# Specificity in the Binding of Aminoglycosides to HIV-RRE RNA†

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ABSTRACT: Quantitative studies of the binding of neomycin B to RRE constructs are carried out to determine the relationship between non-Watson Crick base-paired elements in the RNA and aminoglycoside binding. The RRE region contains two unpaired domains containing a single base bulge and a bubble structure, respectively. Deletion of the single base bulge has no effect on neomycin binding as the site of aminoglycoside binding is localized to the bubble region. Converting the bubble region into an A-form duplex gradually abolishes neomycin B binding in 3−5-fold steps in affinity over a 75-fold range. Thus, the binding of aminoglycoside is favored at domains in RNA that are nonduplex in nature, but aminoglycoside binding is only graded-specific in that affinities are enhanced gradually as the structure further deviates from a duplex form. It is likely that high-affinity aminoglycoside binding does not occur in duplex RNA because the major groove is too narrow to allow for aminoglycoside access and that structural perturbations that allow widening of the groove facilitate access. However, these interactions are only graded-specific with respect to both aminoglycoside structure and RNA domain structure.

Aminoglycoside antibiotics operate as antibacterial agents by binding to specific regions of rRNA in prokaryotes, causing mistranslation and premature termination of protein biosynthesis (*1*). The A-site decoding region of prokaryotic rRNA is thought to be the functional target for aminoglycosides (*2*). This decoding region contains an internal bubble structure which allows access of aminoglycosides to the major groove of the RNA (*3*).

Of particular interest here is the question of what types of local RNA structures allow high-affinity and specific interactions with aminoglycosides. Aside from the decoding region, many other RNA molecules have been found to bind aminoglycosides (*4*−*6*). These include group I introns (*7*), a hammerhead ribozyme (*8*), and the RRE[1] transcriptional activator region from HIV which contains the binding site for the Rev protein (*9*). Moreover, in vitro selection against aminoglycosides generates a variety of RNA aptamers that are able to bind these antibiotics (*10*−*12*). The various RNA molecules that were uncovered which bind aminoglycosides exhibit little apparent sequence homology, suggesting that there are many structural solutions available for aminoglycoside binding. In the specific instance of the RRE transcriptional activator region, the aminoglycoside binding domain appears to be confined to an asymmetrical bubble region containing noncanonical purine-purine base pairs (*9*, *13*).

An important question to address is what structural features in RNA molecules are essential for specific aminoglycoside binding. As part of this issue, it is also important to determine whether there are common secondary structural features shared by the various aminoglycoside binding domains. These questions are part of a broader one relating to the specificity of RNA−ligand interactions, and whether highly specific antagonists for RNA molecules can be designed. In this article, the specificity of aminoglycoside binding to the HIV-RRE region is explored. The binding of aminoglycoside is favored at domains in RRE RNA that are nonduplex in nature, but aminoglycoside binding is only graded-specific in that affinities are only gradually enhanced as the structure further deviates from a duplex form.

The wild type RRE sequence (RRE1) is a 30-nucleotide fragment termed RBE3 (*14*) in the literature which contains the high-affinity Rev binding site of the RRE (*15*). NMR studies of the RBE3 sequence, both when free and when bound to the Rev peptide, show that the internal bubble in this sequence is partially closed by two purine-purine base pairs and adopts a widened major groove for Rev binding (*14*, *16*). Moreover, footprinting studies with the 67-nucleotide domain II of RRE suggest that neomycin B binds to this G-rich internal bubble of RRE (*9*). This region consists of two noncanonical purine-purine base pairs (G·G and G·A) and a single bulged uridine. Although neomycin B appears to prefer the internal bubble region of RNA, the general rules underlying neomycin B−RNA recognition are still unknown. To determine the nature of the neomycin B binding site in the wild type RRE RNA, we performed a series of mutational studies in which each unnatural element was removed from RRE (Scheme 1) and then quantitatively determined the binding affinities of each construct for neomycin B.

## MATERIALS AND METHODS

*Materials.* Kanamycin B sulfate, neomycin sulfate, and paromomycin sulfate were purchased from Sigma and were

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[1] Abbreviations: nt, nucleotide; RRE, HIV-Rev response element; CRP, tetramethylrhodamine-labeled paromomycin.

