Bacteriophage P22 Antitermination boxB Sequence Requirements Are Complex and Overlap with Those of \( \lambda \)

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Transcription antitermination in phages \( \lambda \) and P22 uses N proteins that bind to similar boxB RNA hairpins in regulated transcripts. In contrast to the \( \lambda \) N-boxB interaction, the P22 N-boxB interaction has not been extensively studied. A nuclear magnetic resonance structure of the P22 N peptide boxB_{left} complex and limited mutagenesis have been reported but do not reveal a consensus sequence for boxB. We have used a plasmid-based antitermination system to screen boxBs with random loops and to test boxB mutants. We find that P22 N requires boxB to have a GNRA-like loop with no simple requirements on the remaining sequences in the loop or stem. U:A or A:U base pairs are strongly preferred adjacent to the loop and appear to modulate N binding in cooperation with the loop and distal stem. A few GNRA-like hexaloops have moderate activity. Some boxB mutants bind P22 and \( \lambda \) N, indicating that the requirements imposed on boxB by P22 N overlap those imposed by \( \lambda \) N. Point mutations can dramatically alter boxB specificity between P22 and \( \lambda \) N. A boxB specific for P22 N can be mutated to \( \lambda \) N specificity by a series of single mutations via a bifunctional intermediate, as predicted by neutral theories of evolution.

\( \lambda \) and P22 share a closely related system of regulating the expression of early lytic genes by allowing transcription past terminators in the P_{left} and P_{right} operons (47). The recognition of the boxB RNA hairpins of nut (\( N\)-utilization) sites by the binding domains of the viral N proteins (Fig. 1A, B, and C) initiates the assembly of an antitermination complex. This complex contains N, host factor NusA, RNA polymerase, and other host factors bound to the viral nut site boxB and the nonhairpin boxA, allowing transcription to proceed through downstream transcription termination signals (32). The four wild-type nut sites of \( \lambda \) and P22 are similar in sequence but differ even between boxB_{left} and boxB_{right} in the same virus. Notably, both P22 boxBs have a C in the loop where both boxBs have an A, P22 boxB stems are longer than those of \( \lambda \) by 1 base pair, and P22 boxB_{left} appears to have a noncanonical C:C base pair. boxBs bind noncognate N peptides poorly in vitro (2, 8, 44). Likewise, noncognate N-nut interactions do not function in vivo (14, 28), and noncognate N proteins do not rescue N-deficient viruses (10).

The details of the \( \lambda \) N-boxB interaction have been revealed by extensive genetic and biochemical work (47) and by solution state nuclear magnetic resonance (NMR) structures of the arginine-rich domain of the N protein bound to boxB_{left} (29) and boxB_{right} (39) RNA. Genetic and biochemical studies of the P22 interaction are less complete (13, 44) but are supported by the solution state NMR structure of the arginine-rich domain of the N protein bound to P22 boxB_{right} (5).

\( \lambda \) and P22 boxBs bind their N peptides as hairpins in which 4 of 5 bases adopt a GNRA-like tetraloop structure (Fig. 2). Tetraloops are frequently found in RNAs serving structural roles as stable caps to stems and as motifs recognized by proteins; GNRA tetraloops are noted as particularly frequent and thermodynamically stable (9). The \( \lambda \) and P22 boxB loops adopt distinct conformations: the fourth nucleotide (nt) of the boxB tetraloop is extruded from the GNRA tetraloop (4-out), while in the P22 boxB it is the third nucleotide that is extruded (3-out). As in the canonical GNRA tetraloop, there is a non-standard, sheared G:A base pair and extensive stacking. The bound peptides adopt helical conformations, occupy the major grooves, and make contacts with boxB stems and loops.

NMR data support a model in which the N peptides recognize a specific conformation of boxB, with little direct recognition of the sequence (5, 29, 39). Most observed contacts are not sequence specific but are made to the backbone or are hydrophobic in nature. The only certain, base-specific hydrogen bonds detected by NMR are to the guanine of the standar, sheared G:A base pair at the base of the loop. No other proposed peptide-base hydrogen bond is supported by mutagenesis. Cai et al. (5) report possible hydrogen bonding between a P22 N lysine and the base pair adjacent to the loop of P22 boxB_{left}. Legault et al. (29) propose hydrogen bonds between the \( \lambda \) N peptide and a base adjacent to the loop of \( \lambda \) boxB_{left}, while Scharpf et al. (39) have evidence only for hydrogen bonds to the guanine of the sheared G:A base pair of \( \lambda \) boxB_{left}. \( \lambda \)-P22 specificity may reflect conformational energetics, likely governed by subtle base-stacking interactions.

