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In vitro selection and characterization of streptomycin-binding RNAs: Recognition discrimination between antibiotics

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ABSTRACT

As pathogens continue to evade therapeutical drugs, a better understanding of the mode of action of antibiotics continues to have high importance. A growing body of evidence points to RNA as a crucial target for antibacterial and antiviral drugs. For example, the aminocyclitol antibiotic streptomycin interacts with the 16S ribosomal RNA and, in addition, inhibits group I intron splicing. To understand the mode of binding of streptomycin to RNA, we isolated small, streptomycin-binding RNA aptamers via in vitro selection. In addition, bluensomycin, a streptomycin analogue that does not inhibit splicing, was used in a counter-selection to obtain RNAs that bind streptomycin with high affinity and specificity. Although an RNA from the normal selection (motif 2) bound both antibiotics, an RNA from the counter-selection (motif 1) discriminated between streptomycin and bluensomycin by four orders of magnitude. The binding site of streptomycin on the RNAs was determined via chemical probing with dimethylsulfate and kethoxal. The minimal size required for drug binding was a 46- and a 41-mer RNA for motifs 1 and 2, respectively. Using Pb^{2+} cleavage in the presence and absence of streptomycin, a conformational change spanning the entire mapped sequence length of motif 1 was observed only when both streptomycin and Mg^{2+} were present. Both RNAs require Mg^{2+} for binding streptomycin.

Keywords: antibiotics; Mg^{2+}; RNA aptamers

INTRODUCTION

Streptomycin, a member of the aminocyclitol glycoside antibiotics, interferes with translation by interacting with ribosomal RNA. The antibiotic has been shown to suppress missense and nonsense codons in vivo, and to induce misreading of the genetic code in vitro (Davies et al., 1964, 1965; Pestka et al., 1965; Gorini, 1974). Interaction of streptomycin with RNA was proposed following mutational analysis of the 16S ribosomal RNA in *Escherichia coli*, where a C to U base change at position 912 or an A to C change at position 523 resulted in a streptomycin-resistant phenotype (Montandon et al., 1986; Melancon et al., 1988). Chemical modification assays performed on ribosomes bound with streptomycin further support an interaction between streptomycin and ribosomal RNA. These assays revealed strong protections in the 915 region, which lies at the central part of the 16S rRNA structure, as well as weak protections in the 1400 region, located at the decoding site of the 16S rRNA (Moazed & Noller, 1987).

Streptomycin was also found to interact with group I intron RNA, thereby inhibiting self-splicing (von Ahsen & Schroeder, 1991). Group I intron splicing is initiated by the binding of an exogenous guanosine to the guanosine binding site in the catalytic core of the intron (Michel et al., 1989; Cech, 1993). Guanosine binds to the G-binding site via its guanidino group (Bass & Cech, 1984, 1986). Several small molecules that contain guanidino groups, but that cannot promote splicing, compete with guanosine for the G-binding site and thus inhibit splicing. Competitive inhibitors of group I intron splicing are dideoxy-guanosine, deoxyguanosine (Bass & Cech, 1986), the amino acid arginine (Yarus, 1988), streptomycin (von Ahsen & Schroeder, 1991), and the peptide antibiotic viomycin (Wank et al., 1994). Interaction sites between streptomycin and the intron RNA were determined by chemical probing (von Ahsen & Noller, 1993) and mutational analysis (von Ahsen & Schroeder, 1991).

Bluensomycin (Davies, 1967) is a streptomycin analogue differing from streptomycin in that it contains a

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carbamido group instead of a guanidino group in the *para*-position to the glycosidic bond of the streptose. Bluensomycin does not inhibit group I intron splicing of the T4 thymidylate synthase gene (von Ahsen & Schroeder, 1991). The structural formulae comparing streptomycin, dihydrostreptomycin, and bluensomycin are shown in Figure 1.

In order to understand the mode of action of RNA-binding antibiotics, a detailed analysis of their modes of interaction with RNA is required. Structure determination at the atomic resolution level is the method of choice to understand binding. However, the natural target sites of streptomycin are too large to undertake structural characterization of their streptomycin complexes. There are two ways of circumventing this size problem. (1) If the ligand-binding site is part of an autonomous RNA domain, then the RNA can be dissected and structural analysis can be undertaken. This has been demonstrated elegantly for the 16S rRNA A-site (Purohit & Stern, 1994). A small RNA derived from the A-site binds the aminoglycosides in a similar way as the RNA in the context of the 30S subunit. The structure of this A-site model RNA complexed with paromomycin was solved by NMR (Fourmy et al., 1996). (2) The second way consists of isolating small RNAs with antibiotic binding properties from random-sequence RNAs. This approach does not directly address how the antibiotic binds to its natural target sites, but provides information on how RNA can accommodate the ligand.

Several in vitro selections have already been undertaken to isolate antibiotic-binding RNAs: these selections identified aptamers with specific binding to the aminoglycosides tobramycin (Wang & Rando, 1995), lividomycin and kanamycin A (Lato et al., 1995), neomycin B (Wallis et al., 1995), and the peptide antibiotic viomycin (Wallis et al., 1997). Characterization of the neomycin, viomycin, and tobramycin aptamers revealed fundamental features of the RNA–antibiotic interaction. NMR structural analysis of the tobramycin-binding aptamer has been reported recently (Jiang et al., 1997). Tobramycin binds to the RNA major groove at a stem-loop junction site with part of the antibiotic being clasped between the major groove and a looped-out cytosine residue. Similar to that found for RNA binding tobramycin, a widened major groove was found in most of the selected RNAs to accommodate neomycin (Wallis et al., 1995). The selection of the viomycin-binding RNAs revealed that all selected RNAs formed pseudoknots, as do the natural viomycin-binding sites (Wallis et al., 1997).

We used in vitro selection with affinity chromatography to isolate streptomycin-binding RNAs. Additionally, to isolate RNAs that can discriminate between streptomycin and bluensomycin (like the group I intron), we counter-selected with bluensomycin. This counter-selection should provide RNA that recognizes streptomycin via its *para*-positioned guanidino group.

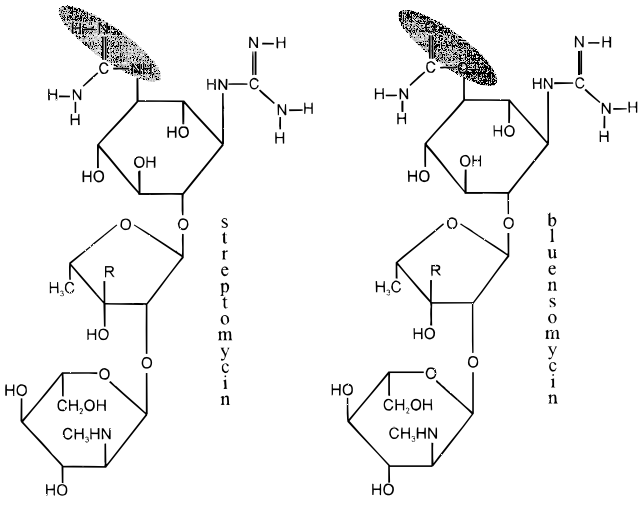


FIGURE 1. Chemical structures of streptomycin (R = -CHO, streptomycin) and bluensomycin (R = CH_2OH, dihydrostreptomycin, bluensomycin). The differing elements between the two are shaded.