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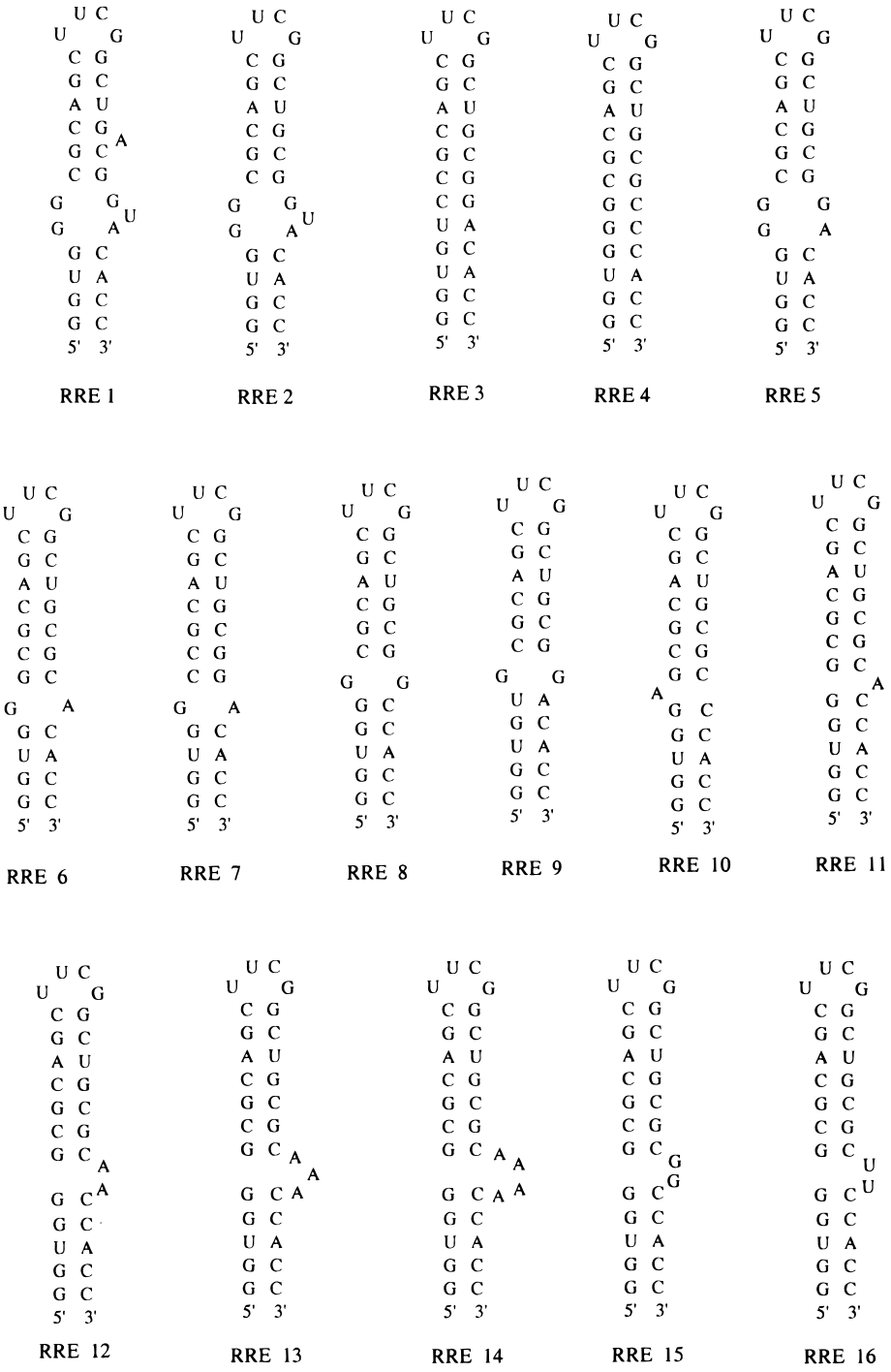
Scheme 1: Secondary Structures of RRE1−16 Derivatives




used without further purification. 5-Carboxytetramethylrhodamine succinimidyl ester was purchased from Molecular Probes, Inc. 5-Carboxytetramethylrhodamine-labeled paromomycin (CRP) was prepared as previously reported (*17*). Oligonucleotides were obtained from Oligo's, Etc., Inc. Polymerase chain reactions (PCRs) were carried out using the Gene Amp PCR kit with AmpliTaq DNA polymerase from Perkin-Elmer. RNA transcripts were generated using the RiboMAX large-scale RNA production kit with T7 RNA polymerase from Promega.

*RNA Synthesis and Purification.* RRE RNAs were transcribed in vitro by T7 RNA polymerase using synthetic oligonucleotide templates (*18*). All RNA contained GG at the 5′-end to increase the efficiency of transcription. Purified RNA was resuspended in sterile deionized water. The RNA was renatured by incubating in binding buffer [150 mM NaCl, 2 mM $MgCl_2$, and 20 mM HEPES (pH 7.5)] for 1 min at 80 °C followed by slow cooling to 25 °C.