The regulatory role of RNA-protein recognition in important biological systems has elicited much interest (16). The diversity of known RNA-protein complexes raises the question as to which evolutionary mechanisms are capable of finding new recognition strategies. Kimura’s neutral theory of molecular evolution contends that for any given genotype, there are sufficient mutants of neutral fitness to create smooth paths to different phenotypes (26). Though computational modeling of evolving RNA secondary structure strongly supports neutral theories (21, 38, 45), few experimental tests using biologically
active RNAs have been reported. Studies of small hairpin RNAs that bind arginine-rich peptides have shown that single substitutions and base pair changes are enough to create changes of specificity (23, 41), suggesting that neutral paths between distinct activities can be found when sequences are small. In the case of protein-binding RNAs, mutations leading from relaxed-specificity sequences to sequences with distinct binding preferences would be equivalent to speciation.

We examined the requirements of boxB recognition by P22 N using a plasmid-based reporter system that reconstitutes antitermination in *Escherichia coli* (14). boxBs active with P22 N were obtained from two randomized loop libraries. Assays of selected and designed boxBs reveal that P22 N recognition requires a GNRA boxB pentaloop to form double strands with PstI and BamHI overhangs to replace the entire nut site, such that differences between reporters were confined to boxB. boxB cloning oligonucleotides were designed to form double strands with PstI and BamHI to replace the entire nut site of P22 N fusion plasmid. Each reporter plasmid was constructed with the boxB of interest replacing only the boxB of the parent plasmid. boxB reporter plasmids were confirmed through sequencing.

**MATERIALS AND METHODS**

**General.** Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were obtained from Roche (Germany). Bacterial-medium components were obtained from Oxoid (United Kingdom). Fine chemicals were obtained from Amersham (United Kingdom), Sigma (United States), and Amresco (United States).

**Bacterial strains, plasmids, and viruses.** *Escherichia coli* (NC_001416) and P22 (accession no. NC_002371) were obtained from GenBank. Each reporter plasmid was constructed with the boxB of interest replacing only the boxB of the parent plasmid. boxB cloning oligonucleotides were designed to form double strands with PstI and BamHI to replace the entire nut site of P22 N fusion plasmid. Each reporter plasmid was confirmed through sequencing.

**Construction of boxB reporter and library plasmids.** Wild-type sequences of lambda (accession no. NC_001416) and P22 (accession no. NC_002371) were obtained from GenBank. Each reporter plasmid was constructed with the boxB of interest replacing only the boxB of the parent plasmid. boxB cloning oligonucleotides were designed to form double strands with PstI and BamHI to replace the entire nut site of P22 N fusion plasmid. Each reporter plasmid was confirmed through sequencing.

**DNA preparation and sequencing.** For each boxB reporter, mixtures of four or more minipreps were separated into lambda N, P22 N, and HIV Rev N strains of *E. coli*, and minipreps were selected for each construct.

**DNA preparation and sequencing.** For each boxB reporter, mixtures of four or more minipreps were separated into *lambda N*, P22 N, and HIV Rev N strains of *E. coli*, and minipreps were selected for each construct.
We first confirmed N-boxB activity and specificity using a two-plasmid reporter system of Franklin (14). This reporter system reconstructs λ N-mediated antitermination in E. coli and has extensively been used for the study of λ N-boxB interaction and heterologous RNA-protein interactions. Four wild-type boxB reporters (Fig. IA) were constructed in which λ and P22 boxBs were presented in the context of the λ nutleft sites to avoid influence from variations in boxA and the remainder of the nut sites (Fig. IB). λ N was expressed as the full-length protein, whereas the P22 N RNA-binding domain was expressed as a fusion to the λ activation domain (Fig. IC). The P22 N fusion limited the differences between N proteins to the well-characterized RNA-binding domains and avoided the high level of background activity displayed by a full-length, wild-type P22 N construct (15; data not shown). The P22 N fusion supplier was tested and found to have low activity on a heterologous HIV RRE nut reporter and strong activity on its cognate boxB reporters as expected, though its absolute activity was substantially less than that of the λ N supplier. The P22-λ N fusion supplier plasmid rescues the P22 N-null phase (λimm) [P22-Nam24 (13)] but not the λ N-null phase (λNam7 am53 [13]; data not shown). Thus, while the P22 N fusion activity likely directly reflects boxB-peptide affinity, the lambda N activity likely reflects the ability of the boxB loop to bind lambda N in the proper 3-out conformation that recruits NusA and allows optimal antitermination (49). The P22-λ N fusion supplier was used for all further experiments.