RESULTS AND DISCUSSION

In vitro selection of streptomycin aptamers

Starting with a pool of 10^{15} different DNA molecules, in vitro selection procedures were performed to select for RNAs with a high affinity to streptomycin. The pool DNA molecules consisted of a 74-nt long randomized region flanked upstream and downstream with constant regions (Famulok, 1994). Affinity chromatography was performed using dihydrostreptomycin coupled to sepharose. The coupling reaction between antibiotic and sepharose occurs in 10 mM NaOH. Under such high pH conditions, streptomycin undergoes irreversible hydrolysis. Dihydrostreptomycin does not, and was therefore used to couple to the column matrix (J. Davies, pers. comm.). Using this coupling procedure, both hydroxyl and amino groups are linked to the epoxy-derivatized sepharose such that, during selection, RNAs that recognize different sets of functional groups are obtained. By using the ligand to elute the RNAs from the column, binding to the ligand in solution is ensured. To ensure that the selected RNAs bind to a defined set of functional groups, a counter-selection with an analogue of the ligand, which lacks the specific functional group, is performed.

RNA from the starting pool was applied to the dihydrostreptomycin column and was specifically eluted with 5 mM streptomycin. After four rounds of selection, an enrichment was detected and the pool was split. Although one half of the pool continued to follow the selection scheme described (deemed "normal selection"), the other half was subjected to counter-selection conditions using bluensomycin (Fig. 2).

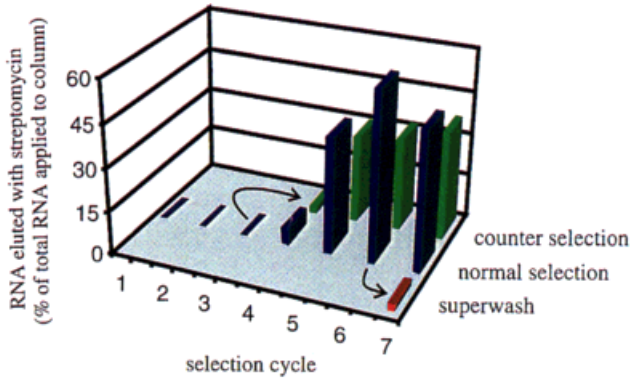


FIGURE 2. Enrichment of RNAs with affinity to streptomycin. RNA specifically eluted with 5 mM streptomycin from the dihydrostreptomycin column is plotted as a function of the corresponding selection cycle. Arrows originate from pools that were split, and point to the corresponding selection scheme at the round indicated.

In the counter-selection scheme, RNAs were washed off the column with equilibration buffer followed by a 3 mM bluensomycin wash. Streptomycin-binding RNAs were then specifically eluted using 5 mM streptomycin. Three final cycles following the counter-selection scheme were performed before a plateau in the enrichment curve was detected, and selection was terminated (Fig. 2).

The normal selection scheme continued for two more cycles before it was again split. While one half of the sequences was subjected to another round of selection before being cloned, the other half was subjected to the third selection procedure. Instead of washing three times before specifically eluting, the column was "superwashed" with 30 column volumes of equilibration buffer, followed by a 5 mM streptomycin elution (Fig. 2). RNAs from the three final pools were reverse-transcribed, PCR-amplified, cloned, and subjected to sequence analysis.

Sequence analysis of streptomycin aptamers

Of 99 clones sequenced (33 from each selection procedure), 43 were unique, with no primary sequence motif dominating the pool. The large number of different RNAs obtained might, on the one hand, reflect different groups recognized by the RNA and, on the other hand, there are most probably several solutions for how RNA can accommodate a specific ligand. From the counter-selection, we expect the selected RNAs to recognize the guanidino group in *para* position to the streptose and from the normal selection several groups might be the recognition determinant.

Two motifs shown in Figure 3 were found in different locations within different clones and were chosen for further investigation. Motif 1 is 22-nt long, has no sequence variation within the motif, and originates from the high-stringency, counter-selection scheme. Motif 2 is 26-nt long, with sequence variation of two bases found within the motif (A to G and C to A, shown in Fig. 3). One clone, B47, contains a subdomain of motif 2. Unlike motif 1, motif 2 originates from the superwash and normal selections and was not found in the counter-selection.

In order to confirm the ability of RNAs containing motifs 1 and 2 to bind streptomycin, single clones were transcribed and subsequently subjected to affinity chromatography. When compared to the elution profile from

Motif 1 (5' → 3')

C # 107 (1x)
ggagcucagccuucacugc - uguaacucuaggcucccGUGGAUCGCAUUUGGACUUCUGCCuaacgcgugugcuagagagcaggcaaacaagug - ggcaccacggucggauccac

C # 128 (1x)
ggagcucagccuucacugc - accccuuugugggaacgcaaaacuaaaggaagaucucaagaaagGUGGAUCGCAUUUGGACUUCUGCCcagggu - ggcaccacggucggauccac

B # 73 (1x)
ggagcucagccuucacugc - acaacgcgucacugcucuagggcgcgcgugauagcaggaguaGUGGAUCGCAUUUGGACUUCUGCCgcacacaua - ggcaccacggucggauccac

Motif 2 (5' → 3')

B # 84 (5x)
ggagcucagccuucacugc - cagacaguagagggaagugugagcuaucaccuCAAGGAAAACGCUUCAGAAAGGGACUuaggugaugauagugu - ggcaccacggucggauccac

B # 63 (3x)
ggagcucagccuucacugc - guaugcacgcaaguuggaguagggugcuaauguugugCAAGGAGAACGCUUAAGAAAGGGACUaguacgauuaa - ggcaccacggucggauccac

B # 47 (3x)
ggagcucagccuucacugc - agcgcgcGAAAACGCUUCAGAAAGGGAgccgcagauagcauuaccaccguuaguagcugcuuguaucaacauaua - ggcaccacggucggauccac

FIGURE 3. Sequences of clones containing motif 1 and motif 2. The number in parenthesis indicates how often a clone was found among the 99 sequenced clones. Motifs are capitalized and shaded. B = superwash selection; C = bluensomycin counter selection. Circles and arrows indicate bases that are different within motif 2.

the final selection cycle, the single clones showed similar binding characteristics (data not shown).