*Fluorescence Measurements.* 5-Carboxytetramethylrhodamine-labeled paromomycin (CRP) concentrations were determined spectroscopically at 550 nm using a molar extinction coefficient of $6.00 \times 10^4$ $M^{-1}$ $cm^{-1}$. Fluorescence anisotropy measurements were performed on a Perkin-Elmer LS-50B luminescence spectrometer equipped with a thermostat accurate to ±0.1 °C. The tracer solution was excited at 550 nm, and monitored at 580 nm. The integration time

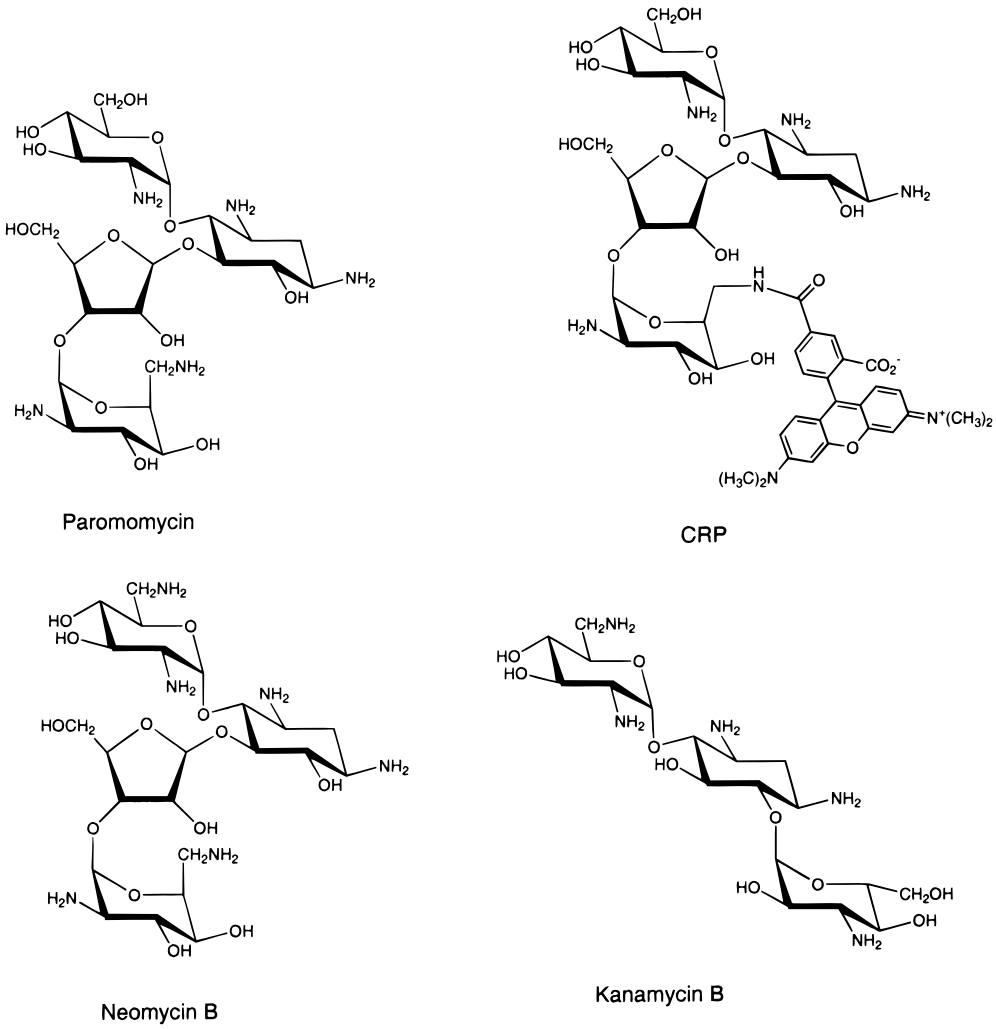



FIGURE 1: Aminoglycosides used in the binding assays.

was 5 s. For every point, six measurements were taken, and their average values were used for calculations. Measurements were performed in a buffer solution containing 150 mM NaCl, 2 mM $MgCl_2$, and 20 mM HEPES (pH 7.5).

