A non-canonical base pair within the human immunodeficiency virus Rev-responsive element is involved in both Rev and small molecule recognition

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Background: Human immunodeficiency virus (HIV) replication depends on the interaction of an HIV regulatory protein, Rev, with a viral RNA element (the Rev-responsive element, RRE). The high affinity RRE core region contains a non-canonical base pair (G48:G71) that is important for Rev recognition. Aminoglycoside antibiotics, specifically neomycin B, bind to the RRE and selectively block Rev–RRE interactions *in vivo* and *in vitro*. We attempted to generate an *in vitro* model for the establishment of HIV-1 resistance to neomycin B.

Results: We have used *in vitro* genetic selection to evolve RRE variants that bind to Rev in the presence of neomycin B. Most of the RRE variants selected in the presence of 10 μM neomycin B contain a G48:G71 to A48:A71 substitution. Those selected in 100 μM neomycin B contain either C:A or A:A substitutions at this position. Binding constants for the interaction of neomycin B with the wild-type RRE and a subset of the selected RRE variants were determined using a novel ultrafiltration procedure.

Conclusions: A purine–purine base pair within the bulge region of the RRE core element is critical for neomycin B binding as well as Rev binding. RRE variants that survive in high concentrations of neomycin do so either by binding Rev better than wild-type (this correlates with the sequence A48:A71) or by binding neomycin poorly (correlating with the sequence C48:A71). Other sequences must also influence both Rev and neomycin B binding.

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Introduction

Productive HIV infection is dependent on the interaction of the regulatory protein, Rev, with a specific RNA structure known as the Rev-responsive element or RRE (reviewed in [1]). The RRE is a 234-nucleotide RNA sequence embedded within the viral *env* coding region. The high affinity Rev binding site, or core element, within the RRE consists of a stem–bulge–stem structure corresponding to stem IID and stem–loop IIB of RRE domain II (Fig. 1, [2–5]). The intervening bulge region is predicted to contain two non-Watson–Crick base pairs, G48:G71 and G47:A73 [6–8].

Rev activates *env* expression by facilitating the redistribution of unspliced and singly spliced HIV mRNA out of the nucleus by a mechanism that is not fully understood [9,10]. Because the Rev–RRE interaction is essential for viral replication, and is known to limit replication in some circumstances [9,10], Rev is an attractive target for antiviral therapy. Several strategies have been attempted to reduce intracellular Rev levels or to block its function. These include, for example, the expression of antisense nucleic acids directed against Rev mRNA [11], dominant-negative Rev mutants [2,12], and Rev-specific single-chain antibodies [13].

Small molecules can be useful antiviral agents because they are relatively easy to deliver. One potential limitation of such therapy, however, is that the virus may rapidly evolve drug-resistant strains. HIV-1 is notorious for the 'malleability' observed when it develops resistance to small molecules that target HIV reverse transcriptase [14] or protease [15].

Neomycin B and other aminoglycoside antibiotics can block the Rev–RRE interaction *in vitro* and *in vivo*, by binding to the RRE core [16]. RNA-binding drugs such

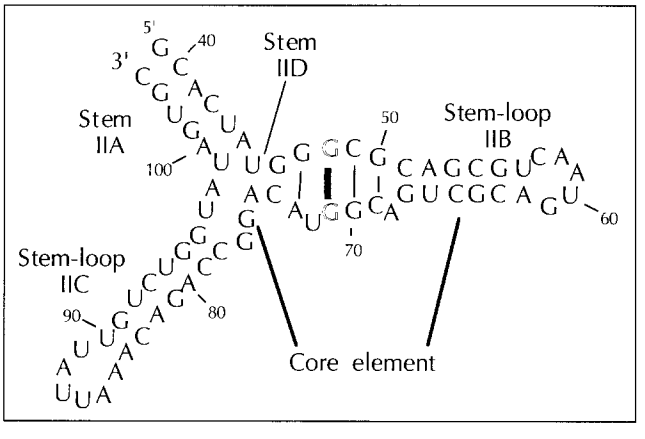

Fig. 1. Domain II of the HIV RRE. Vertical lines indicate base pairing within the RRE core element and the G48:G71 base pair is outlined.

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as neomycin B are thus attractive lead compounds for antiviral therapies. But is it possible to generate mutations within the RRE that retain Rev binding but are resistant to neomycin B inhibition? Here we use an *in vitro* selection technique [17–19] to mimic the evolution of neomycin B-resistant RREs. We find that neomycin-resistant RNAs can evolve in two ways, either by increasing their affinity for Rev or by decreasing their affinity for neomycin B. At high concentrations of drug, the latter mechanism appears to be more important.

Results

Selection for neomycin resistance leads to changes at the G48:G71 base pair

We initiated the selection with a previously described RNA pool that was biased toward the wild-type (WT) RRE [20]. The pool contains 10^{13} variants of the 67 nucleotides of domain II of the WT RRE, and was constructed such that each base had a 65 % chance of being WT, 30 % chance of being substituted, and a 5 % chance of being deleted.