Three clones (one from each selection procedure) not containing the primary sequence found in the chosen motifs were also tested for streptomycin binding. In all cases, RNA from the clones bound to the dihydrostreptomycin column and could be specifically eluted by streptomycin. Based on these results and considering the size and the number of possible binding sites found on streptomycin, we concluded there are several ways RNA can bind to the antibiotic.

RNA boundary mapping

The minimal sequence necessary to bind streptomycin was determined by subjecting RNAs from clones containing motifs 1 and 2 to partial alkaline hydrolysis followed by affinity chromatography. Analysis of two clones containing motif 1 mapped the nucleotides required to bind streptomycin from the 5′ end of the motif to the 3′ end of the constant flanking region (Fig. 4A,B). Seven nucleotides located at the 5′ end of motif 1 (5′-guggauc-3′) are complementary with ones at the 3′ end of the flanking region (5′-gauccac-3′) and have the potential to base pair. Analyses of two clones containing motif 2 indicate that, like motif 1, the conserved nucleotides comprising motif 2 are necessary but not sufficient for binding to streptomycin. However, unlike motif 1, the essential sequences include nonconserved nucleotides both upstream and downstream of motif 2 (Fig. 4A,B). The additional nonconserved sequences contain nucleotides that are complementary, suggesting the formation of a stem.

We investigated whether the small RNAs determined by the boundary mapping experiment retained the ability to bind streptomycin. Small RNAs comprising the essential sequences, a 46-mer RNA for motif 1 and a 41-mer RNA for motif 2, were synthesized. The binding abilities of the small RNAs were tested by affinity chromatography. Elution profiles using increasing concentrations of streptomycin and bluensomycin compared with the profile using equilibration buffer without antibiotic are shown in Figure 5. Motif 1 RNA was elutable with 1 μM streptomycin, but not with 1 mM bluensomycin nor with the equilibration buffer. This result was the first indication that RNA-containing motif 1 has a high affinity for streptomycin and strongly discriminates between streptomycin and bluensomycin.

The small RNA-containing motif 2 was specifically elutable with 10 μM streptomycin solution. In contrast to motif 1, motif 2 was also elutable with 10 μM bluensomycin. This implies that RNA-containing motif 2 has a lower affinity for streptomycin than RNA-containing motif 1, and that it binds both antibiotics, although not equaly well. Motif 2 RNA has some preference for streptomycin over bluensomycin.

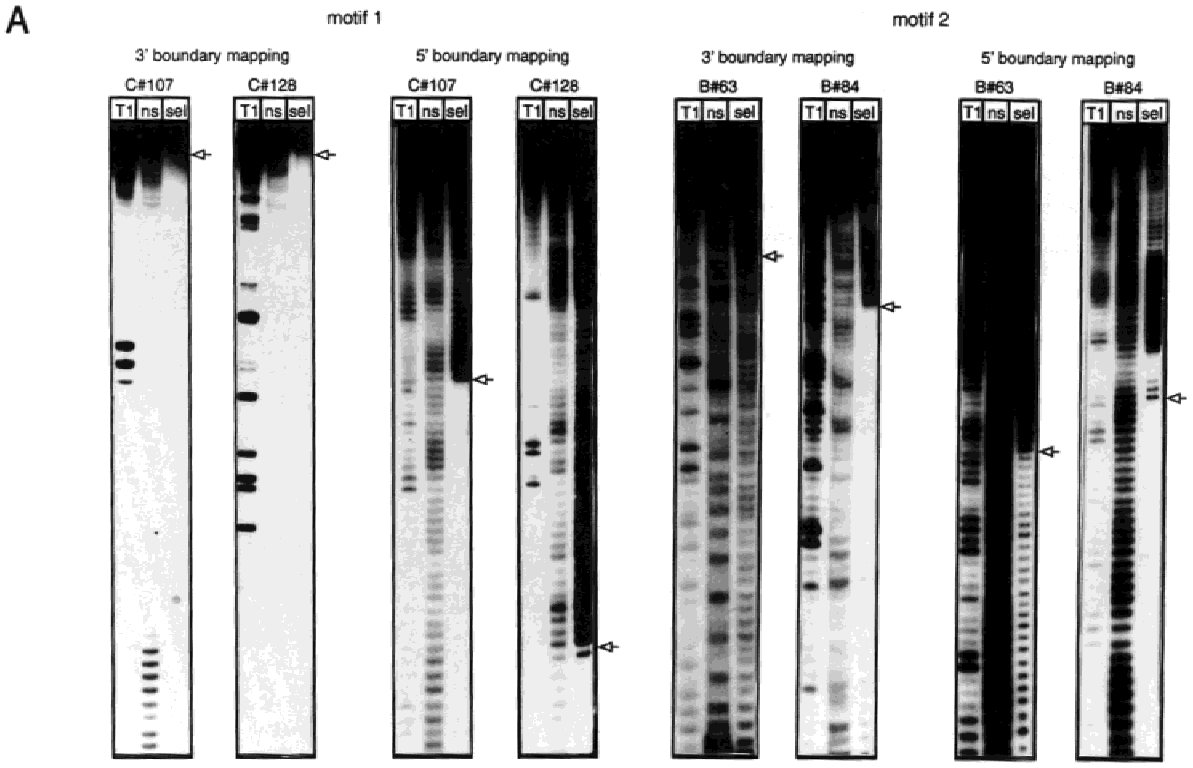
These data indicate that the counter-selection scheme was effective. RNAs found in the final pool from the counter-selection scheme were subjected to selective pressure to bind streptomycin and ignore bluensomycin. RNA-containing motif 1 is a product of the counter-selection scheme. By being specifically eluted with streptomycin and not with bluensomycin, motif 1 responded to the selective pressure exactly as expected. RNA-containing motif 2 was not found in the clones from the counter-selection scheme and, without the counter-selection pressure, motif 2 recognizes both antibiotics. These results sparked further investigation in order to confirm motif 1's streptomycin–bluensomycin discrimination and motif 2's ability to bind both antibiotics.

Mg^{2+} dependence of streptomycin binding

Mg^{2+} is known to be essential for the correct folding of several natural RNAs (Pan et al., 1993). For many aminoglycoside antibiotics, Mg^{2+} is essential to determine binding specificity. In the absence of Mg^{2+}, aminoglycosides bind to RNA unspecifically (Famulok & Huttenhofer, 1996; Hendrix et al., 1997). The equilibration buffer used in the in vitro selection schemes contained 250 mM NaCl and 5 mM Mg^{2+}. We investigated whether binding is dependent on the presence of Mg^{2+} or whether Mg^{2+} competes with the antibiotic. RNAs containing motifs 1 and 2 were tested for binding characteristics on streptomycin sepharose using equilibration buffer containing 250 mM NaCl and 0, 5, or 10 mM Mg^{2+}. As shown in Figure 6, in the absence of Mg^{2+}, neither motif was able to bind to the column. Furthermore, the RNAs bound better with 10 mM Mg^{2+} than under the original selection condition of 5 mM Mg^{2+}. This indicates that Mg^{2+} does not compete with streptomycin for binding to the RNA aptamers, suggesting that the binding is specific and that Mg^{2+} is an essential cofactor for the interaction of streptomycin to these RNAs.