We measured the activities of the four wild-type boxB reporters with the λ N supplier pBR-pE-λN and with the P22 N fusion supplier pBRNP22Na using a solution assay with the β-galactosidase reporter gene (31) (Table 1). The boxBs display specificity for their cognate N proteins; the P22 boxBlight reporter displays very high specificity. In vitro experiments indicate substantial affinity between λ boxBlight and the P22 N peptide (44) and between P22 boxBlight and the λ N peptide (7). Other work in vivo shows that λ boxBlight has significant activity with P22 N in vivo (13, 19) and that P22 boxBlight is highly specific (13, 28). Despite the uniform λ context of the boxB-peptide interaction, the specificities displayed by our boxB reporters largely agreed with published data, though there were unresolved discrepancies between published boxB-N peptide specificities in vitro (2, 7, 43, 44).

Screening of a boxB library. In order to reveal the loop consensus for P22 boxB, we constructed a plasmid library

### Table 1. Cognate N-boxB antitermination activities

| Reporter | Sequence<sup>a</sup> | β-Galactosidase units<sup>b</sup> | % of cognate's activity<sup>c</sup> |
|----------|----------------------|---------------------------------|----------------------------------|
| λ boxB<sub>left</sub> | GCCCU GAAAG AAGGC | 9,400 ± 2,100 | 24 ± 7.2 | 4.5 ± 0.47 | 110 | 25 | 4.8 |
| λ boxB<sub>right</sub> | GCCCU GAAAG AAGGC | 8,300 ± 1,000 | 29 ± 2.9 | 4.2 ± 1.6 | 100 | 30 | 5.4 |
| P22 boxB<sub>left</sub> | UGGCCU GACAA AGGCG | 1,600 ± 290 | 97 ± 4.0 | 5.1 ± 0.93 | 20 | 100 | 5.5 |
| P22 boxB<sub>right</sub> | ACCGCC GACAA AGGGU | 3.5 ± 0.43 | 97 ± 8.2 | 3.3 ± 0.53 | 0.04 | 100 | 3.8 |
| HIV-1 RRE IIB | -UGGCCCACA--UAGGCAAUA- | 3.5 ± 0.30 | 37 ± 0.55 | 93 ± 28 | 0.04 | 3.8 | 100 |

<sup>a</sup> All RNAs were assayed as boxB replacements in the context of the λ nut<sub>left</sub> site. Gaps flanking the loop are for clarity, and only the high-affinity site of the RRE boxB replacement is shown.

<sup>b</sup> β-Galactosidase units were assayed from saturated cultures in tryptone medium and with continuous 0.5 mM IPTG induction. Values represent averages ± standard deviations for three or more experiments.

<sup>c</sup> The percentage of the cognate's activity is calculated as the activity of the reporter relative to that of the cognate reporter (λ boxB<sub>left</sub> for wild-type λ N, P22 boxB<sub>left</sub> for P22 N, and HIV-1 RRE for Rev N).

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**RESULTS**

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We measured the activities of the four wild-type boxB reporters with the λ N supplier pBR-rtac-λN and with the P22 N fusion supplier pBRNP22Na using a solution assay with the β-galactosidase reporter gene (31) (Table 1). The boxBs display specificity for their cognate N proteins; the P22 boxBlight reporter displays very high specificity. In vitro experiments indicate substantial affinity between λ boxBlight and the P22 N peptide (44) and between P22 boxBlight and the λ N peptide (7). Other work in vivo shows that λ boxBlight has significant activity with P22 N in vivo (13, 19) and that P22 boxBlight is highly specific (13, 28). Despite the uniform λ context of the boxB-peptide interaction, the specificities displayed by our boxB reporters largely agree with published data, though there are unresolved discrepancies between published boxB-N peptide specificities in vitro (2, 7, 43, 44).

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colonies were picked, pooled, and used for DNA preparation. Five clones were assayed the same day.

The boxBleft. The library pool was sequenced and showed no bias in the presence of P22 N divided by the boxBleft units in the presence of Rev N.