Neomycin B concentrations of 0.1 to 1 μM can quantitatively inhibit Rev binding ([16], this study). We carried out three separate selections, using 0, 10 or 100 μM neomycin B. In each selection, RNA molecules bound to Rev were separated from unbound sequences by a combination of nitrocellulose-filter binding and mobility-shift gel electrophoresis. Bound RNAs were eluted, amplified by the reverse-transcriptase polymerase chain reaction (RT-PCR), and transcribed into RNA. A total of nine rounds of selection and amplification were carried out

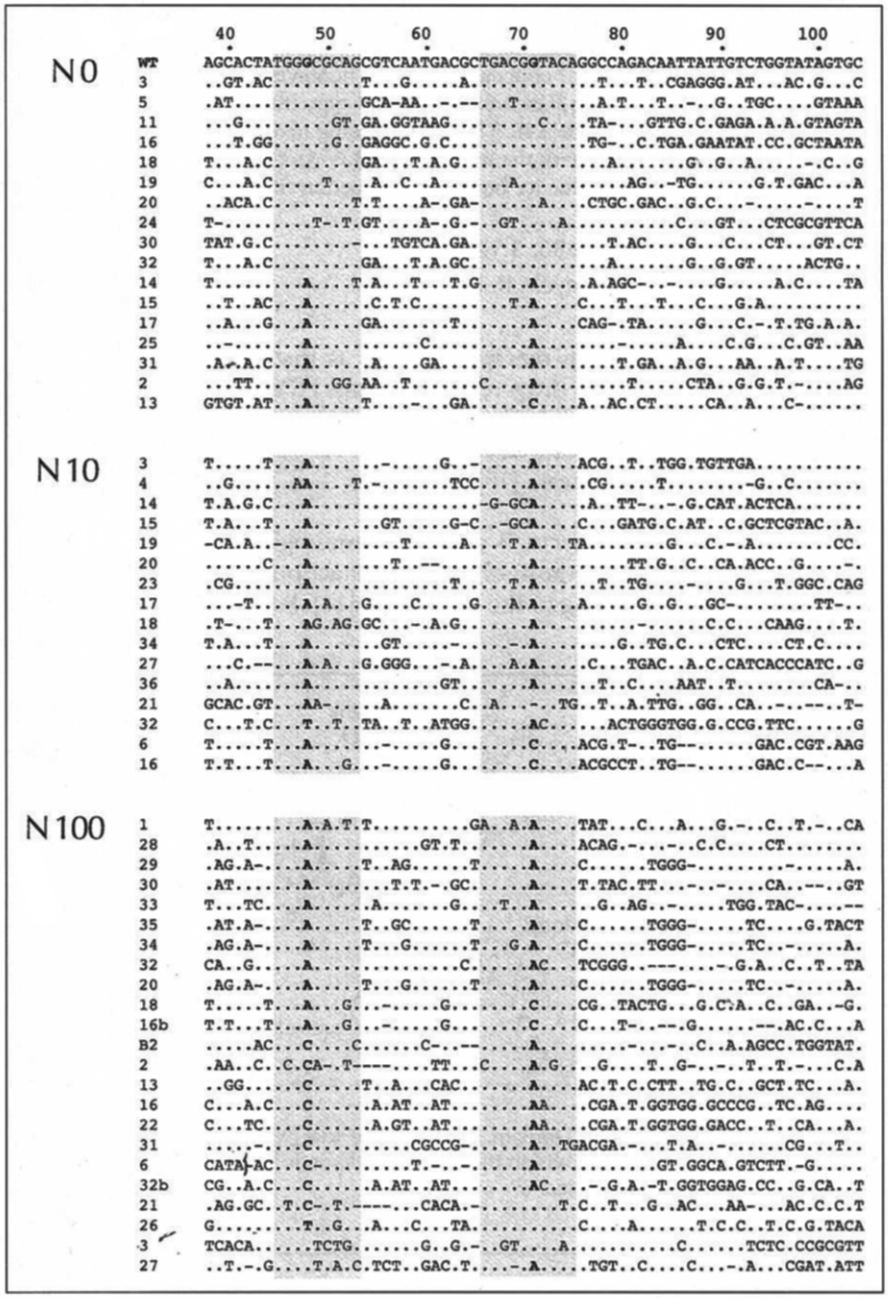

Fig. 2. The sequences of RRE variants selected in the presence or absence of neomycin B. Pools N_0, N_{10} and N_{100} were selected for Rev binding in the presence of 0, 10 and 100 μM neomycin B, respectively. The sequences are aligned with the RRE domain II region (WT). Nucleotides are numbered according to Malim *et al.* [2,3]. Bases that differ from the WT sequence are indicated, bases that correspond to WT are designated by a period (.) and deleted bases are indicated by a dash (–). Within each pool, the sequences are grouped according to the identity of the 48:71 base pair. All of the selected sequences contain two relatively conserved blocks of sequence (shaded) that correspond to the RRE core domain upon folding. RRE variants, especially those selected in the presence of neomycin B (N_{10} and N_{100}), diverge from the WT sequence at positions 48 and 71, which corresponds to the G:G base pair within the WT RRE core region. Sequences selected in 10 μM neomycin B predominantly contain A48:A71 while those selected in the presence of 100 μM neomycin B contain either an A:A or C:A base pair at this position. The DNA sequence is given here; for the RNA sequence, read U for T.

and members of each pool were sequenced at various stages during the selection.

Two characteristics of the selected RRE variants are evident upon comparison with the WT RRE (Fig 2). First, all of the selected sequences contain two relatively conserved blocks of sequence that correspond to the RRE core domain. Sequences outside of the core element, especially those corresponding to stem IIA and stem–loop IIC, diverge greatly from WT. This is expected since the RREs were selected by virtue of their affinity for Rev. Second, RRE variants selected in the presence of 10 μM neomycin B (N_{10}) and 100 μM neomycin B (N_{100}), diverge from the WT sequence, at positions 48 and 71, and these bases seem to covary.

The covariation of bases 48 and 71 supports a large body of evidence that a purine–purine base pair is required at this position for efficient Rev binding [20–22]. Of the 17 sequences determined from the N_0 pool, 10 are G48:G71 (WT) and 6 are A48:A71. The A:A substitution has previously been shown to support Rev binding [20,21]. Selection in the presence of neomycin B appears to strongly favor RRE variants containing A48:A71 or C48:A71; 75 % of the N_{10} pool sequences contain an A:A at this position and the N_{100} pool consists of a mixture of A:A (39 %) and C:A (35 %) containing variants. Remarkably, none of the N_{10} or N_{100} RNAs contain a G48:G71 base pair.