RNA structural probing using Pb^{2+}-induced RNA cleavage

Pb^{2+} induces cleavage of the RNA backbone preferentially in single-stranded regions and in regions of high flexibility and is thus a good method to probe RNA secondary structure (Gornicki et al., 1989). By performing Pb^{2+}-induced cleavage in the absence and presence of streptomycin and Mg^{2+}, we were able to detect conformational changes in the RNA upon addition of both ligands. RNA cleavage of clone C#128, containing motif 1, was performed with increasing amounts of Mg^{2+} ranging from 0 to 10 mM. Lanes 2–6 in Figure 7A show that, without streptomycin, no change in the cleavage pattern could be observed. RNA incu-



B

Motif 1 (5' → 3')

C # 107 (75 mer)

-cc**gu** GGAUCGCAUUUGGACUUCUGCCuaacgcgugugcuagagagcaggcaaacaagug-ggcaccacggucggaucc ac

C # 128 (46 mer)

-cgcaaaacuaaaggaagaucucaagaaag**gu** GGAUCGCAUUUGGACUUCUGCCcagggu-ggcaccacggucggaucc ac

Motif 2 (5' → 3')

B # 63 (38 mer)

-ggaguagggugcuaaugu ugugCAAGGAGAACGCUUAAGAAAGGGACUaguacgau uaa-ggcaccacggucggauccac

B # 84 (41 mer)

-agugugagcu aucaccuCAAGGAAAACGCUUCAGAAAGGGACUuaggugau gauagugu-ggcaccacggucggauccac

FIGURE 4. 5′ and 3′ boundary mapping using partial alkaline hydrolysis and affinity chromatography of motif 1 and motif 2. **A:** Lanes on all gels are the same: T1, partial RNase T_1 digestion showing the position in the RNA which are guanosines; ns, partially hydrolyzed, nonselected RNA; sel, partially hydrolyzed, specifically eluted RNA with arrow indicating the extreme ends. **B:** 5′ and 3′ mapping results. Motifs 1 and 2 are shown in bold and capital letters and are shaded. Arrows indicate the RNA boundaries.

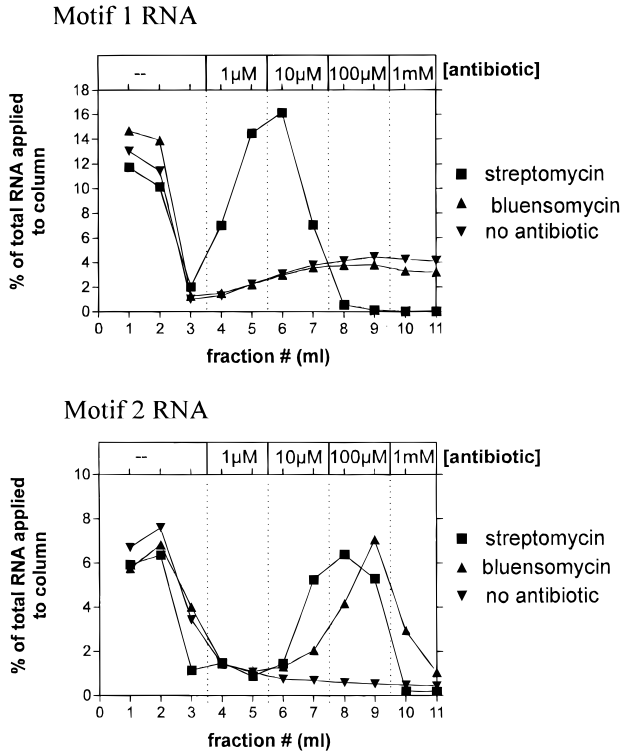


FIGURE 5. Affinity chromatography of small RNAs constructed from the information obtained from boundary mapping. Elution profiles of motif 1 and motif 2 are shown. Curves represent percent of total RNA loaded onto the column as a function of collected fractions. Fractions contained either different concentrations of streptomycin or bluensomycin, or no antibiotic.

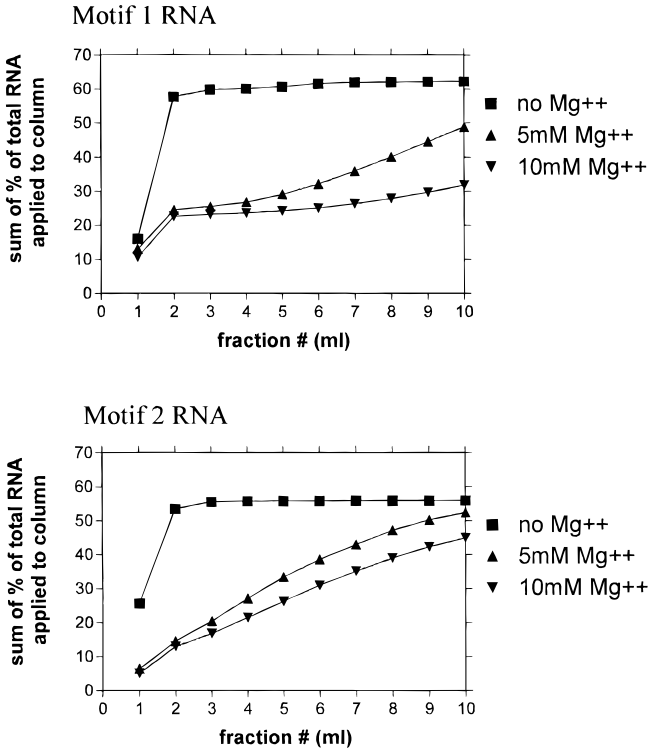


FIGURE 6. Column-washing profile of clones containing motif 1 (clone C#128) and motif 2 (clone B#84). The sum of the percent of the total RNA loaded onto the streptomycin column is plotted as a function of the fraction number. Different curves within the same graph indicate different concentrations of Mg^{2+} in the washing buffer containing 250 mM NaCl and 50 mM Tris-HCl, pH 7.6.

bated with streptomycin without Mg^{2+} (Fig. 7A, lane 8) gave the same Pb^{2+}-cleavage pattern as the reaction containing no antibiotic. The first indication of a major alteration in the RNA conformation is seen only when both ligands, streptomycin and Mg^{2+}, are present (Fig. 7A, lane 7). The protection of the RNA from Pb^{2+} in the presence of both ligands is an indication that the RNA looses flexibility.

