Streptomycin inhibits splicing of group I introns by competition with the guanosine substrate

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Received March 7, 1991; Revised and Accepted April 16, 1991

ABSTRACT

Streptomycin is an aminocyclitol glycoside antibiotic, which interferes with prokaryotic protein synthesis by interacting with the ribosomal RNA. We report here that streptomycin is also able to inhibit self splicing of the group I intron of the thymidylate synthase gene of phage T4. The inhibition is kinetically competitive with the substrate guanosine. Streptomycin and guanosine have in common a guanidino group, which has been shown to undergo hydrogen bonds with the ribozyme (Bass & Cech, Biochemistry *25*, 1986, 4473). The inhibitory effect of streptomycin extends to other group I introns, but does not affect group II introns. Mutating the bulged nucleotide in the conserved P7 secondary structure element of the *td* intron alters the affinity of the ribozyme for both guanosine and streptomycin. Myomycin, an antibiotic with similar effects on protein synthesis as streptomycin, is also able to inhibit splicing. In contrast, bluensomycin, which is structurally related to streptomycin, but contains only one guanidino group does not inhibit splicing. We discuss these findings in support of an evolutionary model that stresses the antiquity of antibiotics (J. Davies, Molecular Microbiology *4*, 1990, 1227).

INTRODUCTION

The group I intron of the thymidylate synthase (*td*) gene of phage T4 follows the splicing pathway described for the Tetrahymena rRNA intron (1). Splicing occurs via a two step reaction: a first transesterification cleaves the 5′ splice site and covalently adds the substrate guanosine to the first nucleotide of the intron. In a second transesterification the 3′ splice site is cleaved and the exons are ligated (Fig.1B; 2). The first step, cleavage of the 5′ splice site, is initiated by guanosine, which acts as a substrate. Bass & Cech (3) have suggested that guanosine binds to the G-binding site (guanosine binding site) via hydrogen bonds using the guanidino, the 6-keto and most probably the ribose hydroxyl groups. This hypothesis is strongly supported by the findings of Michel et al. (4), who mapped the G-binding site to the G264 C311 pair of the conserved P7 stem in the Tetrahymena rRNA intron. They proposed two hydrogen bond interactions between the G264 nucleotide of the ribozyme and the C_2-NH_2 and N_1-H of the substrate guanosine. Furthermore, the amino acid arginine, which contains a guanidino group, competes with guanosine for the G-binding site (5).

Streptomycin is an aminocyclitol glycoside antibiotic which interferes with prokaryotic protein synthesis. Its main effects are induction of misreading of the genetic code and inhibition of translational initiation. Streptomycin-induced miscoding is believed to be the result of interference with a proof-reading step in translation (6−8). Moazed and Noller (9) reported that streptomycin interacts with functional sites in the 16S ribosomal RNA of Escherichia *coli*. They observed protection of residues U911, C912 and adenosines 913−915 from attack by methylating agents, when streptomycin is bound to the 70S ribosome. This is consistent with the finding that a mutation at position 912 (C to U) confers resistance to streptomycin (10). Mutations that result in streptomycin resistance or dependence also occur in ribosomal proteins S12 (11, 12) and S4 (13). Both steps in which the antibiotic interferes with protein synthesis are dependent on GTP binding and hydrolysis.

Recently J. Davies (14) conceived a model in which antibiotics are likely to be ancient metabolites, and antibiotic binding sites in RNA molecules are suggested to be very old with respect to essential functions in the evolution of the translation mechanism. Furthermore, we have shown that streptomycin, which contains two guanidino groups, inhibits splicing of group I introns by competing with the substrate guanosine (Fig. 3B; 15). We therefore analyzed the effect of streptomycin, myomycin and bluensomycin on splicing of group I introns *in vitro* and discuss the possibility that both RNAs, the ribosomal and the intron RNAs, which are thought to have catalytic activity, may have had a common ancestor and may have been modulated by secondary metabolites during biochemical evolution.