The percentage of P22 activity is normalized to the activity of P22 boxBleft assayed the same day.

Based on P22 boxBleft where the 7 nt comprising the pentaloop and adjacent base pair were randomized. The NMR structure of P22 boxB bound to the P22 N peptide reveals boxB adopting a 3-out GNRA-like pentaloop, with a role for the boxBleft stem.

Only GNRA-like pentaloops are found in a randomized loop library. As expected of GNRA-like pentaloops, all selected sequences contained a G1:C5 pair (Table 2). All four nucleotides occurred at positions 2, 3, and 4, violating a strict GNRA-like consensus, assuming that all boxBs adopt the expected 3-out conformation when bound. Purines are common at positions 2 and 4 in strongly active boxBs, suggesting that base-stacking interactions are important. In the P22 boxBleft NMR structure (5), A4 fulfills the purine role with an intramolecular hydrogen bond, but no prior mutagenesis in boxB had indicated its importance. Consistent with these data, GNRA-like pentaloops are known to exist without a purine in this role (12, 42).

A series of mutant boxBs were constructed to explore the determinants of P22 N activity (Table 3). The A2→G mutation of P22 boxBleft (clone D1) does not reduce activity, as was expected from published in vitro data (44). The C3→A mutation (D2) reduces activity to 85%, while a G2→A double mutation (D3) reduces activity to 36%. In contrast, C3→A A1→G mutations (D4) maintain moderate to wild-type activity, as was also expected from published data (44). The low frequency of positive sequences, their similarity to GNRA tetraloops, and the lack of any observed hydrogen bonds between the peptide and bases are consistent with a model where the ability of boxB to adopt the required conformation depends on favorable base-stacking interactions (24), as well as activity on X-Gal plates. Nineteen clones with activities similar to that of P22 boxBleft were sequenced, yielding 14 unique sequences. These were assayed for antitermination (Table 2). Neither P22 boxBleft nor any sequence resembling P22 boxBright was recovered, indicating that our screen was not exhaustive. Indeed, only 1 of the 14 selected was a boxBleft point mutant.

### TABLE 3. Activities of designed boxB mutants

| Clone | boxB sequence | β-Galactosidase units | Fold activation | % of P22 boxBleft activity |
|-------|--------------|-----------------------|----------------|--------------------------|
|       |              | P22 N | Rev N            |               |                          |
| D1    | UGCCGCU GCGCGG | 120±9.2 | 6.8±0.55 | 18±1.4 | 100                     |
| D2    | UGCCGCU GCGCGG | 90±11  | 11±0.52 | 8.3±0.98 | 85                     |
| D3    | UGCCGCU GCGCGG | 38±7.4 | 9.5±1.4 | 4.0±0.78 | 36                     |
| D4    | UGCCGCU GCGCGG | 87±15.1 | 11±1.0 | 7.9±1.4 | 82                     |
| D5    | UGCCGCU GCGCGG | 110±4.3 | 8.0±0.32 | 14±0.54 | 100                    |
| D6    | UGCCGCU GCGCGG | 38±9.4 | 4.2±0.26 | 9.0±2.2 | 64                     |
| D7    | UGCCGCU GCGCGG | 7.9±1.1 | 4.2±0.10 | 1.9±0.27 | 13                     |
| D8    | UGCCGCU GCGCGG | 11±0.41 | 3.9±0.21 | 2.9±0.11 | 14                     |
| D9    | UGCCGCU GCGCGG | 11±1.7 | 4.2±0.76 | 2.7±0.39 | 15                     |
| D10   | UGCCGCU GCGCGG | 4.4±0.17 | 3.5±0.28 | 1.2±0.05 | 5.7                    |
| D11   | UGCCGCU GCGCGG | 5.7±0.94 | 3.8±0.43 | 1.5±0.25 | 7.3                    |
| D12   | UGCCGCU GCGCGG | 3.3±0.17 | 4.1±0.22 | 0.8±0.04 | 4.3                    |
| D13   | UGCCGCU GCGCGG | 140±9.7 | 3.4±0.12 | 41±2.9 | 140                   |
| D14   | UGCCGCU GCGCGG | 110±22 | 3.4±0.61 | 31±6.4 | 110                   |
| D15   | UGCCGCU GCGCGG | 30±6.7 | 3.1±0.53 | 9.8±2.2 | 31                     |

* a Reporters are designed boxB replacements in the context of the λ mutS site.