Similar to motif 1, for clone B#84, containing motif 2, no change in the Pb^{2+} cleavage pattern is observed with increasing amounts of Mg^{2+} (Fig. 7B, lanes 2–6). Nevertheless, contrary to motif 1, upon addition of streptomycin to the reaction containing Mg^{2+}, no conformational change spanning the entire motif could be observed. Instead, an enhancement in the Pb^{2+} cleavage of two nucleotides (adenines 13, 14) was detected (Fig. 7B, lane 7).

Chemical probing using dimethylsulfate (DMS) and kethoxal and secondary structure model

Chemical probing using DMS and kethoxal was performed to (1) propose a secondary structure model, (2) locate the region of RNA–antibiotic interaction, (3) ascertain whether the motifs can discriminate between bluensomycin and streptomycin, (4) determine an approximate dissociation constant (K_d) between the RNA and the drug, and (5) verify the Mg^{2+} dependence of the RNA and antibiotic interaction. Reverse transcription analysis of DMS-modified RNA reveals non-base paired adenosines and cytosines, whereas kethoxal modification reveals non-base paired guanosines (Stern et al., 1988).

The boundary mapping data enabled us to focus on those regions that are necessary to bind the antibiotic. Bases that are accessible to DMS (Fig. 8A,B) and kethoxal (data not shown) modification and that were accessible to the Pb^{2+} cleavage were allotted to loops or bulges within the RNA. Finally, by inputting the small RNAs (46-mer containing motif 1 and the 41-mer containing motif 2) into Mfold (Zuker, 1989) and by applying the restrictions obtained from the chemical modification and Pb^{2+}-cleavage, a model for the secondary RNA structures in the absence of streptomycin is proposed (Fig. 8C).

By comparing the chemical modification pattern of RNA in the absence of antibiotic to that in the presence of antibiotic, the region of RNA–streptomycin interaction was located. Comparison of lanes 2 and 5 in Figure 8A shows streptomycin-induced protection of C7, A8, and C18 within motif 1, as well as protection of

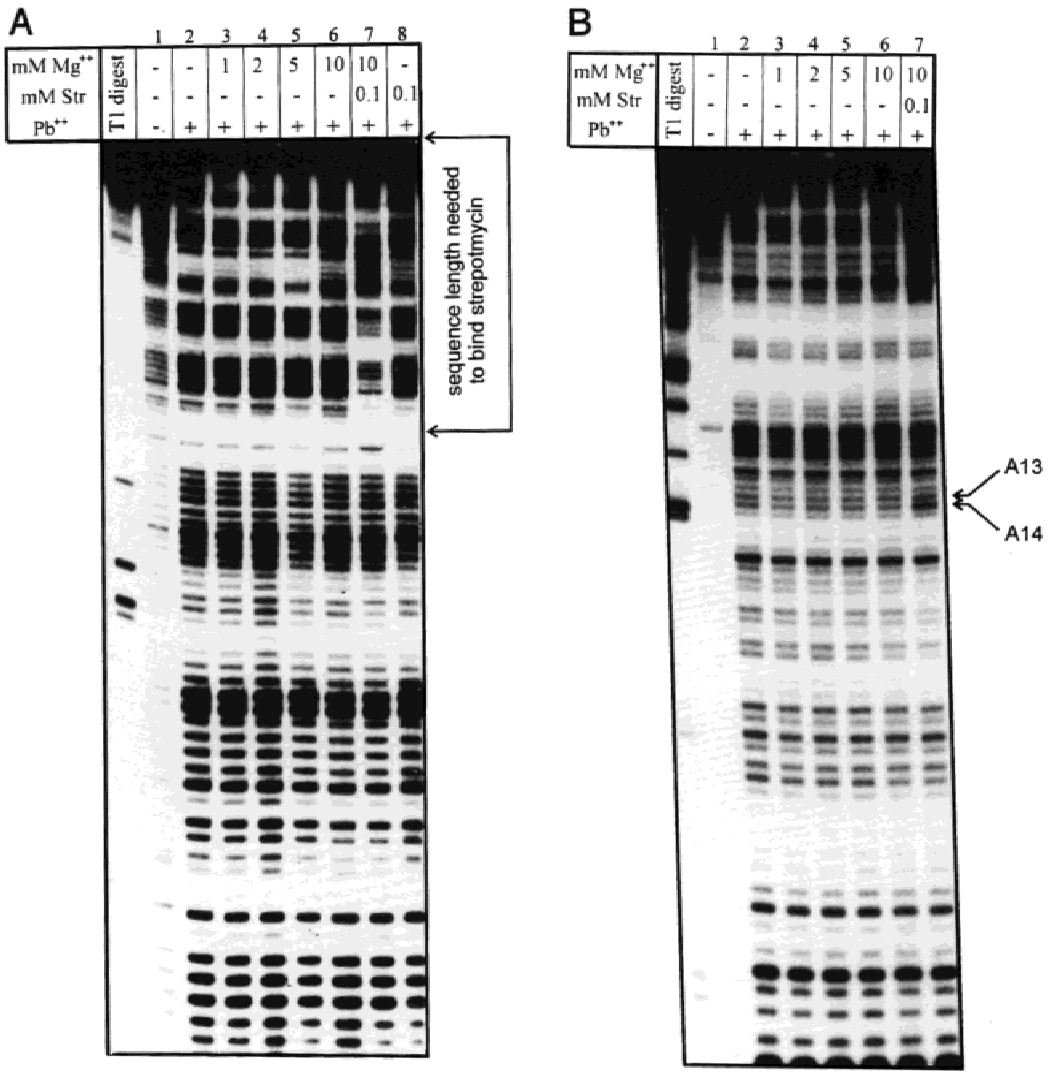


FIGURE 7. Autodiagram of gels after Pb^{2+}-induced cleavage on RNA from clones containing motifs 1 (**A**) and 2 (**B**); T1 digest, nuclease-induced cleavage used to indicate position of guanosines. Different lanes represent Pb^{2+}-cleavage performed under the indicated conditions. Changes in the cleavage pattern upon the addition of streptomycin and Mg^{2+} are indicated.

C33, A35, and C36 within the 3′ flanking region from DMS modification. Comparison of lanes 2 and 5 in Figure 8B demonstrates protection from DMS modification at A14, A15, A16, C17, and A31 within motif 2. Figure 8C shows the proposed secondary structures and the positions of the protected bases. No streptomycin protection from kethoxal modification using either motif was detected (data not shown). The observed protections may result from direct interaction with the antibiotic, but also from conformational changes induced upon binding of the ligand.

The specificity of the RNA–antibiotic interaction was investigated by replacing streptomycin with bluensomycin in the chemical modification reaction. Comparison of lanes 5 and 7 in Figure 8A demonstrates that, even in 100-fold molar excess, bluensomycin cannot produce the chemical protection pattern caused by streptomycin. This result is consistent with that obtained from the affinity chromatography experiment using small RNAs. Both results indicate motif 1 distinguishes between streptomycin and bluensomycin. Motif 2, on the other hand, cannot distinguish between the two antibiotics. As seen in comparison of lanes 5 and 7 in Figure 8B, when equal amounts of streptomycin and bluensomycin are added to the modification reactions, the protection patterns are comparable. This is also consistent with the affinity chromatography results using motif 2, which show that RNA can bind both antibiotics.