*Determination of Dissociation Constants.* The following equation (eq 1) was used for the determination of the dissociation constant for the interactions between RNA and CRP ($K_d$).

$$A = A_0 + \Delta A\{[\mathrm{RNA}]_0 + [\mathrm{CRP}]_0 + K_d - [([\mathrm{RNA}]_0 + [\mathrm{CRP}]_0 + K_d)^2 - 4[\mathrm{RNA}]_0[\mathrm{CRP}]_0]^{1/2}\}/2 \quad (1)$$

where $A$ and $A_0$ are the fluorescence anisotropy of CRP in the presence and absence of RNA, respectively, and $\Delta A$ is the difference between the fluorescence anisotropy of 1 nM CRP in the presence of an infinite concentration of RNA minus the fluorescence anisotropy in the absence of RNA. $[\mathrm{RNA}]_0$ and $[\mathrm{CRP}]_0$ are the initial concentrations of RNA and CRP, respectively.

In the competitive binding assay, eq 2 is used for the calculation of the $K_D$ values.

$$[\mathrm{aminoglycoside}]_0 = [K_D(A_\infty - A)/K_d(A - A_0) + 1][[\mathrm{RNA}]_0 - K_d(A - A_0)/(A_\infty - A) - [\mathrm{CRP}]_0(A - A_0)/(A_\infty - A_0)] \quad (2)$$

where $K_D$ is the dissociation constant for dissociation of the RNA and the aminoglycosides. $[\mathrm{aminoglycoside}]_0$ is the initial concentration of the aminoglycosides. Both $K_d$ and $K_D$ were determined by nonlinear curve fitting using the equations described above and are presented as a mean value of three indepedent measurements.

## RESULTS

*Characteristics for Binding of 5-Carboxytetramethylrhodamine-Labeled Paromomycin (CRP) to RRE and Derivatives.* To identify the essential structural elements which are required for the specific interaction with neomycin B, nine different sequences related to RRE (RRE1−9) (Scheme 1) were prepared and studied with respect to their abilities to specifically bind to the aminoglycoside. The predicted secondary structures shown in Scheme 1 were obtained using M-fold (*19*). Fluorescence methods were used for the quantitative determination of aminoglycoside binding and required validation here before proceeding. For the quantitative measurement of binding affinities and stoichiometries of binding, 5-carboxytetramethylrhodamine-labeled paromomycin (CRP), shown in Figure 1, was used in a fluorescence anisotropy assay according to a previously reported procedure (*17*). The binding assays were performed by titrating a CRP solution (10 nM) with aliquots of the RNA solution. As expected, the fluorescence anisotropy of CRP

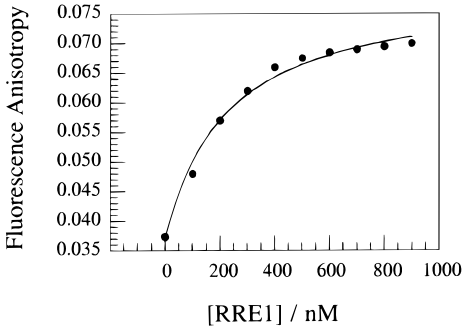



FIGURE 2: Fluorescence anisotropy of CRP (10 nM) as a function of RRE1 concentration.

Table 1: Binding Constants of CRP with RRE Mutants

| RRE mutant | $K_d$ ($\mu$M) | RRE mutant | $K_d$ ($\mu$M) |
|---|---|---|---|
| RRE1 | 0.48 ± 0.06 | RRE4 | 0.29 ± 0.07 |
| RRE3 | 0.40 ± 0.09 | RRE5 | 0.27 ± 0.06 |

increased upon the addition of RNA, and the corresponding fluorescence anisotropy values were then optimally fit to the binding data by nonlinear curve fitting to afford binding constants ($K_d$) (*17*). CRP exhibited comparable binding affinities (approximately 0.4 $\mu$M) with all of the RRE derivatives under consideration here. The binding constants for binding of CRP to several representative RRE constructs (Scheme 1) are shown in Table 1, and a representative binding isotherm for CRP with RRE1 is shown in Figure 2. This binding isotherm exhibits saturable behavior, and is fit to a 1:1 stoichiometry mode of binding (*17*). Therefore, the observed binding is well-behaved and allows for the determination of dissociation constants for the binding of aminoglycosides to RRE constructs. In subsequent competition experiments, the CRP was used to determine thermodynamic data for the binding of neomycin B to various RRE derivatives. Typical data for the fluorescence competition assay with three different RRE constructs are shown in Figure 3. From these data, $K_D$ values are readily calculated (*17*).