* b Gaps flank the loop; underlining indicates nucleotides different from those of P22 boxBleft.
hydrophobic interactions with the N protein (4) and ancillary factors (49).

A:U and U:A are preferred adjacent to the loop. The absence of a Watson-Crick base pair adjacent to the loop of P22 boxB right suggests either a tolerance of nonpaired bases or C:C pairing. The loop of phage 21 boxB adopts a structurally related conformation, and NMR-based molecular modeling suggests C:C pairing (7). Our library's random region encompassed these positions in order to examine their importance. Only U:U (wild type) and A:U base pairs were found in strongly positive clones, while the only boxB with a C:G base pair had reduced activity (Table 2, reporter L13). To address the roles of the bases adjacent to the loop, we constructed a series of boxBs with substitutions at these positions (Table 3, D5 to D12). The placement of A:U into boxB left did not reduce activity (D5), though it did affect boxB right (D6, D7). Interestingly, C:C, C:G, G:C, and wobble pair replacements strongly reduced activity to 15% or less in the context of boxB left (D8 to D12).

How does boxB right tolerate the apparent C:C pair? The U:A substitution adjacent to the loop of boxB right increased activity (D13), indicating that U:A is also preferred in this context and suggesting that the two base pair differences between boxB left and boxB right at the beginning of the stem contribute to boxB right activity. The separate placement of two boxB right base pairs into a boxB left context (D14, D15) increased and decreased activity, respectively. These data suggest that the contiguous stacking from the beginning of the stem through the loop allows distal mutations to modulate the backbone conformations required for binding P22 N.

Screening a hexaloop library yields few active boxBs. Since the GNRA motif is found imbedded in larger loops (1), we constructed a library in which the 5-nt loop of boxB left was replaced with 6 random nt. This library should contain 4,096 unique sequences. Approximately 10,000 colonies were screened in N567 P22 N on X-Gal plates, and 287 blue colonies were picked, pooled, and screened against Rev N. Twelve colonies of the negative clones (90%) were collected and sequenced, yielding eight unique sequences. Of these, three were hexaloops and five were pentaloops. The unselected library pool was sequenced and showed no significant contamination with random pentaloops (data not shown), suggesting that active hexaloops are far rarer than pentaloops. Interestingly, the three selected hexaloops are all related by single insertions into high-activity boxBs (Table 4). A designed hexaloop (D16) and heptaloop (D17) were found to have minimal activity. We speculate that larger loops are more likely to accommodate extruded bases where they sterically hinder protein binding. Indeed, two hexaloops and one heptaloop with structures resembling GNRA tetraloops (20) found in a Haloarcula marismortui large ribosomal subunit (27) accommodated the extra nucleotides where they would interfere with N protein binding.

Mutations alter boxB specificity toward P22 N and λ N. The diversity of boxB variants active with P22 N suggested that some may also function with λ N. Though the recognition strategies of P22 and λ N are distinct, the origin of specificity is not obvious, and some sequences might be able to participate in both recognition strategies. Tan and Frankel (44) reported a boxB (equivalent to D4 here) that binds both P22 N and λ N peptides in vitro. We first designed and assayed some additional reporters with λ boxB stems (Table 5), which showed that the boxB stem did not abrogate P22 N activation. We measured the activities of all described boxB reporter with P22 and λ N proteins (Table 6). Using the ratio of the percentage of P22 N to the percentage of λ N activity to represent specificity, a wide range of specificities is apparent (Fig. 3A). While a few boxBs had higher specificity to λ N than to wild-type λ boxBs, none were found to be more specific to P22 N than to P22 boxB left. Possibly because most variants were in a P22 boxB left context and P22 boxB right has extremely high specificity. Remarkably, many boxBs have relaxed specificity and maintain at least moderate activity. Several boxBs selected for P22 N activity have more than 50% activity with λ N, suggesting that bifunctional boxBs may be relatively common.