Streptomycin is not a chromophor, and radioactively labeled streptomycin is unavailable. K_d's were therefore estimated by the titration of increasing amounts of streptomycin into the DMS modification reaction (Wallis et al., 1995). Beginning with 0.01 μM strepto-

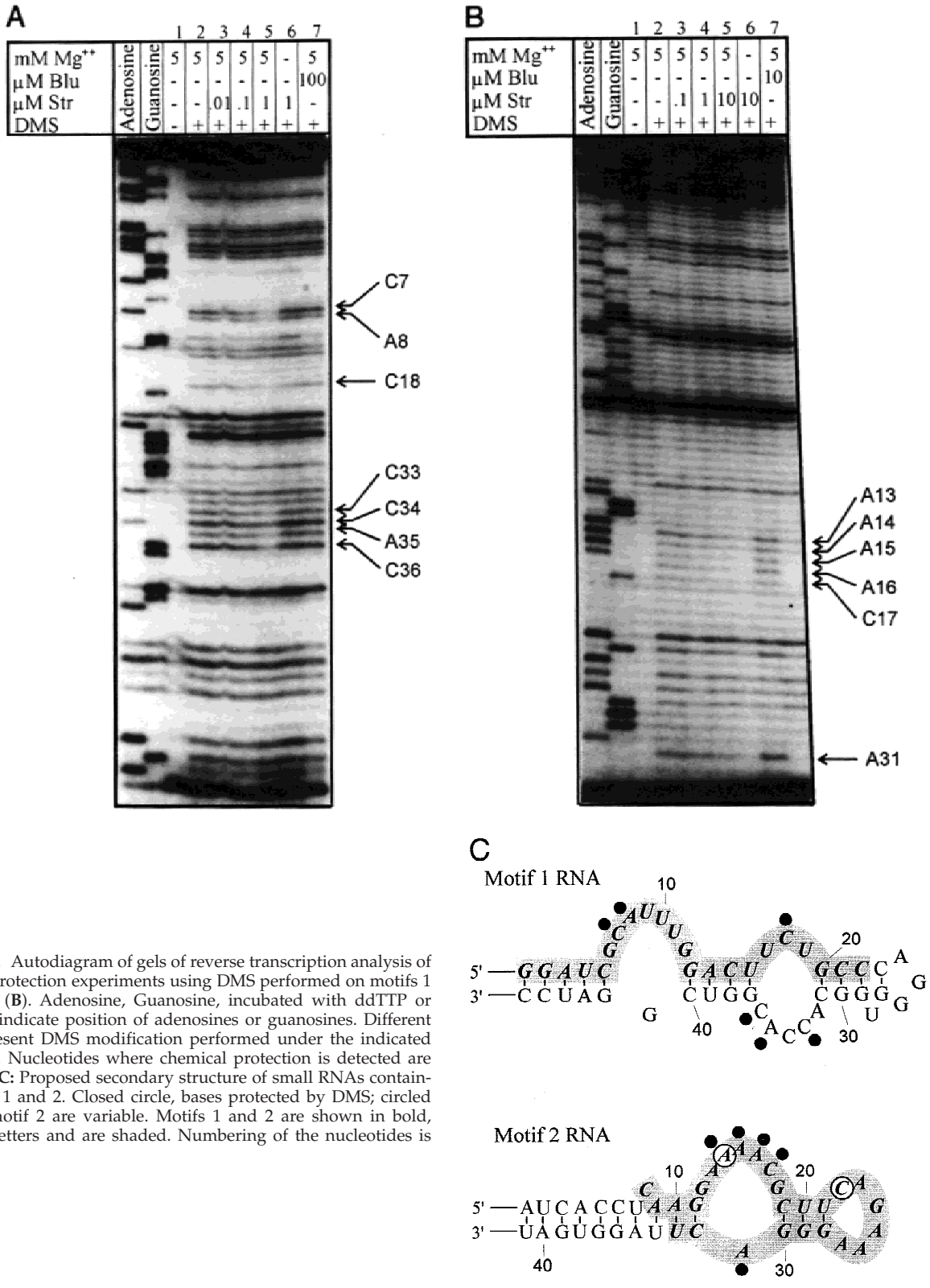


FIGURE 8. Autodiagram of gels of reverse transcription analysis of chemical protection experiments using DMS performed on motifs 1 (**A**) and 2 (**B**). Adenosine, Guanosine, incubated with ddTTP or ddCTP to indicate position of adenosines or guanosines. Different lanes represent DMS modification performed under the indicated conditions. Nucleotides where chemical protection is detected are indicated. **C:** Proposed secondary structure of small RNAs containing motifs 1 and 2. Closed circle, bases protected by DMS; circled bases in motif 2 are variable. Motifs 1 and 2 are shown in bold, italicized letters and are shaded. Numbering of the nucleotides is indicated.

mycin and increasing the antibiotic concentration 10-fold per lane (Fig. 8A, lanes 3–5), chemical protection within motif 1 was first detected at 1 μM. This result is in perfect agreement with the affinity chromatography (Fig. 5), where RNA-containing motif 1 was elutable from the column with 1 μM streptomycin. Upon titration of streptomycin into the reactions using motif 2 RNA (Fig. 8B, lanes 3–5), the DMS chemical protection first appears at a concentration of 10 μM. As with motif 1, the approximate K_d range of motif 2 is

in exact accordance with the affinity chromatography experiment (Fig. 5).

The chromatography experiment using different amounts of Mg^{2+} in the washing buffer indicated the necessity of Mg^{2+} for both motifs to bind streptomycin. The Pb^{2+}-induced cleavage experiment indicated a conformational change that occurs only in the presence of both streptomycin and Mg^{2+}. Although structural probing with lead gives folding and molecule flexibility information, and not binding information, this observation, along with the chromatography results, strongly suggests the binding of streptomycin to be dependent on the presence of Mg^{2+}. Streptomycin was added to two chemical modification reactions: one reaction contained Mg^{2+}, the other did not. Comparison of lanes 5 and 6 in both Figure 8A and B indicates the Mg^{2+} must be present in order for streptomycin to protect the RNA from DMS chemical modification.