Neomycin B sensitivity of individual RRE variants

Binding of Rev and neomycin B is competitive [16] and thus our selection procedure will identify RNAs that are better able to discriminate between Rev and neomycin than the original pool. Improved discrimination could occur because the affinity for Rev has increased, the affinity for neomycin B has decreased, or both. We therefore examined the affinity of each variant for Rev in the absence of drug (Table 1), and chose representative members from each of the three pools to examine in binding assays with Rev in the presence of 0, 10 or 100 μM neomycin B.

Figure 3a shows that each N_0 RNA tested was sensitive to neomycin B inhibition in a dose-dependent manner; inhibition was greater at 100 μM than 10 μM drug. Some of these N_0 RNAs were less sensitive than the WT RRE to neomycin B, probably due to their higher affinity for Rev.

The RNAs from the N_{10} pool were much less sensitive than the WT RRE or the N_0 RNAs to neomycin B inhibition (Fig. 3b); binding of the N_{10} RNAs to Rev in 10 or 100 μM neomycin B was generally comparable. Like the N_0 RNAs, all but one of the N_{10} RNAs (N_{10}-21) bound Rev with a higher affinity than did the WT RRE.

All of the N_{100} RNAs were neomycin B-resistant (Fig. 3c); Rev binding was comparable in drug concentrations from 0 to 100 μM. Unlike the N_0 and N_{10} RNAs, the N_{100} RNAs bound to Rev with a wide range of affinities, and, surprisingly, several (N_{100}-13,16,21,22 and 26) bound less well than the WT RRE.

Table 1. Comparison of Rev and neomycin B binding affinities.

RRE variant[a]	Position 48:71[b]	K_d^{Neo} (nM)[c]	K_d^{Rev} (nM)[d]	$\frac{K_d^{Neo}}{K_d^{Rev}}$
WT RRE	G:G	82 ±3	1.0	82
RRE-AA	A:A	230 ±20	0.7 ±0.2	330
RRE-CA	C:A	320 ±30	1.4 ±0.1	230
N_0-16	G:G	20 ±3	0.4 ±0.3	50
N_0-25	A:A		0.5 ±0.6	
N_0-19	G:G		0.5 ±0.4	
N_0-14	A:A		0.5 ±0.1	
N_0-18	G:G		0.5 ±0.1	
N_0-31	A:A		0.6 ±0.1	
N_0-32	G:G		0.6 ±0.1	
N_0-30	G:G		0.8 ±0.3	
N_0-13	A:C	61 ±6	0.8 ±0.2	76
N_{10}-23	A:A	28 ±1	0.4 ±0.3	70
N_{10}-16	A:C	53 ±24	0.5 ±0.5	110
N_{10}-14	A:A		0.7 ±0.5	
N_{10}-3	A:A		0.6 ±0.6	
N_{10}-19	A:A		0.6 ±0.6	
N_{10}-20	A:A	160 ±20	0.6 ±0.5	270
N_{10}-21	A:-	810 ±80	3.2 ±0.1	250
N_{100}-31	C:A	180 ±40	0.4 ±0.3	450
N_{100}-35	A:A		0.5 ±0.2	
N_{100}-1	A:A	92 ±9	0.7 ±0.4	130
N_{100}-6	C:A	120 ±30		
N_{100}-34	A:A		0.8 ±0.2	
N_{100}-13	C:A	560 ±270	0.8 ±0.3	700
N_{100}-22	C:A	720 ±190	1.1 ±0.3	650
N_{100}-16	C:A	990 ±300	1.6 ±0.1	620
N_{100}-21	C:G	450 ±140	3.3 ±0.2	110
N_{100}-26	U:G	450 ±60	4.3 ±0.1	100
N_{100}-22GG	G:G	110 ±10	0.8 ±0.1	140
N_{100}-26GG	G:G	60 ±7	4.0 ±0.2	15

[a]RRE variants are listed within each pool according to decreasing affinity for Rev.
[b]Identity of bases 48 and 71 when the variant sequences are positioned to give maximum alignment with the wt RRE.
[c]Binding constants (shown as the mean value ± the standard error of the mean of at least four independent experiments) were determined using the ultrafiltration/NPT II assay.
[d]Estimate derived from the K_d of Rev for the WT RRE (1 nM [36,37]) divided by the Rev-binding ratio (see Materials and methods). Reported K_d values for the Rev–WT RRE interaction range from 0.3 to 15 nM.

Together, these results indicate that the RREs selected in the presence of neomycin B (pools N_{10} and N_{100}) were refractory to neomycin B inhibition, whereas RRE sequences selected in the absence of drug (pool N_0) retained sensitivity.

Role of the 48:71 base pair in neomycin B resistance

We next examined whether the increased resistance of the neomycin B-resistant variants is due to the substitution at positions 48:71. We used RRE variants containing the point mutations A48:A71 (RRE-AA) and C48:A71 (RRE-CA) in an otherwise WT background (Fig. 3d). In