MATERIALS AND METHODS

Plasmids

In vitro splicing experiments were performed with a truncated version of the *td* gene, containing 79 nt of exon I, 265 nt of intron sequences containing only the catalytic core (delta P6-2) and 21 nt of exon II (16, 17). Mutant U870 has been described earlier

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(17). The plasmids containing the Tetrahymena rRNA intron and the yeast mitochondrial cob intron 1 were pBGST7 (18) and pBSbI1 (19), respectively.

In vitro splicing assays

DNA was prepared by the alkaline-lysis procedure and linearized with Hind III. For the truncated version of the intron, the plasmid was linearized with Pvu II, which cuts at nucleotide 968. Transcription was performed in a total volume of 20 µl at 30°C for 1 hour in buffer containing 40 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 0.4 mM spermidine, 0.5 mM of each NTP, 5 mM DTT, 10 units T7 RNA polymerase (Boehringer Mannheim), 10 µCi [alpha-^{35}S]-CTP and 10 units RNAse inhibitor (Boehringer Mannheim). The reaction mixture was loaded on a 5% polyacrylamide/7M urea gel. Precursor RNA was cut out of the gel and soaked in 400 µl elution buffer (10 mM Tris-HCl pH 7.5, 2 mM EDTA, 3.5 M NH_4OAc, 0.1% SDS) for two hours. After ethanol precipitation, the RNA was dissolved in H_2O. Before splicing experiments were performed the RNA was incubated in splicing buffer (40 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 0.4 mM spermidine) for 2 min at 56°C. Splicing was initiated by addition of guanosine at the appropriate concentration at 37°C. When splicing was to be inhibited by streptomycin, the antibiotic was added immediately before the guanosine. Splicing reactions were performed at 37°C for 10', where not otherwise indicated. The reactions were stopped by adding 45 µl of stop solution (2.5 mM EDTA, 0.1 mg/ml yeast tRNA) and 150 µl of 0.3M NaOAc/ETOH to a 5 µl reaction volume. Samples were separated on 5% acrylamide/7 M urea gels. Kinetic measurements were performed by incubating pre-RNA with increasing amounts