*Minimization of Unnatural Structural Elements in Wild Type RRE RNA.* With the quantitative fluorescence methodology established, the binding of neomycin B to the constructs shown in Scheme 1 was investigated to establish the importance of the nonduplex elements for aminoglycoside binding. In addition to the G-rich internal bubble, wild type RRE (RRE1) has a single A bulge. Initial experiments were performed to determine what, if any, role this bulge has in mediating neomycin binding. Deletion of this single bulge (RRE2) (Scheme 1 and Table 2) had little effect on the neomycin B binding. This result is consistent with the footprinting experiments carried out by Green and co-workers to show that the neomycin B binding site is localized to the G-rich internal bubble (*9*). To investigate the neomycin B binding site in the wild type RRE, we constructed three RRE mutants (RRE3−5). In the RRE3 and RRE4 mutants, the G-rich internal bubble is completely disrupted. The RRE5 mutant still had two purine-purine base pairs, but the single bulged uridine was removed. The RRE5 mutant exhibited a 3-fold decrease in the extent of binding to neomycin B compared to that of wild type RRE1, while RRE3 and RRE4 mutants exhibited very weak binding (6.4−7.5 $\mu$M) of neomycin B (Table 2). These results suggest that the two

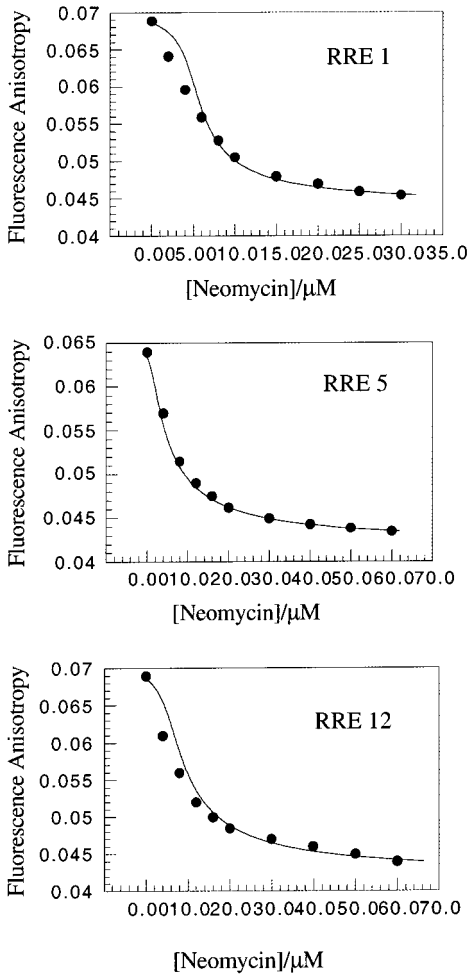



FIGURE 3: Representative competition binding isotherms for RRE derivatives.

Table 2: Dissociation Constants for Dissociation of Neomycin and RRE1−23 Derivatives

| A | | | |
|---|---|---|---|
| RRE derivative | $K_D$ ($\mu$M) | RRE derivative | $K_D$ ($\mu$M) |
| RRE1 | 0.15 ± 0.04 | RRE9 | 2.74 ± 0.83 |
| RRE2 | 0.16 ± 0.03 | RRE10 | 1.25 ± 0.16 |
| RRE3 | 7.54 ± 1.70 | RRE11 | 0.65 ± 0.07 |
| RRE4 | 6.40 ± 0.80 | RRE12 | 0.54 ± 0.17 |
| RRE5 | 0.48 ± 0.07 | RRE13 | 0.65 ± 0.08 |
| RRE6 | 1.18 ± 0.32 | RRE14 | 0.61 ± 0.09 |
| RRE7 | 2.93 ± 0.85 | RRE15 | 0.44 ± 0.08 |
| RRE8 | 1.75 ± 0.57 | RRE16 | 0.52 ± 0.13 |

| B | | | |
|---|---|---|---|
| mutant | original | mutant | $K_D$ ($\mu$M) |
| RRE17 | G4·C27 | C4·G27 | 0.57 ± 0.07 |
| RRE18 | G5·C26 | U5·A26 | 0.63 ± 0.12 |
| RRE19 | G5·C26 | C5·G26 | 0.51 ± 0.09 |
| RRE20 | G6·C23 | A6·U23 | 0.84 ± 0.17 |
| RRE21 | G6·C23 | C6·G23 | 3.40 ± 0.83 |
| RRE22 | C7·G22 | A7·U22 | 0.60 ± 0.09 |
| RRE23 | G8·C21 | U8·A21 | 0.49 ± 0.10 |

purine-purine base pairs in and of themselves allow for binding to neomycin B.

To determine whether both of the two purine-purine base pairs were required for neomycin B binding, the four different RRE mutants (RRE6−9) in which each purine-purine base pair was replaced by the corresponding Watson−Crick base pair were prepared (Scheme 1) and studied.