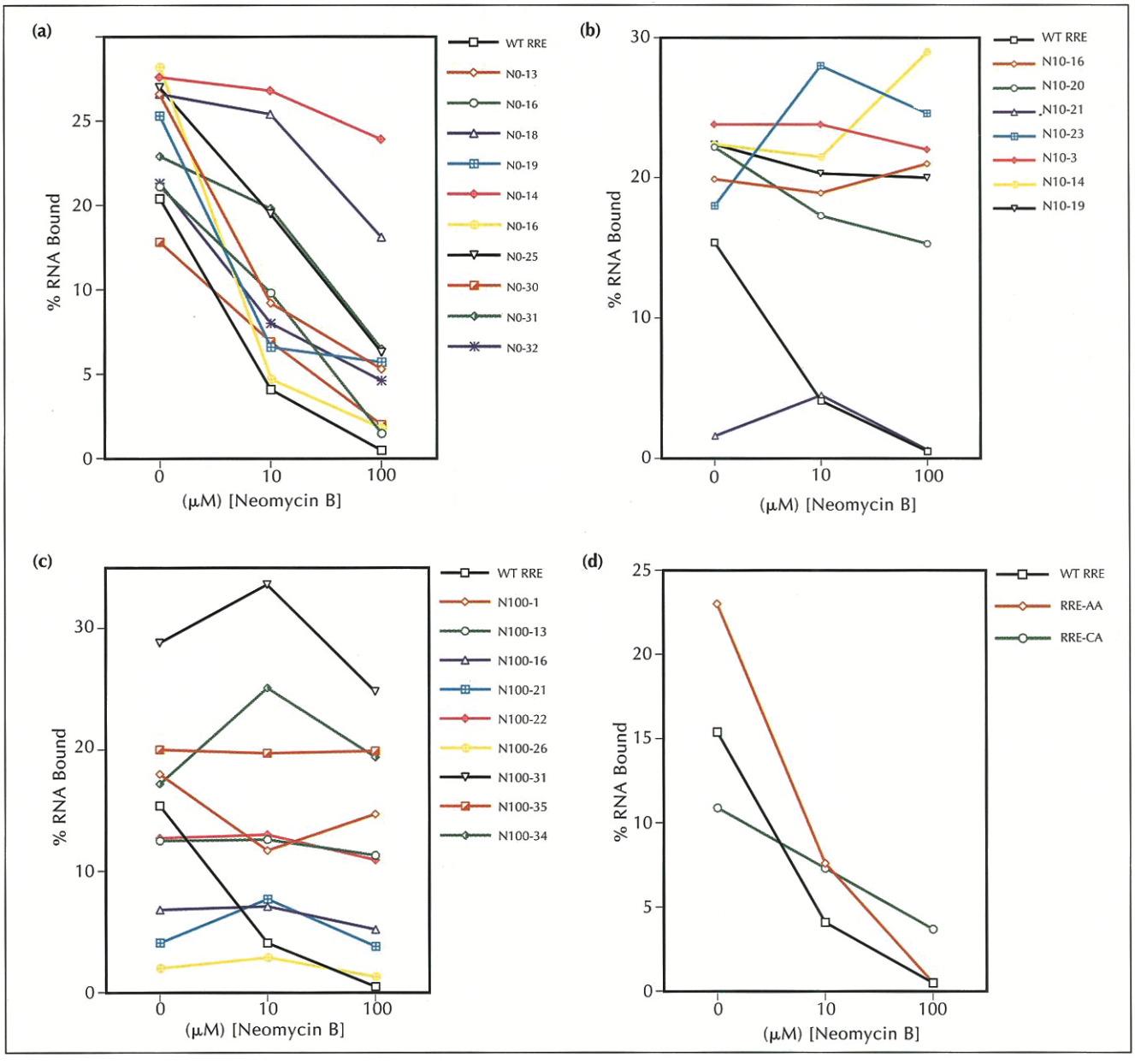

Fig. 3. Rev binding to RRE variants in the presence of 0, 10 and 100 μM neomycin B. Individual RRE variants were tested for their sensitivity to neomycin B inhibition of Rev binding. ^{32}P-labeled RRE variants were incubated with Rev and various concentrations of neomycin B. Bound RNA was isolated by nitrocellulose filter binding and quantitated by scintillation counting. Binding of the WT RRE is compared to individuals selected from **(a)** pool N_0, **(b)** pool N_{10}, **(c)** pool N_{100} and **(d)** the RRE mutants RRE-AA and CA (point mutants consisting of the WT RRE sequence with a G48:G71 to A:A or C:A substitution). Each data point represents an average of three independent experiments.

the absence of drug, Rev bound with a 1.5 fold higher affinity to RRE-AA than to the WT RRE, and RRE-CA had the lowest relative affinity for Rev. In 10 μM neomycin B, the relative affinity of Rev for RRE-AA increased only slightly — not enough to explain the predominance of A48:A71, and the lack of G48:G71, in the variants in the N_{10} pool. In 100 μM neomycin B, binding of Rev to the WT RRE and to RRE-AA was effectively blocked. Most importantly, in 100 μM neomycin B, RRE-CA retained significant Rev binding activity.

Thus, the identity of the 48:71 base pair in the wild-type background can be important for both Rev binding and neomycin B sensitivity. In particular, an A48:A71 base pair increases Rev affinity but does not greatly affect neomycin B sensitivity. A C48:A71 base pair modestly decreases affinity for Rev but dramatically decreases neomycin B sensitivity. The results of Figure 3d explain the preponderance of C48:A71 variants in the N_{100} pool (see Fig. 2).

A novel assay for quantitating neomycin B–RNA interactions

The RNA binding data (Fig. 3) clearly show that sequences outside the core region are also involved in neomycin B resistance. For example, N_0-14 contains an A48:A71 substitution but is significantly less sensitive to neomycin B than RRE-AA. Furthermore, N_{100} RNAs such as N_{100}-16,

which contains the C48:A71 base pair, and N_{100}-35, which contains the A48:A71 base pair, are more resistant to neomycin B inhibition than would be expected from this one mutation alone (compare Fig. 3c and Fig. 3d). These results suggest that neomycin B resistance can be affected by sequence elements other than the base pair at 48:71.

To test this idea directly, binding constants were determined for the interaction of neomycin B with individual RRE variants. The small size of the drug relative to RNA permits the use of an ultrafiltration technique [23–26] to separate the free and RNA-bound forms of the drug (Fig. 4). Bound and free neomycin B concentrations were quantitated by radiolabeling with [γ^{32}P]-ATP and neomycin phosphotransferase II (NPT II) [27].