Natural versus in vitro-selected streptomycin-binding sites

When using random RNA pools to isolate antibiotic-binding aptamers, a large number of different molecules are obtained that have to be compared to the natural binding sites. Our previously selected aptamers, which bind the aminoglycoside neomycin B or the peptide viomycin, clearly contained structural features in common with the respective natural binding sites; most neomycin-binding aptamers had stems with elements that widen the major groove (Wallis et al., 1995) and all viomycin-binding aptamers were pseudoknots, as are the natural viomycin-binding sites (Wallis et al., 1997). Two natural streptomycin-binding sites are known, one in the core of the 16S rRNA and one in the core of self-splicing group I introns. The streptomycin-binding site in the 16S rRNA is not well defined, given that both footprints and mutations resulting in resistance against the antibiotic are spread over three regions of the RNA (the 530, 912, and 1415 regions). The binding site is probably composed of several secondary structure elements that converge in space. The streptomycin-binding site in group I introns is the G-cofactor binding site, which is a G-C pair located in a stem, immediately following a semi-conserved bulged nucleotide (Michel et al., 1989). Again, this binding site might be composed of several elements scattered over the two-dimensional structure (Wang & Cech, 1992). Because no obvious similarities can be deduced between the natural and the in vitro-selected RNAs, a much more elaborate structural analysis of both is required. Interestingly, as for the selected aptamers, the binding of streptomycin to the intron RNA might involve Mg^{2+} ions, as does the binding of the guanosine cofactor (Sjögren et al., 1997). Mutation of the bulged nucleotide 5′ to the G-cofactor binding site results in increased Mg^{2+} requirement and in hypersensitivity to streptomycin (Schroeder et al., 1991).

Discriminative binding of motif 1 RNA to streptomycin is accompanied by a conformational change

We were interested in how RNA-containing motif 1 is able to discriminate between streptomycin and bluensomycin. Both antibiotics are relatively large and contain several electron acceptors and donors. The variance of the guanidino group on streptomycin and a carbamido group on bluensomycin represents a difference of two electron acceptors to two electron donors (Fig. 1). As shown by two experiments in this report, the carbamido group on bluensomycin renders it virtually invisible to the RNA, whereas the para-guanidino group on streptomycin is the antibiotic's ticket to recognition. Bluensomycin also contains a guanidino group, but in ortho-position to the streptose, which is ignored by the RNA. We therefore conclude that the RNA, in order to discriminate between the antibiotics, makes additional contacts with other parts of the molecule and that the recognition of a guanidino group alone is not sufficient. Furthermore, both antibiotics are flexible molecules, which can adopt different conformations. Discrimination between the two could therefore also occur if bluensomycin is not able to adopt the conformation that binds to the motif 1 RNA.

When analyzing the binding of RNA-containing motif 1 to streptomycin, it is important to bear in mind that the RNA–antibiotic interaction is dynamic. This is demonstrated when streptomycin is added to the Pb^{2+}-cleavage experiment. Upon binding of motif 1 RNA to streptomycin, a conformational change spanning the entire motif occurs. Figure 9 shows the quantitative comparison of lanes 6, 7, and 8 from Figure 7A. The comparison indicates that, upon addition of both ligands, nucleotide regions $P1_{3'}$–J3/2 and $P3_{5'}$–$P1_{5'}$ are less accessible to Pb^{2+} cleavage, whereas region $P3_{3'}$–L3 is more accessible. Quantitative analyses from the rest of the clone (six nucleotides of which are shown 5′ of the arrow in Fig. 7A) show the conformational change is exclusively located within the 46 nt identified in the RNA boundary mapping. The relatively large number of protections from DMS modification in the presence of streptomycin and Mg^{2+}, shown in Figure 8A, might not all derive from direct interaction with the ligand, but most probably also from base–base contacts that form after ligand binding, when the RNA adopts a new conformation.

The conformational change in the RNA observed when discrimination between two related ligands occurs is reminiscent of many other in vitro-selected RNA aptamers (Eaton et al., 1995; Cech & Szewczak, 1996). An RNA aptamer that binds the bronchodilator theophylline but not the chemically similar compound,