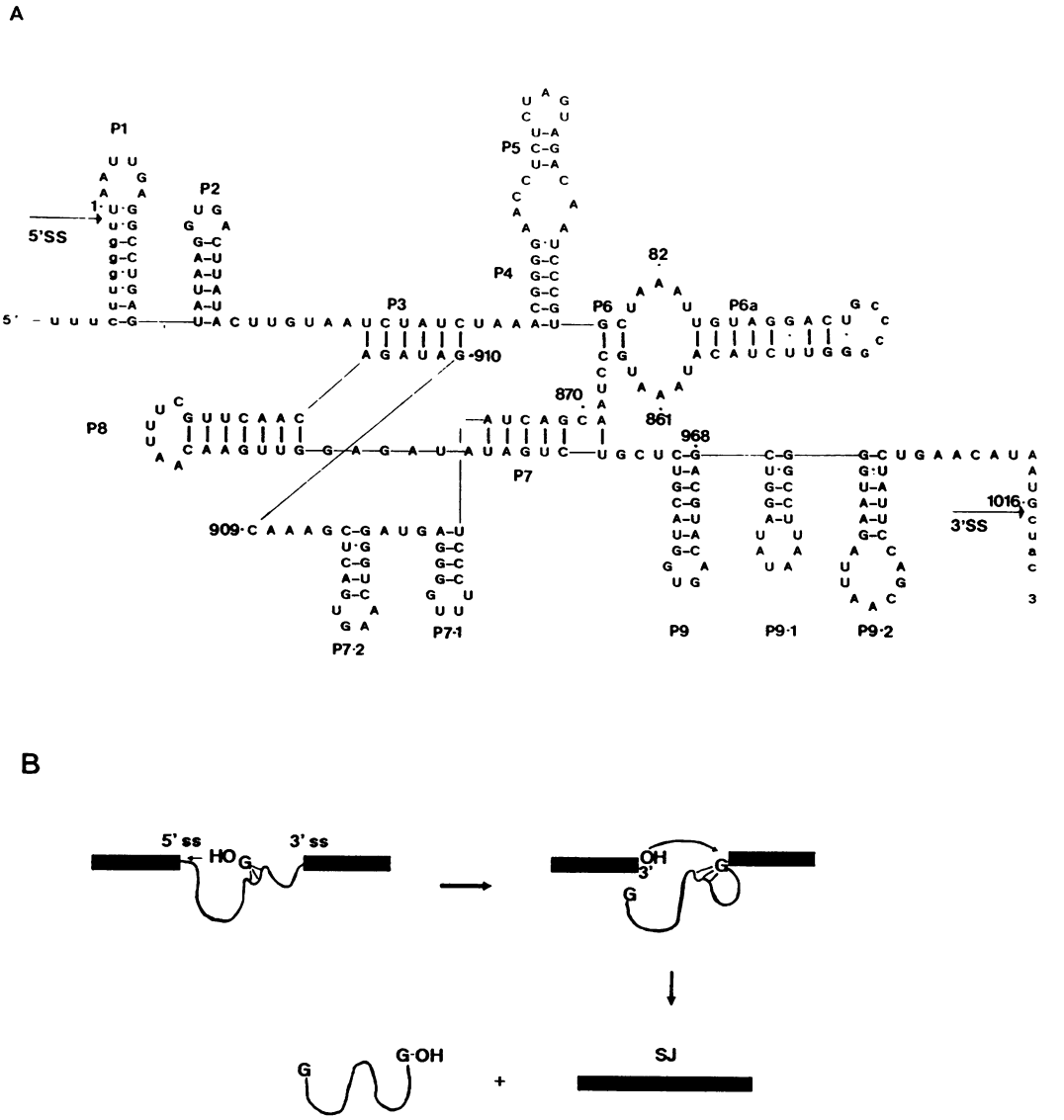

Figure 1. A. Secondary-structure map of the group IA2 intron of the T4 phage *td* gene (20, 33, 34). Intron sequences are in upper case letters and exon sequences in lower case letters. Secondary structure elements are labeled P1 to P9.2. Small numbers indicate intron residues. The sequence shown is deleted for extraneous sequences in P6a (16). The 5' and 3' splice sites (5'ss and 3'ss) are indicated by an arrow. B. Splicing pathway of group I introns: solid bars indicate exons, folded lines indicate intron sequences and SJ represents the splice-junction. An exogenous G is in the G-binding site and initiates splicing by nucleophilic attack. The 5' splice-site is cleaved while the exogenous G becomes covalently bound to the first nucleotide of the intron. The free 3'OH of the upstream exon attacks the 3' splice-site, the intron is released and the exons are ligated.

of GTP, as indicated in the text, with different streptomycin concentrations (4, 10 and 20 mM for the wild type; 0.5, 2 and 5 mM for the U870 mutant) for 2 and 5 minutes. Autoradiograms were scanned on a DU8 Beckman spectrophotometer. The reciprocal of initial velocity was plotted versus the reciprocal of GTP concentration in the presence of fixed concentrations of the inhibitor. Myomycin and bluensomycin were gifts from Dr. J. Davies and from Dr. D. White, respectively.

RESULTS

Streptomycin inhibits splicing in vitro

To demonstrate inhibition of splicing by streptomycin we incubated gel purified *td* pre-RNA with increasing amounts of streptomycin and 1 μM GTP (its K_m concentration) for 10 min as described in the Materials and Methods (Fig. 2A). Streptomycin started to inhibit splicing at a concentration of 4 mM, and at 10 mM no splicing products could be detected (Fig. 2A, lanes 2–6). To investigate, whether the inhibition is competitive we performed splicing reactions with 20 mM streptomycin and increasing amounts of GTP (Fig. 2A, lanes 7–10). Splicing was partially restored at a concentration of 10 μM GTP and reached almost uninhibited levels at 100 μM GTP. The mechanism of inhibition was determined by measuring the initial velocities of the splicing reaction at various GTP and streptomycin concentrations. Pre-RNA was incubated for 2 and 5 min with 1, 5 and 25 μM GTP in the presence of 0, 4, 10 and 20 mM streptomycin. The results are represented in a Lineweaver-Burk plot (Fig.2B). From the change of slope and fixed intercept on the y axis we deduced that the mechanism of inhibition is competitive and that the dissociation constant for streptomycin is 5 mM for a Mg^{2+} concentration of 8 mM.