Figure 5a shows that binding of drug to the WT RRE is saturable when ~80 % of the RNA is bound. Under identical conditions, very little *Escherichia coli* tRNA was bound by drug, indicating that interaction with neomycin B is specific, consistent with previous results [16]. A Scatchard plot reveals a single neomycin B binding site in the WT RRE, with a K_d of 82 nM (Fig. 5b). Consistent with this result, previous studies have shown that under some conditions neomycin B inhibits Rev binding at concentrations as low as 100 nM ([16] and data not shown), and that 100 nM neomycin B protects the RRE from chemical attack [16].

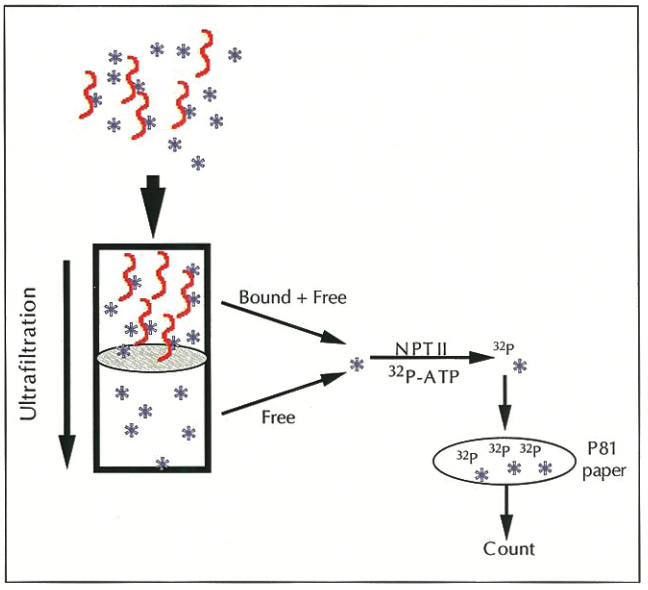

Fig. 4. Summary of the procedure used to determine neomycin B binding constants. Binding reactions containing 0–800 nM neomycin B and 0–200 nM RRE (or RRE variant) were partitioned by ultrafiltration through a 10 000 MW cut-off filter into fractions containing both free and RNA-bound neomycin B or free neomycin B alone. The neomycin B in each fraction was radiolabeled in a reaction with [γ^{32}P]-ATP and NPT II [27]. Radiolabeled neomycin B was isolated on P81 paper and quantitated by scintillation counting. The red lines represent the RRE variant RNAs and the blue asterisks represent neomycin B.

Affinity of neomycin B for WT RRE and variants

Is the increased resistance to neomycin entirely, or only partially, due to changes in the affinity of the RNA for the drug? The N_0 pool RRE variants were sensitive to neomycin B inhibition (Fig. 3a) and therefore are predicted to have a relatively high affinity for the drug. The two N_0 RNAs tested (N_0-16 and N_0-13) did indeed have a slightly higher affinity for neomycin B than the WT RRE. These variants also bound Rev with higher affinity than that of WT (estimated K_d values of 0.4 and 0.8 nM, respectively; see Table 1).

In contrast, the N_{10} pool RREs were relatively resistant to neomycin B inhibition (Fig. 3b). In these RNAs, two mechanisms for resistance were evident: N_{10}-23 and N_{10}-16 both showed somewhat higher affinity Rev binding than WT RRE, but bound neomycin B with K_d values similar to WT (Table 1). Thus, these RNAs were selected in 10 μM drug by virtue of their high affinity for Rev (K_d^{Rev} of 0.4 and 0.5 nM, respectively). In contrast N_{10}-21 had a relatively low affinity for Rev (K_d^{Rev} of 3.2 nM), most probably resulting from deletion of the base at position 71. But N_{10}-21 was even more severely compromised for binding to neomycin B (K_d = 810 nM). For all of these RNAs except N_{10}-23, the level of discrimination between neomycin and Rev was higher than that of WT.

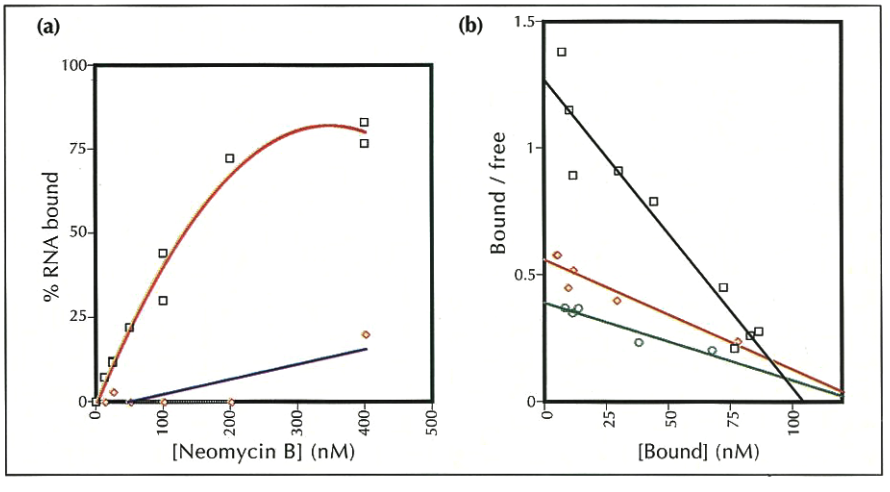

Fig. 5. Determination of the Rev–RRE binding constant. Ultrafiltration was used to separate free neomycin B from binding solutions containing the RRE variant RNA (or tRNA) and various concentrations of neomycin B. Bound and free drug concentrations were determined relative to standards using a neomycin phosphotransferase II (NPT II) assay. Representative data from a typical experiment are plotted in the form of **(a)** a saturation curve and **(b)** a Scatchard plot. The K_d values for the WT RRE (82 ± 3 nM), RRE-AA (230 ± 16 nM) and RRE-CA (320 ± 27 nM) were determined by least-squares analysis of at least four independent experiments.