Table 3: Binding of Aminoglycosides to RRE Derivatives ($K_D$, $\mu$M)

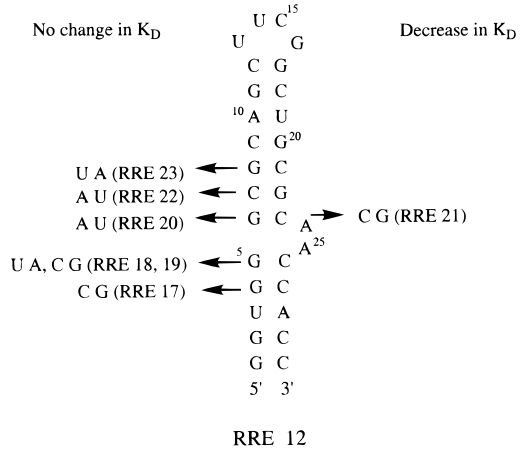
| RRE derivative | neomycin | paromomycin | kanamycin B |
|---|---|---|---|
| RRE 1 | 0.15 ± 0.04 | 8.3 ± 1.1 | 5.60 ± 0.83 |
| RRE 4 | 6.40 ± 0.80 | 25 ± 2.5 | 12.5 ± 1.28 |
| RRE 5 | 0.48 ± 0.07 | 15.5 ± 3.6 | 6.30 ± 0.72 |
| RRE 12 | 0.54 ± 0.17 | 18.7 ± 3.4 | 13.3 ± 2.5 |

Fluorescence competition experiments showed that the affinity of neomycin B binding decreased to 1.2−2.9 $\mu$M. Interestingly, mutations disrupting three consecutive G bases had more significant effects on neomycin B binding (RRE7 and RRE9). These results indicate that high-affinity neomycin B binding requires two purine-purine base pairs.

*Bubble Structure and Neomycin B Binding.* The studies described above show that modifying the RRE structure lowers the binding affinities for aminoglycosides. Since the neomycin B binding site in wild type RRE has two unnatural elements (noncanonical purine-purine base pairs and a single bulge), it is not clear whether an asymmetric bulge by itself can create a neomycin B binding site. To determine if a bulge structure can be a minimal structural element allowing for aminoglycoside binding, new RNA sequences were prepared in which an asymmetric bulge was added to the regular A-form duplex RRE4. In this study, we chose A bulges because the bulged A bases frequently appear in protein binding sites in rRNAs (*20*) and bulged purines are known to destabilize A-form RNA more than bulge pyrimidines (*21*). The secondary structures of the relevant RRE derivatives as calculated by M-fold (*19*) are shown in Scheme 1, and the data for neomycin B binding to these RNA constructs are summarized in Table 2A. In this series, RRE10 and RRE11 (Scheme 1) were prepared to study the effect of a single A bulge on neomycin B binding. Addition of a single A bulge to the regular A-form duplex increased the affinity of neomycin B binding by 5−10-fold. Interestingly, RRE11, where a single A bulge is inserted on the side opposite three consecutive G bases, exhibited enhanced neomycin B binding compared to RRE10. RRE12−14 were prepared so the effect of the asymmetric bulge size on neomycin B binding could be studied (Scheme 1). Fluorescence binding assays showed that the neomycin B binding affinities were not greatly affected by the bulge size. In this series, a two-base bulge proved to be slightly more efficacious with respect to neomycin B binding than the other sized bulges. Further studies with the two-base bulged RNA series (RRE15 and RRE16) showed that the two-base bulge (A24 and A25) in RRE12 could be changed to GG or UU with insignificant effects on neomycin B binding. This observation suggests that the bases in the bulge are probably not directly involved in neomycin B binding.

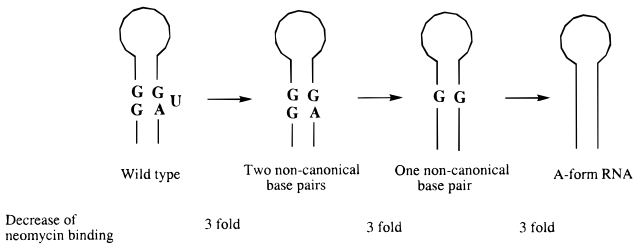
*Binding of Aminoglycosides to RRE and Derivatives.* Four representative RRE derivatives (RRE1, 4, 5, and 12) were tested for binding to aminoglycosides other than neomycin B (Table 3). Paromomycin and kanamycin B exhibited weak affinities in the range of 5.6−18.7 $\mu$M. Interestingly, RRE5 and -12 discriminate by a factor of approximately 35-fold between neomycin B and paromomycin. These specificities of these RNAs for neomycin B binding were comparable to that of the wild type RRE1. In contrast, RRE4 (the regular A-form duplex) exhibited little specificity of binding among the aminoglycosides.