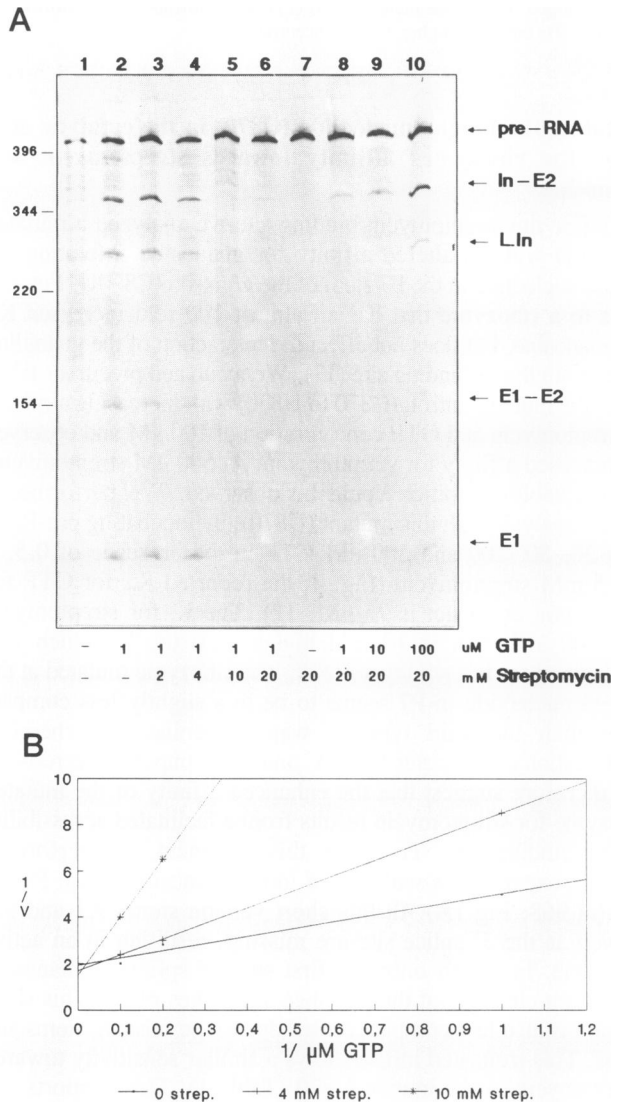

Figure 2. Splicing inhibition by streptomycin. A. Pre-RNA of the *td* intron was incubated with 1 μM GTP and increasing amounts of streptomycin (lanes 1 through 6;). Increasing the GTP concentration restored splicing (lanes 8–10). The sizes in nucleotides of RNA markers are indicated to the left. RNA species are as follows: pre-RNA = precursor RNA; In-E2 = intron-exon II; L.In = linear intron; E1-E2 = ligated exons; E1 = exon I. B. Lineweaver-Burk plot: the reciprocal initial velocities of formation of splicing products is plotted versus 1/[GTP] for different, but constant amounts of the inhibitor as indicated on the graph.

The effect of streptomycin extends to other group I introns, but does not affect group II introns

If the streptomycin binding site is equivalent to the guanosine binding site and if it is phylogenetically conserved, the effect of the antibiotic should extend to other group I introns, but should not affect group II introns, which do not bind guanosine. We performed the same experiments with the precursors of the Tetrahymena rRNA, a group IC1 intron according to Michel and Westhof (20) and the yeast mitochondrial cobI1, a self-splicing group II intron (19). As expected, streptomycin inhibited splicing of the Tetrahymena rRNA group I intron but not of the group II cobI1 intron (Table 1).

Streptomycin-analogs as splicing inhibitors

Myomycin, an aminocyclitol glycoside antibiotic, which has been reported to have the same inhibitory effects on protein synthesis as streptomycin (21), was used in a splicing assay. It did indeed inhibit splicing at 20 mM concentration (Table 1). Myomycin has a structure that differs significantly from streptomycin, but it has one guanidino group that could potentially interact with the G-binding site of the ribozyme (Fig. 3D).

