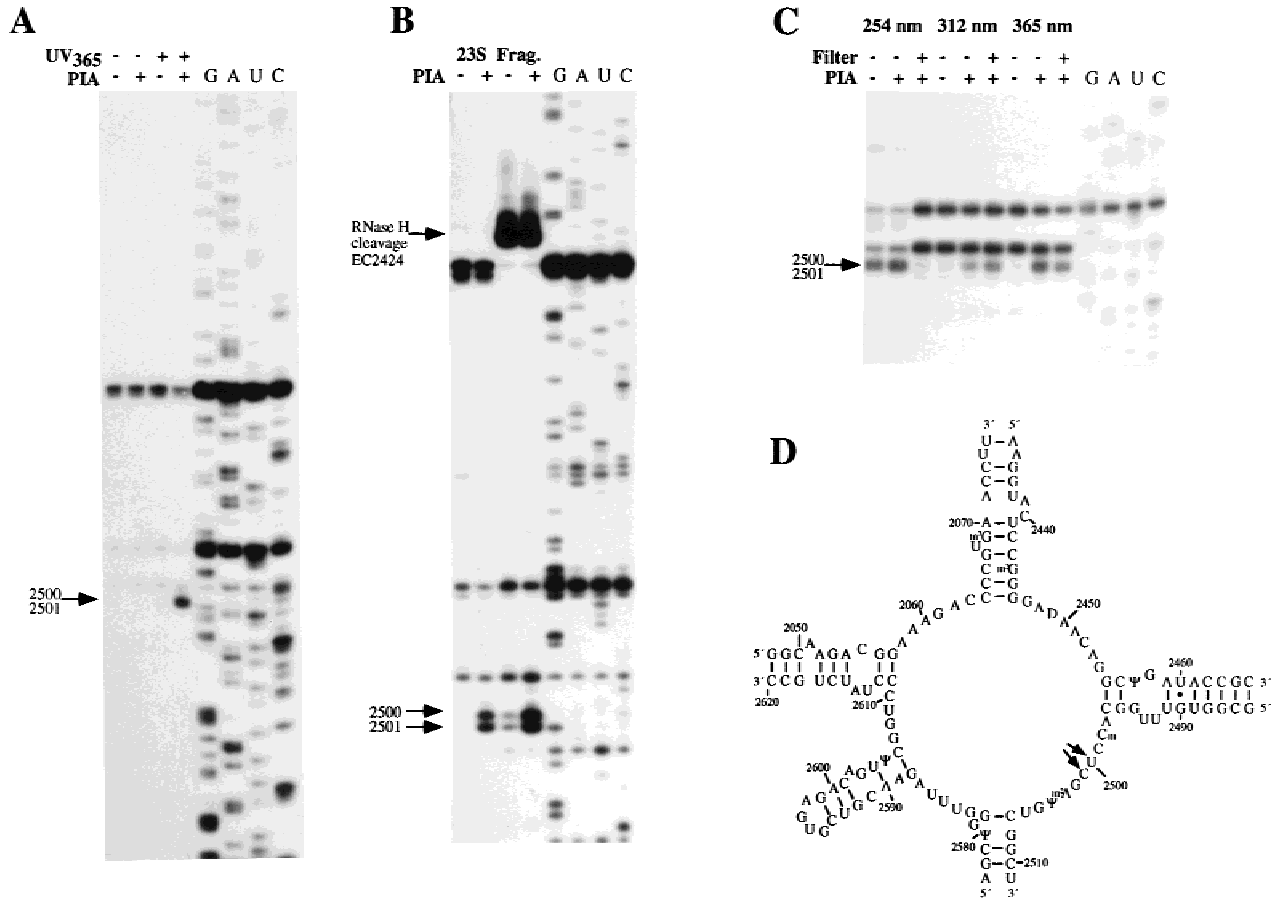

FIGURE 6. Binding of PIA to protein-free 23S rRNA isolated from *E. coli* 50S subunits. **A**: 23S rRNA (0.15 μM) was complexed with PIA (50 μM) and irradiated for 15 min at 365 nm. The rRNA was treated as described in Materials and Methods and analyzed by extension from primer EC2621. Control samples were prepared in the absence of PIA with (+) and without (−) UV irradiation. **B**: The experiment was performed as in **A**. One half of each sample (23S) was analyzed directly by primer extension using EC2521. The other half of the sample was treated with RNAse H in the presence of oligonucleotides EC2424 and EC2563. The released fragment (~124 nt, Frag.) was gel-purified and subjected to extension from primer EC2521. Weak bands at positions 2500/2501 (lane 3) derive from UV scanning of the gel containing RNAse H bands at 254 nm (see text). **C**: Wavelength dependence: Complexes were formed as described above and irradiated at 254 nm, 312 nm, or 365 nm for 2 min and, when indicated (+), a petri dish was used as filter. **D**: The secondary structure of the peptidyl transferase loop of *E. coli* 23S rRNA where modification sites on protein-free 23S rRNA are indicated by arrows. G, A, U, and C represent nucleotide sequencing tracks.