In general, the neomycin B binding constants for the N_{100} RNAs were higher than those for the N_0 or N_{10} RNAs, ranging from 92 to 990 nM, and a wide range of Rev binding constants was observed (estimated K_d values of 0.4–4 nM). N_{100}-31 and N_{100}-1, which bound Rev with high affinity (estimated K_d values of 0.4 and 0.6 nM) also had the highest affinity for neomycin B (K_d values of 180 and 92 nM, respectively). The variants, N_{100}-22, 16, 21 and 26, all of which bound Rev less well than the WT RRE, also had significantly lower affinity for neomycin B (K_d values of 560, 720, 450, and 450 nM, respectively).

RRE variants selected in the presence of 100 μM neomycin B thus generally had a lower affinity for the drug than those selected in 0 or 10 μM neomycin B. Again, it was possible to see the effect of either enhancing Rev binding without decreasing neomycin binding (N_{100}-1) or decreasing neomycin binding without affecting Rev binding (N_{100}-13, 22). However, it seems to be hard to generate large decreases in neomycin binding without some change in Rev binding. For example, N_{100}-16, 21, 26 and N_{10}-21 all seem to have paid a price in Rev affinity for their decreased neomycin affinity.

The Scatchard data suggested that all but two of the RRE variants contain a single neomycin B binding site. These two exceptions, N_{100}-16 and N_{100}-22, had a relatively low affinity for neomycin B and, when the drug concentration was high enough for binding to be observed, more than one molecule of neomycin B was seen to bind (see Fig. 6). We imagine that at these high concentrations binding of the drug is relatively non-specific.

A G48:G71 substitution increases neomycin B affinity

The RRE-AA and RRE-CA point mutants show three- to four-fold decreases in binding to neomycin compared to wild-type (Table 1), while the selected mutants containing the A:A base pair have affinities ranging from three-fold higher than wild-type to two-fold lower, and the C:A mutants range from 1.5 to 11-fold lower than wild-type. Once again, it is clear that the identity of the 48:71 base pair is not the only factor that determines affinity for neomycin B. To determine the relative importance of this base pair compared to the other changes in the variant sequences, we constructed two additional variants. N_{100}-22GG contains a G48:G71 base pair in the N_{100}-22 background, instead of the selected C:A base pair, and N_{100}-26GG contains a U48:G71 to G:G substitution within the N_{100}-26 background. In the N_{100}-22 variant the C48:A71 to G:G substitution decreased the K_d for neomycin B from 720 nM to 110 nM. In the N_{100}-26 background, the U:G to G:G change decreased the K_d for neomycin B from 450 nM to 60 nM (Table 1). Mutations in this base pair, in either of these variant backgrounds, can thus cause a change in the affinity of the RNA for neomycin B of about seven-fold. This one change can account for essentially all of the differences in neomycin affinity between the variants and wild-type RRE. As expected from these data,

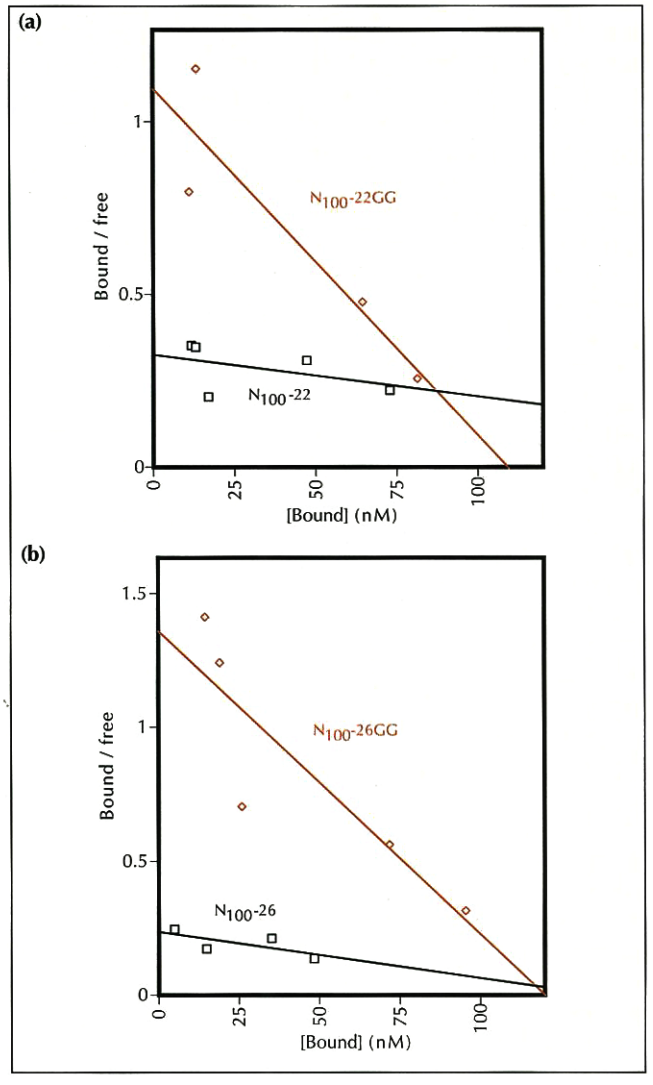

Fig. 6. Representative Scatchard plots of neomycin B binding to N_{100} RRE variants containing a G48:G71 substitution. A G48:G71 base pair was introduced into the N_{100} pool variants, N_{100}-22 (formerly C:A) and N_{100}-26 (formerly U:G). This single base pair substitution shifts the K_d; **(a)** from 720 ± 190 nM (N_{100}-22) to 110 ± 10 nM (N_{100}-22GG) and **(b)** from 450 ± 60 nM (N_{100}-26) to 60 ± 7 nM (N_{100}-26), respectively.