Bluensomycin (22), an antibiotic that differs structurally from streptomycin only by containing one instead of two guanidino groups, was used to answer the question which of the two

Table 1.

Intron	Antibiotics Streptomycin	Myomycin	Bluensomycin	Chloramphenicol
td wildtype group IA2	5 mM*	10 mM	no effect up to 40 mM	no effect up to 10 mM
Tet. rRNA group IC1	2 mM	n.d.	n.d.	n.d.
cobI1, group II	no effect	n.d.	n.d.	n.d.
td-mutant U870	400 μM*	10 mM	no effect	n.d.
td-truncated at PVU II	1 mM	n.d.	no effect up to 10 mM	n.d.

Table 1 summarizes the effect of the antibiotics on the different introns tested in this work. The antibiotic concentrations represent the amount that causes approx. 50% inhibition. Asterix * means that the dissociation constant was determined kinetically. n.d. = not determined.

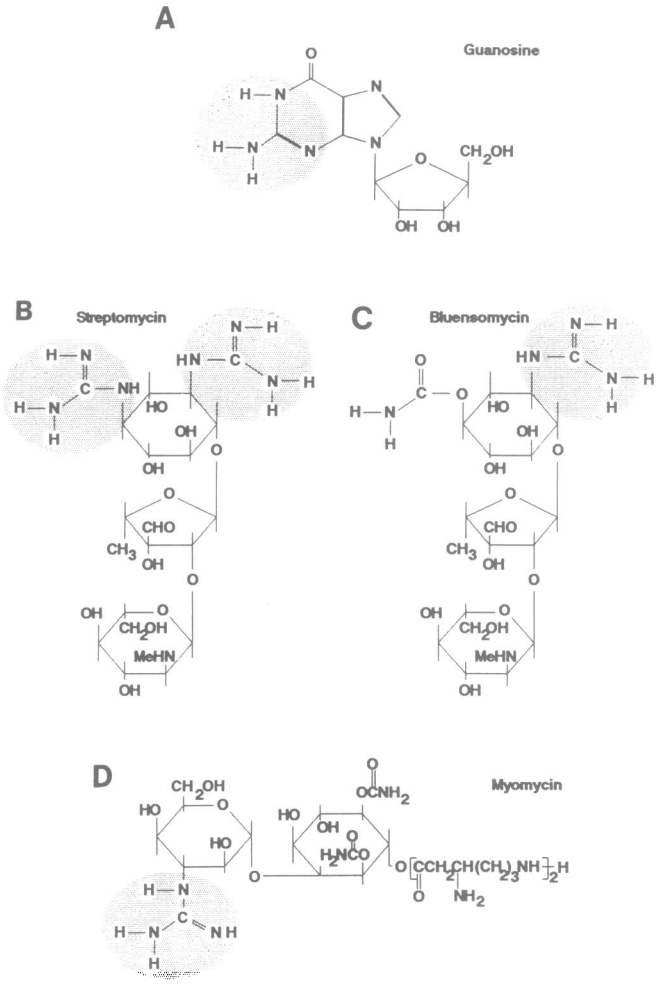

Figure 3. A. The guanosine cofactor. The atoms proposed to be involved in H-bonding to the ribozyme are shaded (3). B. The inhibitor streptomycin. The guanidino groups are shaded. C. Bluensomycin, which differs from streptomycin by containing an carbamido group in para position to the glycosidic bond instead of a guanidino group. D. The inhibitor myomycin with the guanidino group shaded.

guanidino groups in streptomycin binds to the ribozyme (Fig. 3B and 3C). As bluensomycin is not able to inhibit splicing at concentrations as high as 40 mM (Table 1), we postulate that the guanidino group that binds to the ribozyme is the one in the para position to the glycosidic bond of the streptose.