Stem and loop position 3 make a clear contribution to specificity. To understand the basis for boxB specificity, nucleotide changes in different boxB contexts were examined. To test whether N left and λ N stems are distinct, the origin of specificity is not obvious, and some sequences might be able to participate in both recognition strategies. Tan and Frankel (44) reported a boxB (equivalent to D4 here) that binds both P22 N and λ N peptides in vitro. We first designed and assayed some additional reporters with λ boxB stems (Table 5), which showed that the boxB stem did not abrogate P22 N activation. We measured the activities of all described boxB reporter with P22 and λ N proteins (Table 6). Using the ratio of the percentage of P22 N to the percentage of λ N activity to represent specificity, a wide range of specificities is apparent (Fig. 3A). While a few boxBs had higher specificity to λ N than to wild-type λ boxBs, none were found to be more specific to P22 N than to P22 boxB left. Possibly because most variants were in a P22 boxB left context and P22 boxB right has extremely high specificity. Remarkably, many boxBs have relaxed specificity and maintain at least moderate activity. Several boxBs selected for P22 N activity have more than 50% activity with λ N, suggesting that bifunctional boxBs may be relatively common.
that the purine role can be fulfilled by C3 in the 4-out GNRA-like pentaloop (6, 11). We cannot account for these discrepancies, but note that we have used a different reporter system and that under higher /H9261N expression, Doelling and Franklin report that the /H9261A3C boxBright has moderate activity (11). In all other cases where comparisons can be made, our results agree well with published data. Interestingly, changing from C3 to A3 in every context shifted specificity from P22 to /H9261N activity, sometimes without a loss of the activity of one or the other N (Fig. 3A).

Published reports indicate that /H9261N is tolerant of mutations in the boxB stem so long as most of the base pairing is preserved (6, 11, 44). We find that exchanging a P22 boxBleft stem with that of /H9261boxB shifted specificity toward /H9261N, again without a necessary loss of activity of one or the other N (Fig. 3B). These coordinate shifts suggest that position 3 and the stem directly contribute to specificity. In contrast, though U:A-to-A:U changes adjacent to the loop are tolerated by P22 N and /H9261 N in their cognate boxBs, the base pair switch causes no consistent alteration of specificity in other contexts (Fig. 3C). These data suggest that this base pair has a complex role coupled with the stem and loop sequence.

**Single-nucleotide mutations can switch specificity between P22 and /H9261 N.** Point mutants of wild-type boxBs have a wide range of specificities, including relaxed specificity (Fig. 3D). Relaxed-specificity boxBs suggest that transitions between P22

### TABLE 6. Specificities of boxB reporters with P22 N and /H9261 N

| Clone | % of the cognate’s activity | Ratio of P22 N activity to /H9261 N activity |
|-------|-----------------------------|-----------------------------------------------|
| LL    | 25 110                      | 0.22                                          |
| LR    | 30 100                      | 0.30                                          |
| PL    | 100 20                      | 5.0                                           |
| PR    | 100 0.04                    | 2.500                                         |
| L1    | 120 93                      | 1.3                                           |
| L2    | 130 75                      | 1.8                                           |
| L3    | 77 26                       | 3.0                                           |
| L4    | 96 0.50                     | 190                                           |
| L5    | 65 0.17                     | 380                                           |
| L6    | 50 1.2                      | 41                                            |
| L7    | 54 62                       | 0.87                                          |
| L8    | 57 5.8                      | 9.8                                           |
| L9    | 51 0.06                     | 850                                           |
| L10   | 64 56                       | 1.1                                           |
| L11   | 72 0.76                     | 96                                           |
| L12   | 40 0.27                     | 150                                           |
| L13   | 37 29                       | 1.3                                           |
| L14   | 28 15                       | 1.9                                           |
| L15   | 54 0.26                     | 210                                           |
| L16   | 24 0.8                      | 30                                            |
| L17   | 17 0.38                     | 46                                            |
| D1    | 100 87                      | 1.2                                           |
| D2    | 85 110                      | 0.77                                          |
| D3    | 36 75                       | 0.48                                          |
| D4    | 82 120                      | 0.69                                          |
| D5    | 100 0.2                     | 510                                           |
| D6    | 64 43                       | 1.5                                           |
| D7    | 13 74                       | 0.18                                          |
| D8    | 14 0.07                     | 218                                           |
| D9    | 15 2.4                      | 6.2                                           |
| D10   | 5.7 0.06                    | 98                                            |
| D11   | 7.3 0.06                    | 120                                           |
| D12   | 4.3 0.05                    | 84                                            |
| D13   | 140 10                      | 14                                            |
| D14   | 110 17                      | 6.2                                           |
| D15   | 31 4.7                      | 6.6                                           |
| D16   | 4.9 0.57                    | 8.7                                           |
| D17   | 3.6 0.47                    | 7.7                                           |
| D18   | 87 100                      | 0.87                                          |
| D19   | 9.3 160                     | 0.06                                          |
| D20   | 24 120                      | 0.20                                          |
| D21   | 25 120                      | 0.21                                          |
| D22   | 57 100                      | 0.57                                          |

* Clone identities are as described in Tables 1 to 5.