rRNA complexes at 254 nm, 312 nm, and 365 nm (Fig. 6C). At 254 nm, where many protein–rRNA and rRNA–rRNA crosslinks are induced in the ribosome (Brimacombe et al., 1990), a spontaneous stop was produced at U2500/C2501 even in the absence of PIA, suggesting that at this wavelength the stop is due to an internal rRNA–rRNA crosslink or cut in the rRNA backbone. This effect can also be seen on those primer-extension analyses of RNAse H fragments (Fig. 2A, lanes 3 and 4, and Fig. 6B, lane 3) where gels were scanned with a UV lamp at 254 nm to detect rRNA bands (see Materials and Methods). In subsequent experiments, when we became aware of this effect, the use of the UV lamp for RNA detection was minimized (see Fig. 7A–C). In contrast, stops induced by UV light at 312 nm or 365 nm were completely dependent on the presence of PIA.

The PIA-induced modification at U2500/C2501 was not affected by PIIA, chloramphenicol, or the macrolides carbomycin, tylosin, and spiramycin (data not shown), all of which interfere with PIA-dependent modification on 70 S ribosomes. This strongly suggests that these drugs do not bind to protein-free 23S rRNA and underlines the special property of PIA.

A "minimal" PIA binding site

PIA was able to bind to, and modify, protein-free 23S rRNA efficiently even though it had not been subjected to any special renaturation procedure. This suggests that the drug may recognize a stable local structure within 23S rRNA. Therefore, in an attempt to define a minimal binding site for the drug on mature 23S rRNA, fragments were excised by oligonucleotide-directed