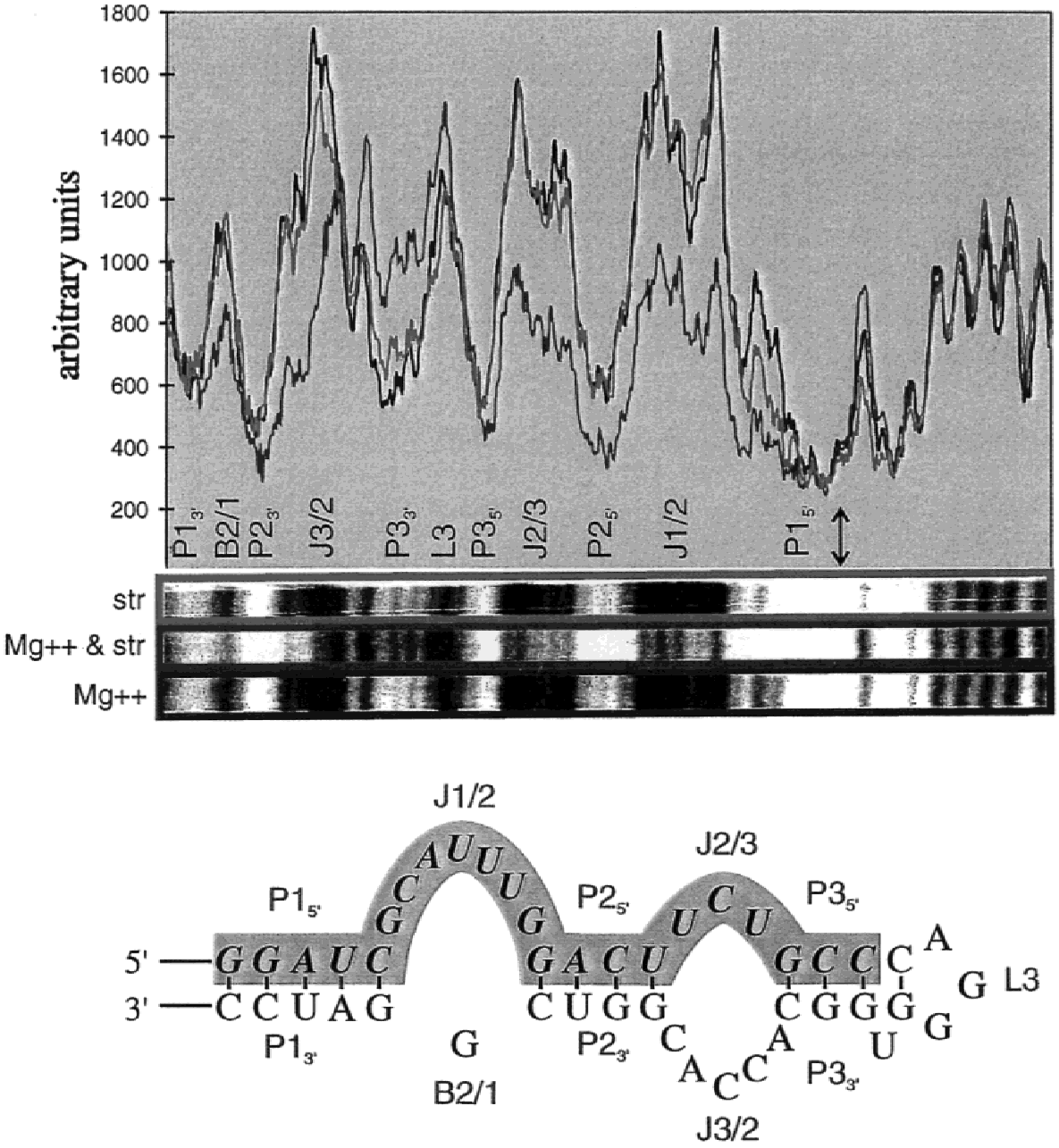


FIGURE 9. Streptomycin/Mg^{2+}-induced conformational change of motif 1 RNA. Above is shown the PhosphoImager-generated graph of Pb^{2+}-induced cleavage from Figure 7A: individual bands (3′ to 5′) from gel lanes 6 (blue), 7 (pink), and 8 (green) are shown as a function of band intensity. For clarification, the graph and gel are separated in regions that correspond to the proposed secondary structure shown below. P1–P3, paired regions with subscripts 5′ or 3′ indicating location in RNA; J1/2, J2/3, and J3/2, unpaired regions; B2/1, bulged guanosine. The minimal sequence length needed to bind streptomycin extends from $P1_{3'}$ to the arrow following $P1_{5'}$. Bold-faced, shaded nucleotides on the secondary sequence depict the conserved sequences of motif 1.

caffeine, discriminates between these two compounds 10,000-fold (Jenison et al., 1994). Upon binding theophylline, the RNA aptamer undergoes a conformational change, adopting a stable structure only after ligand binding. Theophylline is embedded in a direct base-triple interaction that is flanked by two additional triples, forming a theophylline sandwich (Zimmermann et al., 1997). Using a similar strategy, the ATP-binding RNA aptamer sequesters the ligand as the A in a GNRA loop, using the ligand as an essential structure-forming element (Jiang et al., 1996).

In both cases, the RNA used the ligand to adopt a stable conformation. The motif 1 RNA isolated here might follow similar rules, adopting a more rigid conformation after ligand binding. The protections from Pb^{2+} cleavage upon binding streptomycin can be in-

terpreted as a loss of flexibility, i.e., formation of a more rigid structure. It should be noted that, compared to the dynamic binding of motif 1, motif 2's binding to streptomycin is rather bland. As seen in Figure 7B, no conformational change occurs upon the addition of streptomycin to the lead cleavage reaction. Motif 2 cannot distinguish between bluensomycin and streptomycin.

It seems to be a general rule for RNA aptamers that bind their ligands with high affinity and that discriminate between analogous compounds, that the RNA does not present a preformed pocket to the ligand. The RNA is rather unstructured in the absence of the ligand. The aptamer adopts a complex structure only in the presence of the ligand, using it as an essential building block for structure formation.

MATERIALS AND METHODS

Materials

The starting DNA pool was obtained from M. Famulok and was described previously (Famulok, 1994). Bluensomycin was a gift of the Upjohn Company. Streptomycin and dihydrostreptomycin were purchased from Sigma Chemical Company, epoxy-activated Sepharose 6B from Pharmacia Biotech. Synthetic RNAs were ordered from Eurogentec in Belgium.

In vitro selection

Dihydrostreptomycin was coupled to Sepharose 6B following the manufacturer's recommendations. Because the coupling reaction occurs in 10 mM NaOH and streptomycin undergoes irreversible hydrolysis under such high pH conditions, dihydrostreptomycin was used. In order to obtain a final antibiotic concentration of 1 mM on the matrix, and assuming a 33% coupling efficiency (observed efficiency by M. Famulok), coupling reaction was performed in 3 mM dihydrostreptomycin. The random sequence RNA pool was described previously (Famulok, 1994). It consisted of a 74-nt long randomized region flanked by constant regions to allow transcription, reverse transcription, and PCR. The selection protocol was modified from a previously described procedure (Wallis et al., 1995). A bed of 1 mL dihydrostreptomycin-coupled Sepharose was equilibrated with 10 mL equilibration buffer (5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.6, 250 mM NaCl) at room temperature. ^{32}P body-labeled pool RNA was renatured (incubated at 90 °C for 3 min in ddH_2O, allowed to cool to room temperature) and brought to equilibration buffer conditions (final volume of 1 mL) before application to the column. The column was washed with three column volumes of equilibration buffer followed by 3 mL of 5 mM streptomycin. RNA from the streptomycin elution was pooled, precipitated, reverse transcribed, PCR amplified, transcribed, subjected to DNase 1 (1 h at 37 °C), and purified on 10% PAGE before precipitation and completion of the selection cycle by reapplying it to a new dihydrostreptomycin-coupled column. For the counter-selection procedure, the column was washed three times with equilibration buffer then with additional three column volumes 3 mM bluensomycin before the 3-mL 5 mM streptomycin elution. For the superwash selection procedure, instead of washing with three column volumes equilibration buffer, the column was washed with 30 mL before the 3-mL 5 mM streptomycin elution. After sufficient enrichment, PCR fragments were cloned into pGem3Z (Promega) using *Eco*R I and *Bam*H I digestion enzymes and sequenced using T7 sequencing kit from Pharmacia Biotech.

Boundary mapping: Selection of partially hydrolyzed RNA

The procedure used to select the RNA from clones containing motif 1 (C#107 and C#128) and motif 2 (B#63 and B#84) was essentially the same as described earlier (Wallis et al., 1995). After the application of ^{32}P-labeled RNA onto a 1-mL bed of dihydrostreptomycin-coupled sepharose, the column was washed with five column volumes of equilibration buffer before being specifically eluted with 3 mL of 5 mM streptomycin. The three elutions were pooled, precipitated, and analyzed on 8% polyacrylamide gels (20:1).

Pb^{2+}-induced RNA cleavage

Structural probing using Pb^{2+}-induced cleavage and the T1 digestion was performed as described earlier (Wallis et al., 1997) with the following modifications. A titration of Mg^{2+} (0–10 mM) was followed by the addition of 100 μM streptomycin with and without the presence of 10 mM Mg^{2+} (see Fig. 7A,B). Cleaved RNA was analyzed on 8% polyacrylamide gels (20:1).

Chemical probing using DMS and kethoxal

Chemical modification of RNA was essentially performed as described earlier (Wallis et al., 1995), with the exception that NaCl was used instead of NH_4Cl and 10 pmol of RNA was used. Modifying agents used were DMS and kethoxal. DMS methylates N-1 of adenine, N-3 of cytosine, and N-7 of guanine. Kethoxal modifies the N-1 and N-2 positions of guanine.

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