Scheme 2: Mutation Studies of RRE12




RRE 12

Scheme 3: Summary of Mutation Studies of RRE




*Studies on the Binding Site of Neomycin in RRE12 by Mutational Analysis.* Further studies with RRE12 RNA were performed to determine which bases were important for neomycin B binding in this two-base bulged RRE derivative. As shown in Scheme 3, mutations in the duplex regions of RRE12 RNA were studied. Mutations of Watson−Crick base pairs in the duplex region were carried out by reversing each base pair or changing the identity of each base pair to maintain the nucleotide composition and base pairing stability of the duplex region. All the mutations of the base pairs, except for substitution of G6·C23 with a C·G pair, had no significant effects on neomycin binding (Table 2B).

## DISCUSSION

Aminoglycosides readily bind to RNA molecules. On the basis of RNA aptamer studies, it is clear that there are many different structural solutions for binding in the micromolar affinity range (*10*−*12*). This is approximately the affinity range found in the binding of aminoglycosides to the prokaryotic rRNA A-site decoding region and the HIV-RRE region (*13*, *17*, *22*−*24*). The studies presented here were carried out to probe the relationship between RNA structure in the RRE series and the ability to specifically bind aminoglycosides. Previous studies of RNA binding to aminoglycosides have demonstrated that specific aminoglycoside binding is often favored at nonduplex regions (*3*, *9*, *25*). One favored binding motif appears to involve internal bulges or bubbles as part of preferred binding sites (*26*, *27*). The recent NMR study of the complex of the aminoglycoside paromomycin with an A-site rRNA decoding region RNA showed that paromomycin binds in the major groove in a binding pocket formed by noncanonical base pairs and a bulged nucleotide (*3*). Another well-studied aminoglycoside-

binding RNA is the HIV RRE region in which aminoglycosides are thought to bind to an internal bubble region (*6*). The binding of aminoglycosides to this region competes with the binding of the Rev protein to this same site (*9*). Generally, the internal bulges or bubbles consist of noncanonical base pairs and bulge nucleotides. The objective in this study was to specifically probe the importance of internal bulges and noncanonical base pairs in aminoglycoside binding to an RRE region construct.

To accomplish these studies, a sensitive fluorescence anisotropy technique was used to quantitate the binding between the RNA molecule and the aminoglycosides (*17*). CRP, a rhodamine conjugate of paromomycin, binds saturably to the RRE constructs, and the binding is competitive with aminoglycosides (Figure 2). It is a straightforward matter to calculate $K_D$ values from the competition between CRP and various aminoglycosides and, thus, to probe the relationship between the secondary structure, calculated by M-fold (*19*), of a particular RNA molecule and the affinity for a particular aminoglycoside.

It is demonstrated here that CRP stoichiometrically binds to the RBE3 (RRE1) construct (Figure 2). This construct is known to bind to $Rev_{34-50}$, and this binding is competitive with aminoglycoside binding to this region (*9*, *17*). The role of bulge elements in aminoglycoside binding can be readily studied in this system. Removal of the single A bulge did not affect aminoglycoside binding (RRE1 vs RRE2), a result consistent with experiments showing that aminoglycosides bind to the U-bulged region in RRE1 (Table 2). Further experiments were then performed on stem−loop RRE structures in which the A bulge has been deleted. Conversion of the aminoglycoside bulged binding site to duplex RNA structures (RRE3 and RRE4) abolished high-affinity neomycin binding. The binding affinity increased from 0.15 to 7.5 $\mu$M in the case of RRE3 and to 6.4 $\mu$M in the case of RRE4. This result is in contrast with the binding data reported by others using surface plasmon resonance (SPR) to suggest that neomycin B can bind to regular A-form RNA and RBE3 with the same affinity (*24*). The studies reported here, however, are consistent with other previous studies which address some of the same issues. Green and co-workers reported binding constants for the interaction of neomycin B with the RRE variants using a novel ultrafiltration procedure (*13*). On the basis of the binding data, they have proposed that a purine-purine base pair within the RRE core element is critical for neomycin B binding (*13*). These data, along the data described here, support the view that specific aminoglycoside binding to RNA requires nonduplex-containing structural domains, and are inconsistent with results which suggest otherwise (*24*).