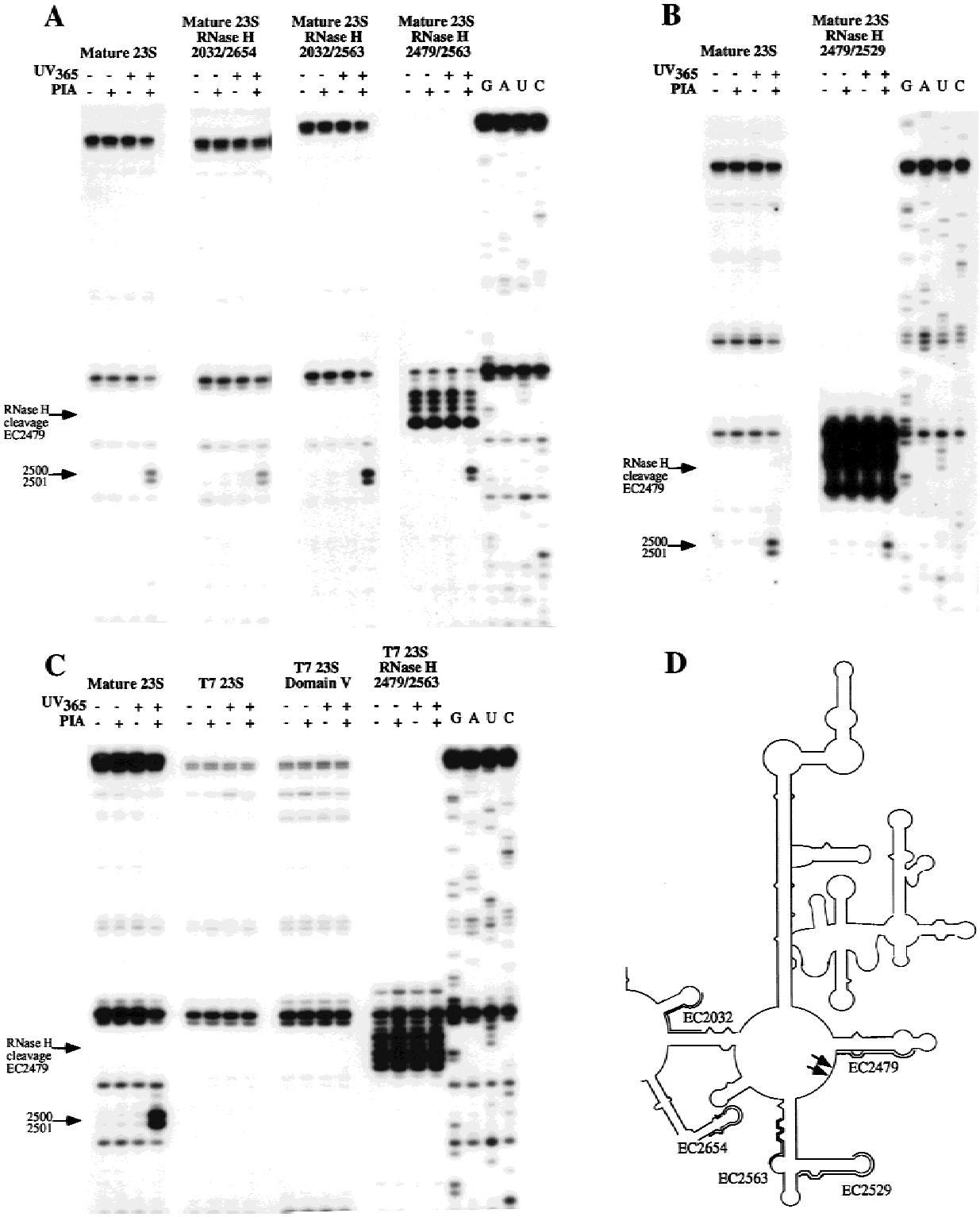

FIGURE 7. Defining a "minimal" binding site for the PIA binding. **A**: PIA was complexed to protein-free mature 23S rRNA or to gel-purified rRNA that was generated by RNAse H cleavage using the indicated pairs of oligonucleotides. RNA (0.15 μM) was complexed to PIA (100 μM) and irradiated for 15 min. Primer extension was performed with EC2521. **B**: As in **A** except that primer extension was performed with EC2512. **C**: PIA was complexed to mature 23S rRNA or to in vitro T7 transcripts of 23S RNA, domain V, or to a gel-purified fragment derived from oligonucleotide-directed RNAse H cleavage of 23S rRNA (T7 transcript) with the indicated pairs of oligonucleotides. Concentrations and primer extension were as described for **A**. **D**: An outline of the secondary structure of domain V of 23S rRNA with flanking regions, showing the sites of hybridization for the oligonucleotides used in the RNAse H analysis. The PIA-dependent modification site is indicated with arrows. G, A, U, and C represent nucleotide sequencing tracks.

RNAse H cleavage, purified on denaturing polyacrylamide gels, incubated with PIA, and irradiated. The first experiments demonstrated that PIA was able to recognize a fragment of only ~71 nt (positions ~2496–2566; Fig. 7A), generated using oligonucleotides EC2479 and EC2563 (Fig. 7D). The mature 23S rRNA was truncated further using the oligonucleotides EC2479 and EC2529 (Fig. 7D), and again PIA-dependent modification occurred efficiently with the gel-purified rRNA (Fig. 7B). Thus the ~37 nt covering positions ~2496–2532 are sufficient for recognition by PIA.

Finally, we tested whether the presence of posttranscriptional modifications had any effect on the PIA modification of protein-free 23S rRNA. T7 transcripts of 23S rRNA and domain V were synthesized in vitro, complexed with PIA, and irradiated. Neither transcript showed any detectable PIA-dependent modification (Fig. 7C). A negative result was also observed for the gel-purified ~71-nt fragment (positions ~2496–2566) prepared as described above (Fig. 7C). Despite several attempts using rRNA renatured by different procedures (see Materials and Methods), we were unable to detect a PIA-dependent modification of in vitro synthesized T7 transcripts of 23S rRNA or fragments thereof. This reinforces the inference that at least one of the posttranscriptional modifications found in the sequence Cm-C-U-C-G-m^2A-Ψ-G_{2505} is important for the interaction with, and/or the PIA-dependent modification of, rRNA.