The antibiotic chloramphenicol has been reported to inhibit splicing in vivo. Accumulation of precursor RNAs in yeast mitochondria from cells grown in the presence of chloramphenicol can be observed and this effect has been connected with protein synthesis (23). A sensitivity in mRNA production has been reported for the *td* intron of phage T4, when the infection takes place in the presence of chloramphenicol. Premessenger and intron RNA do accumulate in the presence of chloramphenicol, while no mature mRNA could be detected, suggesting that intron excision can occur in the absence of phage encoded functions, whereas exon ligation seems to be sensitive to the protein synthesis inhibitor (24). Consistent with a role in protein synthesis rather than a direct inhibition of splicing we found that chloramphenicol up to a concentration of 10 mM had no effect on in vitro splicing of the phage T4 *td* intron (Table 1).

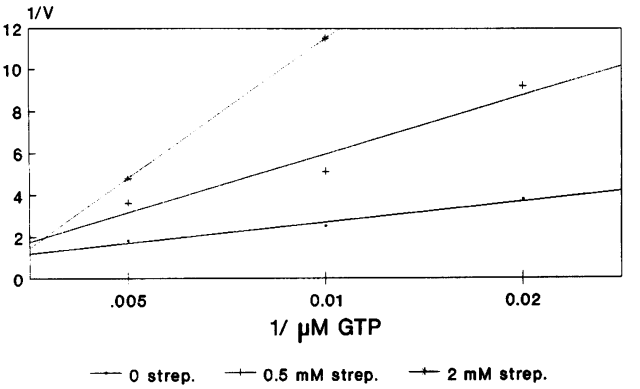

Figure 4. Lineweaver-Burk plot showing the inhibition of splicing by streptomycin for the 'bulged' nucleotide mutant U870 (17). Experiments were performed as in figure 2B, but with higher GTP concentrations.

Mutating the bulged nucleotide (C870) in the catalytic core alters the ribozymes' affinity towards streptomycin and guanosine

To define the streptomycin binding site we analyzed a mutated ribozyme with an altered affinity for guanosine. Mutating the bulged nucleotide in the P7 stem of the *td* intron (C870U; Fig.1A) leads to a ribozyme that has an almost 100 fold increased K_m for guanosine, but does not affect the interaction of the guanidino group with the G-binding site (17). We incubated precursor RNA from this mutated intron (C870 to U870) with increasing amounts of streptomycin at a GTP concentration of 100 μM and observed an increased affinity for streptomycin. At 500 μM streptomycin, a remarkable inhibition could be observed. We performed a kinetic analysis with this mutant (U870), by incubating pre-RNA with 20, 50, 100 and 200 uM GTP in the presence of 0.5, 2 and 5 mM streptomycin (Fig. 4; the reported K_m for GTP for this mutant construct is 75 μM, 17). The K_i for streptomycin was 400 μM; that is 10 fold higher than the K_i which was determined for the wild type RNA. The ribozyme mutated at the bulged nucleotide in P7 seems to be in a slightly less compact form than the wild type as was determined by chemical modification experiments (U.v.A. and R.S.; unpublished results). We therefore suggest that the enhanced affinity of the mutated ribozyme for streptomycin results from a facilitated accessibility to the binding site. To verify this statement, we performed splicing experiments with the *td* intron truncated at the Pvu II site (nt.968; Fig.1A). In this short version stems 9.1 and 9.2 as well as the 3′ splice site are missing, resulting in an active ribozyme, in which only the first step of splicing, guanosine dependent cleavage at the 5′ splice site, takes place. This short version is also less compact folded due to the missing stems and loops. This truncated intron shows a similar sensitivity towards streptomycin as the mutant U870 (Table 1). This supports our theory that the increased sensitivity of the mutated and the truncated versions of the intron are due to a less compact folding of the molecule.

Bluensomycin, which did not inhibit splicing of the wild-type ribozyme, had also no effect on the ribozyme mutated at the bulged nucleotide nor on the Pvu II truncated RNA, suggesting that this antibiotic is not binding to any of the analyzed RNA molecules.