* The percentage of the cognate’s activity is calculated as the activity of the reporter relative to that of the cognate reporter (P22 boxBright for P22 N and boxBright for /H9261 N).

* The ratio of activity is the percentage of P22 N activity divided by the percentage of /H9261 N activity and indicates specificity.

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FIG. 3. Specificities of mutant boxBs. Representative boxB reporters in this study are plotted by their percentages of the activities with P22 N (abscissa) and /H9261 N (ordinate) relative to those with P22 boxBright and boxBright, respectively (Table 6). All clones are as in Tables 1 to 5. (A) boxBs related by C-to-A changes at position 3 are shown connected (D22 to LL, D18 to LR, D1 to LL, D6 to D7, D5 to D6). boxBs with C3 are shown as filled circles, and boxBs with A3 are shown as open circles. (B) boxBs related by P22 boxB left-to-/H9261 boxB stem changes are shown connected (D4 to LL, D2 to LR, PL to D18, L2 to D21, D3 to D20), where boxBs with P22 boxBleft stems are shown as filled circles and boxBs with /H9261 boxBright stems are shown as open circles. (C) boxBs related by U:A-to-A:U changes adjacent to the loop are shown connected (LR to D19, D3 to D7, D2 to D6, D1 to L4, PL to D5), where boxBs with U:A pairs are shown as filled circles and boxBs with A:U pairs are shown as open circles. (D) Selected boxBs related by single-nucleotide changes are shown connected (D4 to LL, D2 to LR, PL to D18, L2 to D21, P3 to D20, PL to D2, PL to D2, PL to D2, PL to L4, D4 to D7, D5 to D6, D6 to D7). Wild-type boxBs are represented by filled circles, and single-substitution mutants are shown as open circles. Four mutant boxBs related by single substitutions are shown as open triangles.
and λ recognition strategies may occur without an intermediate loss of function. We observed no series of single mutations connecting wild-type P22 and λ boxBs, because they differ by a base pair in the stem that was not mutagenized. However, a single mutation transforms P22 N-specific D5 into moderately bifunctional D6, which is a single mutation from λ N-specific D7. Indeed, a single substitution connects P22 N-specific L4 to λ N-specific D7.

**DISCUSSION**

Our results show that P22 N recognizes GNRA-like pentaloops, without strict adherence to the purine requirement. P22 N can also recognize GNRA-like hexaloops with reduced activity. Many boxB variants are recognized, but no simple pattern emerges. The subtle effects of base stacking likely dictate the ability of the pentaloop to adopt the conformation necessary for P22 N binding. P22 N prefers U:A and A:U adjacent to the loop and tolerates the C:C pair of boxBspecific interactions with the cooperation of distal-stem base pairs. Single mutations can dramatically alter boxB specificity between P22 and λ N. Though we found no path between wild-type boxBs, we did find that a series of single mutations connects P22 and λ N-specific boxBs via a relaxed-specificity boxB, as predicted by neutral theories of evolution. Libraries were randomized only in the loop region, and screening was not exhaustive, suggesting that more bifunctional boxBs exist. Our results with P22 N illustrate the complexity of even small RNA-peptide interactions.

While our results relate to the P22 boxB-N peptide interaction, we have recapitulated the interaction in a somewhat heterologous λ antitermination system that has the added complexity of reflecting the ability of host factors to assemble on the boxB-N complex (as reported by Conant et al. [8a]). Mutations that do not affect N binding can affect antitermination (6, 32, 37), and heterologous RNA-protein interactions display substantially lower activities than would be predicted by their in vitro affinities (17). Lower absolute activities of non-λ interactions are consistent with the model where λ N tetrathopan 18 stacks atop the λ boxB 4-out pentaloop, creating a precise conformation sensed by NusA, which then allows optimal antitermination (49). The P22 N fusion presumably does not permit proper NusA binding, since there is no residue to act as λ N tetrathopan 18 and boxB adopts a 3-out conformation when bound to P22 N (4).