Further studies on the bulged region aminoglycoside binding site revealed the secondary structural requirements here. Removal of the A bulge, but retention of the putative noncanonical G·G and G·A base pairs, led to a modest decrease of approximately 3-fold in neomycin binding affinity ($K_D$ = 0.48 $\mu$M for RRE5). With only one noncanonical base pair (RRE6−9), binding affinities increased to approximately 3.5-fold to the low-affinity level (1.5−3 $\mu$M). However, the introduction of an A bulge in a duplex RRE structure (RRE11) restored the binding affinity to the 0.65 $\mu$M range. Increases in the bulge size (RRE12−14) did not enhance this affinity, suggesting that asymmetric bulge size is not an important factor in regulating the affinity for neomycin. Mutations in the RRE12 construct generally showed no influence on neomycin binding affinity, except in RRE21 where a G·C base pair was reversed. Mutation of G6·C23 to a C·G pair decreased the dissociation constant ($K_D$) for neomycin by approximately 6-fold, whereas the G6·C23 to A·U change had a negligible effect on neomycin B binding (Table 2B). In contrast, the G5·C26 base pair, located in the 3′ direction with respect to the bulge, could be mutated to other base pairs with little effect on binding. These results suggest that the adjacent base pair in the 5′ direction to the two-base bulge might be crucial for neomycin binding.

These mutational results can be explained on the basis of structural features of a bulged RNA. Previously, Crothers and co-workers have proposed that the major groove is more accessible in the 5′ direction than in the 3′ direction to the bulge (*28*). This feature of the major groove widening in the bulge region can explain the neomycin binding motif in RRE12. A very similar structural motif was identified in the HIV-1 mRNA transactivation response element (TAR) RNA sequence which adopts a hairpin structure consisting of a hexanucleotide loop and a three-nucleotide bulge (*29*). Recent footprinting studies with the TAR−neomycin complex have shown that the lower stem region of TAR, located in the 5′ direction to the bulge, is the primary neomycin binding site (*25*). On the basis of these results, it appears that neomycin B binding is facilitated by an RNA duplex featuring a widely opened major groove, rather than by a particular nucleotide sequence.

The experiments described here demonstrate a range of approximately 75-fold in neomycin B binding affinity between RRE1 or RRE2, which contain a single internal bubble or bulge structure, and the A-form duplex equivalent RRE3. The question that is addressed is whether the neomycin binding site of RRE2 or RRE3 exhibits high specificity of binding with respect to interactions with neomycin B. This question is answered by determining the abilities of various mutants in the RRE series to bind neomycin B. What is quite clear is that elimination of the unnatural elements in the RRE series gradually decreases the extent of high-affinity neomycin B binding. Elimination of the U mismatch and elimination of the two putative non-Watson−Crick base-paired purine-purine base pairs all individually lead to decreases in binding affinity of between 3- and 5-fold (Scheme 3). Therefore, the specificity of neomycin B binding is less related to sequence than to local structure in this series. This graded specificity can also be observed in the abilities of constructs such as RRE1, RRE4, RRE5, and RRE12 to bind aminoglycosides other than neomycin B. While neomycin B was bound more tightly to the constructs, both paromomycin and kanamycin B also bind. It is interesting to note that in the A-site decoding region construct, neomycin was bound about 10-fold more tightly than aminoglycosides, such as paromomycin and kanamycin (*17*, *23*). The extra amino group in ring A of neomycin B is presumably important for electrostatic interactions with the RNA molecules.

The binding of neomycin B in the RRE series appears to be clearly related to the availability of unnatural structural elements in the RNA molecules themselves. How might these unnatural structural elements facilitate aminoglycoside binding? It is likely that these elements induce openings in the

narrow A-form duplex of the RNA, allowing access to the bulky neomycin molecules. In the absence of groove opening, aminoglycosides may only be able to interact with the phosphate backbone and not interact with the buried bases, except at the loop regions. This notion is consistent with studies on DEPC modification of RNA molecules, where modification only occurred at nonduplex regions of RNA constructs (*28*, *30*).

The limited specificity of aminoglycoside binding to the RRE constructs can be contrasted with the observed highly specific binding of the aminoglycoside tobramycin to certain RNA aptamers (*26*, *27*). High-affinity RNA aptamers can be selected for binding to the aminoglycoside tobramycin with sub-nanomolar affinity (*26*, *27*). In a particularly well-studied aptamer, J6, strong molecular discrimination was found. Aminoglycosides structurally similar to tobramycin were bound with affinities at the nanomolar level or lower (*26*). The binding of tobramycin to J6 while requiring nonduplex structural elements, also involved duplex regions, which were made accessible to tobramycin by the unnatural structural elements (*27*). These studies demonstrated that highly specific aminoglycoside interactions with RNA are possible. In the case of the RRE constructs described here, a similar level of aminoglycoside binding specificity is obviously not observed. Whether it will be possible to design specific antagonists that specifically recognize the RRE bubble remains an open question.

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