DISCUSSION

Pristinamycin IA is a member of the streptogramin B family of closely related ribosome-targeted antibiotics. They constitute one component of the streptogramins that acts synergistically with the A component (Di Giambattista et al., 1989). The B component inhibits peptide elongation in vivo during the early rounds of protein biosynthesis, probably in a similar manner to that of the smaller macrolides such as erythromycin (Vázquez 1979; Gale et al., 1981, Andersson & Kurland, 1987). Moreover, resistance to macrolides, lincosamides, and streptogramin B (MLS_B phenotype) is conferred by methylation or mutation of A2058 within the peptidyl transferase loop (Cundliffe, 1990). The ribosomal binding site of streptogramin B has been partly defined using virginiamycin S affinity-labeled at the 4-oxopipecolinic acid residue (Fig. 1), which crosslinked to proteins L18 and L22 (Di Giambattista et al., 1990). The binding site, together with those of several other peptidyl transferase antibiotics (Moazed & Noller, 1987; Rodriguez-Fonseca et al., 1995 and references therein), has also been probed by chemical footprinting of the rRNAs within the ribosome. Although it yields a relatively complex footprint involving at least 6 nt within the peptidyl transferase loop (Fig. 2C; Porse & Garrett, 1999), this approach cannot distinguish between direct drug–rRNA contacts and drug-induced conformational changes in the ribosome (Christiansen et al., 1990).

The present rRNA modification data suggest that PIA forms a direct crosslink at m^2A2503/Ψ2504. However, we were unable to prove this, first, because no radioactive antibiotic is available and, second, because attempts to detect the drug–rRNA fragment complex by mass spectrometry (Hahner et al., 1997) failed, indicating an upper limit for the putative crosslinking yield in the isolated drug–rRNA fragment complex of 5%. An alternative explanation is that the drug induces a direct crosslink between m^2A2503/Ψ2504 and G2061/A2062. Although this would be compatible with the proposed formation of an antibiotic binding motif, involving base pairing between A2060–G2505 and G2061-Ψ2504, which was deduced from rRNA footprinting studies (Porse & Garrett, 1999), it does not correlate well with either the RNAse analyses or with the differentially enhanced modification at m^2A2503/Ψ2504 that was observed in the presence of PIA and P/P′-site-bound tRNA (Fig. 4). The results do establish, however, that PIA binds very closely to m^2A2503/Ψ2504 and G2061/A2062 on the ribosome, and to U2500/C2501 on the free rRNA, all of which lie close to this putative antibiotic binding motif.

Primer extension produced doublet bands at m^2A2503/Ψ2504 and at G2061/A2062 for the ribosomal complexes and at U2500/C2501 for the rRNA fragment, all within the peptidyl transferase loop (Figs. 2C and 6D). Although this may indicate that both nucleotides become modified, it can also reflect "stuttering" because of modification of the 3′ nucleotide of the doublet with delayed falloff of some reverse transcriptase molecules. Such effects have frequently been observed (Christiansen et al., 1990) and they generally produce a strong 3′ band and a weaker 5′ band, as is observed for A2062-G2061 (Figs. 2B and 4B), but not at U2500/C2501 (see below) where the band intensities are equal (Fig. 7B) suggesting that both nucleotides are modified. For the m^2A2503/Ψ2504 modification, interpretation of the relative band intensities is complicated by the presence of a weak control band arising from the 2-methyl group on A2503 (Figs. 2A and 4B). However, the major enhancement of the m^2A2503 band observed in the presence of PIA (e.g., Fig. 3C), strongly suggests that both nucleotides are modified.

The yield of the PIA-dependent modification was greater at 365 nm than at either 254 nm or 312 nm and suggests that the chemical reaction involves the 3-hydroxypicolinyl group of PIA (Fig. 1), for which the major and minor protolytic forms, at neutral pH, have absorbance maxima at 339 nm and 350 nm, respectively (Clays et al., 1991). This group is also known to produce the natural fluorescence of PIA and its chemical modification can inactivate the antibiotic (Di Giambattista et al., 1984).

Functional site of PIA

The presence of deacylated tRNA (or N-Ac-Phe-tRNA) in the ribosomal P/P′-site increased the PIA-dependent modification at m^2A2503/Ψ2504. Although the functional site of the drug cannot be defined more precisely, the importance of m^2A2503/Ψ2504 is emphasized by the following: (1) mutations at Ψ2504 confer an error-prone phenotype on the ribosome (O'Connor et al., 1995); (2) mutations at m^2A2503 produce reduced levels of peptidyl transferase activity (Porse & Garrett, 1995) and altered sensitivity to the putative A′-site drug chloramphenicol (Vester & Garrett, 1988), which also perturbs the PIA-dependent modification, and (3) the puromycin analog *p*-azido-puromycin crosslinked to G2502 and Ψ2504 on free ribosomes (Hall et al., 1988). These findings all suggest that PIA lies very close to the 3′ terminus of the two peptidyl transferase substrates.