N_{100}-22GG and -26GG are sensitive to drug inhibition (data not shown).

The change in the 48:71 base pair in these variant backgrounds has a negligible effect on Rev binding (Table 1). Significantly, although variant N_{100}-22 seems to bind neomycin B at multiple sites (see above), N_{100}-22GG has a single, higher affinity binding site, consistent with the notion that a purine–purine base pair is an important component of the high-affinity neomycin-binding site.

Discussion

Neomycin B is an aminoglycoside antibiotic that competitively inhibits binding of Rev to the RRE [16]. We have used *in vitro* selection to evolve neomycin B-resistant RRE variants from a diverse sequence pool. Selection is based on the ability of the RNA to bind to

Rev, with the additional selective pressure that the RNA variants must bind Rev in the presence of a drug that normally blocks this interaction.

Each of the three selected RNA pools (N_0, N_{10}, N_{100}) contain distinct sequence characteristics. RRE variants selected in the absence of neomycin B (N_0 pool) contain the WT G48:G71 or the isosteric A48:A71 base pair. The majority of the variants selected in 10 μM drug (N_{10}) contain A48:A71, while those selected in 100 μM drug (N_{100}) predominantly contain an A48:A71 or C:A base pair at this position. The context in which the base pair is found is also important for both Rev and neomycin B affinity. These effects could result from the disruption or formation of direct contacts between the RNA and the protein (or drug) or from indirect effects of RNA structure.

Some of the variants from the N_{10} and N_{100} pools contain regions of non-WT homology, in particular at positions 83–87, that are not found in N_0 RNAs. For example, several of the N_{10} variants (N_{10}-3,6,16,21) contain the sequence U84G85, whereas N_{100} variants (N_{100}-20,29,34,35) contain the sequence UGGG (83–86) and variants N_{100}-16,22,32b contain GGUGG (83–87). These bases are located in the loop region of stem–loop IIC. Molecular simulation modeling predicts that, in the most stable conformation, RRE domain II stem–loop IIC and IIB are stacked [28]. This model is consistent with the idea that bases in stem–loop IIC may affect binding to the RRE core element.

A comparison of Rev and neomycin B binding to the RRE variants from each of the three pools indicates that each pool has distinct protein- and drug-binding characteristics. Variants selected in the absence of drug bound to Rev with high affinity but retained their sensitivity to neomycin B. In fact, the affinities of N_0-13 and N_0-16 for neomycin B are higher than that of the WT RRE. It is possible that high affinity Rev binding sites possess characteristics that also facilitate drug binding. Selection in the presence of 10 μM neomycin B favored sequences that, generally, bound Rev with a higher affinity than the WT RRE. These RNAs typically contain an A48:A71 base pair. One exception is N_{10}-21, which has a deletion of base 71 and binds both Rev and neomycin B poorly. Most of the N_{100} variants showed decreased neomycin B binding affinities, however, suggesting that, for selection in the presence of 100 μM neomycin B, a high affinity for Rev is insufficient.

High affinity Rev binding sites have been identified by several groups [20,21,29]. In general, the results suggest a requirement for a destabilized stem or bulge region that widens the major groove, making it more accessible to Rev [7]. The requirements for neomycin B binding are less clear although, like Rev, it seems to prefer a bulged-helical region in which the major groove is widened [30]. CD spectra and RNA footprinting experiments of the neomycin B–RRE complex suggest that the drug binds within the major groove (L. Ratmeyer *et al.*, personal communication, and [16]). Due to its small size, the drug is probably limited to relatively few contacts with the RRE. Therefore the substitution of a critical base pair is predicted to have a greater effect on drug than on protein binding. This is consistent with the finding that neomycin B binding is restored to WT levels by a G48:G71 substitution in the N_{100}-22 and N_{100}-26 RREs.

Neomycin B binds to a number of RNAs in addition to the HIV RRE, including, prokaryotic ribosomal RNA [31], group 1 introns [32] and hammerhead ribozymes [33]. Artificial, high affinity neomycin-binding RNAs have been selected from random RNA pools ([30], G.W. and M.R.G., in preparation). Together, these studies suggest that neomycin B can recognize and bind to a wide variety of RNAs that seem to be unrelated in terms of primary sequence. Our results suggest that neomycin B recognizes the primary as well as the secondary and/or tertiary structure of RNA. Substituting A48:A71 for the WT G:G base pair in the WT background increased the K_d for neomycin B 2.8-fold, suggesting that the drug may directly contact these residues, and does not merely recognize the structure of the bulged backbone that results from the purine–purine base pair. This is consistent with the observation that neomycin B protects the N1 and/or N2 positions of G48 in RNA footprinting experiments [16].

Significance

We have probed the ability of Rev to interact with RRE variants that are insensitive to a potential anti-viral drug therapy. Our data suggest that neomycin B recognizes and binds to an RNA sequence (or structure) similar to that bound by Rev. Thus, variants that are highly neomycin B-resistant generally show decreased affinity for Rev. This may compromise replication of a virus harboring a neomycin B-resistant RRE variant. Additional constraints may further limit the acquisition of drug resistance. For example, two known constraints are maintenance of a functional *env* open reading frame and the conservation of RRE sequences that interact with cellular factors required for Rev and other functions [34].