DISCUSSION

A specific antibiotic binding site for streptomycin is detectable in the group I intron of the phage T4 *td* gene. The dissociation constant is in the millimolar range and the binding is reversible, suggesting a competitive inhibitory mechanism. A similar competitive inhibition of splicing of the Tetrahymena rRNA intron has been demonstrated for the amino acid arginine, where the dissociation constant is 4 mM (5). Other competitive ribozyme inhibitors are deoxyguanosine and dideoxyguanosine, with dissociation constants of 1.1 mM and 5.4 mM, respectively (3). These four inhibitors all have in common a guanidino group, which has been postulated to interact with the ribozyme. As expected, if the inhibition is caused by competition with guanosine, the inhibition extends to other group I introns. We postulate that streptomycin binds to a subsite within the G-site, which has partially been assigned to the G immediately following the bulged nucleotide in P7 (G264 in the Tetrahymena intron (4); G871 in the *td* intron, Fig.1A).

The splicing of group II introns is not affected by streptomycin, indicating that the inhibition of group I splicing is not due to a more general effect of streptomycin on RNA molecules. Streptomycin has been shown to interfere with nucleic acids and acts as an antagonist to Mg^{2+} and polyamines (25, 26). At 5 mM streptomycin and 10 uM GTP splicing is restored (Fig. 2B), stressing the specificity of the inhibition caused by streptomycin and we assume that the inhibition is not due to a general unspecific interaction of streptomycin with RNA. In the ribozyme mutated at the bulged nucleotide in the P7 stem the increased affinity for streptomycin may be due to a locally less compact folding resulting in a better accessibility of the inhibitor to the binding site.

Streptomycin is a fairly bulky molecule with two guanidino groups, whereas the catalytic core of the ribozyme seems to be very compactly folded (20, 27). To learn more about the accessibility of streptomycin to the G-site we analyzed two antibiotics, that are related structurally (Bluensomycin; Fig.3C) and functionally (Myomycin; 3D) to streptomycin. For bluensomycin, which is very similar to streptomycin, but contains only one guanidino group, no inhibition was obtained up to a concentration of 40 mM. This indicates that the guanidino group in para position to the glycosidic bond to the streptose is the one active in inhibition. Myomycin, for which the same effects on protein synthesis have been reported as for streptomycin (21), inhibits splicing both in the wild type as well as in the mutated ribozyme, stressing the relatedness of these two compounds.

Recently J. Davies suggested an archaic function for antibiotics and proposed that they were implicated in biochemical evolution. He suggests that at one time antibiotics were the functional precursors of modern ribosomal proteins (14).

Finding that an antibiotic has a binding site on a potentially ancient catalytic RNA places antibiotic-mediated evolution to a very early stage. The antibiotic binding sites in the 16S rRNA of *E. coli* reveal the existence of regions of universally conserved nucleotide sequences, that are among 'the most ancient that exist on our planet' (9). Our example is not the first case of an antibiotic interaction with catalytic RNA. The protein synthesis inhibitor puromycin has an inhibitory effect on the catalytic activity of the RNA component of *E. coli* RNase P (M1 RNA; 28). Furthermore, the same G-binding site, which also binds arginine in all reported group I introns, is without exception an arginine codon (29), meaning that streptomycin, which induces misreading in the ribosome binds to an arginine codon in the ribozyme. This is very suggestive and opens the way for many speculative hypotheses on the evolution of the catalytic RNA. The recent discovery of group I introns in the $tRNA^{Leu}$ genes of cyanobacteria strongly support the antiquity of introns (30–32). We may ask the question as to what did a group I intron evolve to. We do believe that a molecule with such a large spectrum of functions should not get lost during evolution, but serve as an ancestor for a variety of biological molecules.

ACKNOWLEDGEMENTS

We want to thank Marlene Belfort and all the members of her laboratory for the constant encouragement and support of our work. We want to specially thank Dr. Julian Davies from the Institut Pasteur for fruitful discussions and for kindly providing myomycin. We thankfully acknowledge Dr. D.R. White from the Upjohn Company in Kalamazoo, Michigan, USA, for the gift of bluensomycin. We very much appreciate the input of Herbert Wank and Markus Wögerbauer into this work.

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