PIA binds directly to isolated 23S rRNA

Previously, it was demonstrated that thiostrepton, which affects the GTPase center on the 50S subunit, and aminoglycosides that perturb the mRNA-decoding center on the 30S subunit can interact with a protein-free rRNA target (Ryan et al., 1991; Thompson & Cundliffe, 1991; Purohit & Stern, 1994; Recht et al., 1996). Strong evidence suggests also that many peptidyl transferase drugs interact with rRNA in the ribosome (Moazed & Noller, 1987; Rodriguez-Fonseca et al., 1995), although it has never been possible to detect direct drug–rRNA binding in the absence of ribosomal proteins. This may reflect either that ribosomal proteins are necessary to maintain the complex structure of the peptidyl transferase loop of 23S rRNA or, for those experiments performed in vitro with T7 transcripts, that posttranscriptionally modified nucleotides are important.

Here we demonstrate, for the first time, the direct interaction of an unmodified peptidyl transferase drug with deproteinized 23S rRNA and small fragments thereof. Moreover, a 3-nt shift was observed for the PIA-dependent modification, from m^2A2503/Ψ2504 in the intact ribosome to U2500/C2501 in the rRNA, and the modification at G2061/A2062 was absent for the latter. The preference for nt U2500/C2501 within free RNA may reflect the fact that they only become accessible to the drug on deproteinization of 23S rRNA (Egebjerg et al., 1990).

The region around the U2500/C2501 and m^2A2503/Ψ2504 is posttranscriptionally modified in mature *E. coli* 23S rRNA with a 2′*O*-methyl-C2498, a 2-methyl-A2503, and Ψ2504 (Kowalak et al., 1995). Two results suggest that one or more of these modifications is important for PIA binding and/or modification. First, PIA-dependent modifications occurred on mature 23S rRNA but not on in vitro T7 transcripts of 23S rRNA, even after renaturating under different conditions. Second, PIA was able to recognize a small ~37-nt fragment of the peptidyl transferase loop of mature 23S rRNA (positions ~2496–2532) that cannot generate any of the secondary structural elements that are known to occur in the mature 23S rRNA (Fig. 7D).

MATERIALS AND METHODS

Preparation of ribosomes and 23S rRNA

Ribosomes and ribosomal subunits were prepared as described by Makhno et al. (1988), except that the final ethanol-precipitation step was replaced by centrifuging in a fixed-angle rotor at 100,000 × *g*. 23S rRNA was generally isolated from 150 pmol of 50S subunits by extracting with phenol containing 0.1% SDS for 20 min, and then chloroform, before precipitating with ethanol. The pellet was resuspended in 300 μL 50 mM Tris·HCl, pH 7.8, 5 mM EDTA, and 0.5% SDS containing 300 μg proteinase K and incubated for 30 min at 37 °C. This procedure was repeated four times, followed by phenol extraction and ethanol precipitation, giving a total of five phenol extractions and four proteinase K treatments. Finally, 23S rRNA was resuspended in double distilled H_2O.

Interaction of PIA with 70S ribosomes, 50S subunits, and 23S rRNA

PIA (at concentrations ranging from 1 nM to 100 μM) was incubated with 50S subunits or 70S ribosomes (30–150 nM) in 20–100 μL of 20 mM Tris·HCl, pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$ for 20 min at 37 °C. In some experiments, deacylated tRNA or N-Ac-Phe-tRNA (1.2 mol/mol ribosomes) was included in the presence of poly-U (1 μg/pmol ribosome; Kirillov et al., 1978). In the antibiotic interference experiments, other peptidyl transferase drugs were added either to preformed PIA–ribosome complexes or directly to 70S ribosomes before adding PIA. After complex formation, samples were maintained as droplets in a microtiter tray, placed on an ice-water bath, and subjected to UV irradiation at 254 nm, 312 nm, or 365 nm in a Stratalinker 1800 (5 × 8 W bulbs, Stratagene, California) for 0.5–60 min. A petri dish was sometimes employed as a filter that efficiently cuts off shorter wavelengths (<300 nm). After irradiation, the samples were extracted with phenol, phenol:chloroform, and chloroform and precipitated with ethanol to remove protein and antibiotics. The rRNA was then subjected to primer extension using AMV reverse transcriptase (Life Sciences, Florida) and different 5′-labeled deoxyoligonucleotides complementary to either 5S, 16S, or 23S rRNA (Christiansen et al., 1990). The extension products were subsequently separated on denaturing polyacrylamide gels and autoradiographed.