This study shows that an *in vitro* genetic selection technique can be used as a model to measure the emergence of drug resistance. This approach is especially useful in the study of highly mutable viruses such as HIV and could be applicable to other potential drug targets. The ability to make predictions about how a target molecule might mutate to become resistant to a potential therapeutic could further our understanding of the basis of the interaction in question and assist in the rational design of newer, more effective drugs.

Materials and methods

Protein and RNA

Rev protein was over-expressed and purified from *E.coli* as described by Zapp *et al.* [35]. Construction of the initial DNA pool containing 10^{13} dRRE domain II variants (pool 0) is described in Bartel *et al.* [20]. The WT RRE domain II was transcribed from the *Hind*III linearized plasmid pGEMZ-94.7 [16]. RRE point mutant RRE-AA, -CA, N_{100}-22GG and N_{100}-26GG were synthesized as single-stranded DNA oligonucleotides. They were PCR amplified with flanking primers and transcribed using T7 RNA polymerase.

In vitro *RNA selection*

Binding reactions for selection were set up as follows: Rev, tRNA and the appropriate concentrations of neomycin B (ICN) were pre-incubated at room temperature (RT) for 10 min in 25 μl of 40 mM Tris, pH 7.9, 50 mM KCl, and 1 mM dithiothreitol (DTT) (binding buffer, BB). Pool RNA in 25 μl of BB was incubated at 80 °C for 3 min and then at RT for 7 min. The RNA and protein/drug solutions were then mixed together (giving final concentrations of 30 nM Rev, 0.1 mg ml^{-1} tRNA, 0.4 μM RRE pool in 50 μl BB) and incubated 1 h at 30 °C. Rev bound RNA was separated from unbound sequences by nitrocellulose filter binding (selection rounds 1,2,3,5,7,9) or by mobility shift (selection rounds 4,6,8).

In the filter-binding assay the mixtures were passed through HAWP 025 00 nitrocellulose filters (Millipore) under vacuum. The filters were washed twice with BB. RNA was eluted in PK Buffer (100 mM Tris pH 7.5, 12.5 mM EDTA, 150 mM NaCl, 1 % SDS) at 65 °C for 40 min. Eluents were phenol/chloroform extracted, ethanol precipitated and then RT-PCR amplified with the primers d40.50 and d22.18 as previously described [20]. To separate bound RNA using the mobility shift assay binding reactions were loaded onto a 5 % polyacrylamide gel (30:1) and run at 250 V for 2.5 h in 0.5 x Tris-borate-EDTA. Shifted bands were resolved by autoradiography, cut out and eluted in PK buffer while shaking for 1 h at room temperature (RT), then amplified as described above.

RRE pools were digested with *Eco*RI and *Hind*III and cloned into the similarly cut pGEM7Zf+ (Promega) vector. Individual clones were sequenced from a T7 primer site within the vector. To transcribe the cloned RRE variants the plasmid was first linearized with *Hind*III and then transcribed with T7 RNA polymerase.

Rev binding affinity and neomycin B sensitivity of individual clones

Binding experiments containing final concentrations of 30 nM Rev, 0.1 mg ml^{-1} *E.coli* tRNA and 0.5 nM WT RRE or RRE variant in BB were incubated for 1 h at 30 °C. Rev-bound RNA was separated using the filter binding assay (described above). Filters were dried and bound RNA was quantitated by scintillation counting. The data were expressed in terms of a Rev-binding ratio which is equivalent to the amount of RRE variant bound divided by the amount of WT RRE bound, then converted to estimated K_d values for Rev using a literature value of 1 nM [36,37] for the WT K_d^{Rev}. Neomycin B sensitivity was determined in similar binding experiments containing 0, 10 or 100 μM neomycin B.

RRE point mutants

The RRE point mutants RRE-AA and RRE-CA were synthesized as oligodeoxyribonucleotides containing the 67-nucleotide sequence from the WT RRE domain II flanked by the primer binding sequences TGGGAGCAGCAGGAA and TATAGTGCAGCAGCAG at the 5' and 3' ends, respectively. These oligonucleotides were PCR amplified with the primers G**TAATACGACTCACTATA**GGGAATTCTTGGGAGCAGCAGGAA and GCAAGCTTCTGCTGCTGCACTATA. The PCR products are then transcribed from the T7 promoter (bold). The point mutants N_{100}-22GG and N_{100}-26GG were constructed in a similar way but using a 67-nucleotide sequence corresponding to those of N_{100}-22 and N_{100}-26, respectively.

K_d determination

Binding reactions containing 0–200 nM unlabeled RRE (or RRE variant) RNA, and 0–800 nM neomycin B were incubated at RT for 1 h in 100 μl solutions containing 10 mM Tris pH 7.6, 10 mM $MgCl_2$, 50 mM NH_4Cl and 1 mM DTT. Free neomycin B was separated from RNA-bound drug by spinning the solution for 30 s in a Microcon 10 filter (Amicon). Three to ten μl of the flow through (containing free drug) or the retentate (containing free and bound drug) were incubated in 21 mM Tris malonate pH 7.1, 130 mM NH_4Cl, 12.5 mM $MgCl_2$, 0.5 mM DTT, 8 pmoles (50 μCi) [γ-^{32}P]ATP, 1 ng neomycin phosphotransferase II (NPT II) (5 prime-3 prime Inc., [27]) in a 50 μl solution. Reactions were incubated at 37 °C for 15 min and then 90 °C for 5 min. Solutions were extracted with one volume of phenol/chloroform and 3–10 μl of the aqueous phase was spotted onto Whatmann P81 paper. The P81 paper was then washed in ddH_20 at 90 °C for 5 min followed by 3 washes in ddH_20 at RT. The paper was dried and counted. Drug concentration was quantitated by scintillation counting relative to standards. Bound and free neomycin B concentrations were determined and the data plotted as a Scatchard plot.

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