The sequence requirements for N binding may be particularly complex because of the relative absence of base-specific hydrogen bonds. In light of the NMR evidence of extensive contacts between P22 N peptide and the 5' backbone of the lower stem (5), even minor conformational changes could drastically affect peptide binding. We speculate that the P22 loop consensus is obscure (besides the G:A pair), because the primary role of loop bases may be to promote a 3-out pentaloop for P22 N binding. Even the extruded C3 makes only hydrophobic interactions with N protein. This model is similar to that proposed for λ boxB, in which the extent of λ peptide binding reflected the proportion of the 4-out pentaloop in unbound boxB (24). The marked preference for U:A and A:U base pairs adjacent to the loop also suggests subtle base-stacking interactions that influence the conformation of the loop and stem. Other examples are known where base pairs influence the stability of proximal loops, such as that of λ boxB (24) and a related tetraloop (46). Interestingly, U:A and A:U base pairs adjacent to the loop of bovine immunodeficiency virus (BIV) trans-acting responsive element indirectly modulate the binding of the BIV Tat peptide to the stem (41). We speculate that the cytosines of P22 boxBspecific are tolerated because they are paired and stacked between the loop and the stem, as proposed for the homologous C:C base pair in phage 21 boxB (7). The complex thermodynamics of base stacking would be unlikely to yield a simple consensus.

What determines specificity between λ and P22 N-boxB interactions? Fluorescence studies support a model where any boxB bound by λ N adopts the 4-out conformation and any boxB bound by P22 N adopts a 3-out conformation (2). The thermodynamic costs of adopting λ- or P22-like loop and stem conformations likely dictate N binding, and specificity may be a simple consequence of disfavoring one conformation. Relaxed-specificity RNAs would be able to adopt either conformation, though at some cost to activity.

Neutral theories of evolution highlight the importance of genetic drift, asserting that mutational paths of neutral fitness exist between genotypes of distinct phenotypes (26, 34, 45). Computational studies of RNA secondary structure strongly support this assertion (21, 38, 45). However, experimental evidence with biologically active RNAs has been limited. Experimental support for the application of neutral theories to RNA activity has come from only a few examples (18, 22, 23, 40). Our findings that single substitutions connect a P22 N-specific boxB via a moderately bifunctional boxB to a λ N-specific boxB support the extension of neutral theories from computationally predictable RNA secondary structures to biologically active RNA phenotypes. However, the existence of bifunctional RNAs linking the N-dependent boxBs of P22 and λ boxBs to those of phage 21 or the structurally unrelated and N-independent RNAs of the HK022 polymerase utilization site (3) seems less likely. Other evolutionary mechanisms exist, such as RNA-protein coevolution and recombination between lambdoid phages (25). Nonetheless, we find that neutral theories apply in this small-model system, where conformation, rather than secondary structure as defined by base pairing, appears to define biological activity.

How common are RNAs that display multiple phenotypes? The intersection theorem of RNA secondary structure states that for any two secondary structures there is at least one sequence that is compatible with both structures (38). The phenotypes of biologically active RNA are typically more complex than the secondary structure, yet genetic drift along neutral paths may lead to new activities. Schultes and Bartel (40) found their intersection sequence able to adopt either of two distinct folds, albeit with severely reduced activity. Riboswitches are observed to undergo changes in secondary structure suggestive of intersection sequences, but few examples of RNAs that can bind different partners with no change in base pairing are known. NMR studies reveal that HIV RRE binds to its cognate peptide HIV Rev and to a selected peptide, RSG1.2, maintaining the same base pairing (50). A mutant hairpin RNA is able to function in either HIV or BIV transcriptional transactivation by binding homologous arginine-rich peptides in different recognition modes (41). These exam-
ple of RNAs able to bind multiple partners suggest that RNAs can serve as intersections of neutral paths between specific phenotypes.

Relaxed specificity may require the ability to adopt distinct conformations. Though plasticity comes with lowered thermodynamic stability, evolution presents many examples of conformational flexibility. Induced-fit interactions are commonly observed in RNA-protein interactions and in arginine-rich peptide-RNA interactions in particular (35). Indeed, the arginine-rich domain of λ N protein is entirely disordered until it is bound to boxB (33), and the boxB loop becomes ordered upon peptide binding (4, 24). The phenomenon of induced fit may increase the likelihood of sequences that can adopt more than one functional conformation, whether to signal binding, to interact with multiple partners, or to serve as intersection sequences. RNAs able to bind multiple partners seem more likely to facilitate the neutral coevolution of protein partners.

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