Attempts to react PIA with protein-free mature 23S rRNA, or to purified in vitro T7 transcripts of 23S rRNA and domain V (Østergaard et al., 1998), were performed essentially as described above except that the RNA samples were either treated directly or first renatured by one of the following approaches: (1) RNA in 70 mM HEPES-KOH, pH 7.8, 270 mM

KCl, 0 or 10 mM $MgCl_2$ was heated for 0.5 min at 95 °C, transferred to 65 °C for 5 min and cooled to room temperature over a period of approximately 45 min and (2) RNA in 50 mM HEPES-KOH, pH 7.5, 400 mM NH_4Cl, 20 mM magnesium acetate was heated for 0.5 min at 95 °C and cooled to room temperature over a period of approximately 60 min.

Isolation of 23S rRNA fragments

PIA was complexed to mature 23S rRNA or 70S ribosomes and irradiated at 365 nm as described above. The samples were subsequently treated twice with phenol and precipitated with ethanol. After resuspending the rRNA (50 pmol) in 20 μL 20 mM Tris-HCl, pH 7.8, 63.5 mM NH_4Cl, one of the following pairs of DNA oligonucleotides was added (75 pmol each): EC2563 + EC2424, complementary to 2563–2577 and 2424–2443, respectively, and EC2234 + EC2032, complementary to 2234–2251 and 2032–2253, respectively. After 5 min incubation at 55 °C, 3 μL 10 mM magnesium acetate and 0.5 U RNAse H (Amersham) were added and incubated for a further 5 min. rRNA was precipitated with ethanol and the RNAse H-released bands were purified on a 5% polyacrylamide gel (containing 7 M urea) and localized by UV shadowing. Gel slices were transferred to 0.3 M sodium acetate (pH 6.0), 2 mM EDTA and extracted with phenol overnight at room temperature. RNA was recovered by ethanol precipitation and resuspended in double distilled H_2O.

Binding of PIA to 23S rRNA fragments

Mature 23S rRNA (200 pmol) or a complete in vitro T7 transcript of 23S rRNA (Østergaard et al., 1998) was subjected to preparative RNAse H digestion using the following sets of oligonucleotides: Mature 23S rRNA: EC2032/EC2654, EC2032/EC2563, EC2479/EC2563, and EC2479/EC2529. T7 transcript 23S rRNA: EC2479/EC2563. The resulting fragments were purified on 5–8% polyacrylamide gels as described above and resuspended in double distilled H_2O. Complexes of rRNA (0.15 μM) and PIA (100 μM) were prepared in 20 μL Tris·HCl, pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$ by incubation at 37 °C for 20 min. UV irradiation at 365 nm was performed as described above.

The following oligonucleotides were used for the isolation of RNAse H fragments. The complementary positions of *E. coli* 23S rRNA are given in parentheses.

1. EC2032: 5′-CGC GGG TAC ACT GCA TC (2032–2048)
2. EC2234: 5′-CCA GTC GAA CTA CCC ACC (2234–2251)
3. EC2424: 5′-GGA GTA CCT TTT ATC CGT TG (2424–2443)
4. EC2479: 5′-GCC AAA CAC CGC CGT CGA (2479–2496)
5. EC2529: 5′-ATA CCC TTG GGA CCT ACT TC (2529–2548)
6. EC2563: 5′-TCG CGT ACC ACT TTA (2563–2577)
7. EC2654: 5′-CTC CGG TCC TCT CGT ACT (2654–2671)

RNAse H cleaves close to the 3′ end of the RNA in a RNA–DNA hybrid after which an exonucleolytic trimming event occurs that stops ~4 bases from the 5′ end of the RNA in the hybrid (Crooke et al., 1995, and references therein). For EC2479, the main cleavage event occurred at a position corresponding to the penultimate nucleotide at the 3′ end of the hybridized RNA. Thus the dominant fragment released by oligonucleotides EC2479 and EC2563 would correspond to positions 2496–2566 of 23S rRNA.

ACKNOWLEDGMENTS

We are grateful to Dr. D. Beyer at Rhône-Poulenc Rorer for providing the pristinamycins, to Dr. V.I. Makhno at Petersburg Nuclear Physics Institute for purification of ribosomal particles, and to Finn Kirpekar, Odense University, for help with mass spectrometry. The research was supported by the Danish RNA Regulation Center and by a grant from the Novo Nordisk Foundation to B.T.P.

Received November 12, 1998; returned for revision December 17, 1998; revised manuscript received January 25, 1999

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