An RNA enhancer in a phage transcriptional antitermination complex functions as a structural switch

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Antitermination protein N regulates the transcriptional program of phage λ through recognition of RNA enhancer elements. Binding of an arginine-rich peptide to one face of an RNA hairpin organizes the other, which in turn binds to the host antitermination complex. The induced RNA structure mimics a GNRA hairpin, an organizational element of rRNA and ribozymes. The two faces of the RNA, bridged by a sheared GA base pair, exhibit a specific pattern of base stacking and base flipping. This pattern is extended by stacking of an aromatic amino acid side chain with an unpaired adenine at the N-binding surface. Such extended stacking is coupled to induction of a specific internal RNA architecture and is blocked by RNA mutations associated in vivo with loss of transcriptional antitermination activity. Mimicry of a motif of RNA assembly by an RNA-protein complex permits its engagement within the antitermination machinery.

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A general feature of protein-RNA recognition is costabilization of novel binding surfaces (Tan and Frankel 1995). The structural diversity of RNA—a rich repertoire of nonstandard base pairing and backbone organization—underlies its biological role in macromolecular assembly and catalysis (Cate et al. 1996). Protein binding is often accompanied by large-scale rearrangement of RNA structure; examples are provided by the Tat and Rev proteins of mammalian immunodeficiency viruses (Puglisi et al. 1993, 1995; Battiste et al. 1994, 1996; Ye et al. 1995). Induced RNA structures exhibit nonstandard base pairing and stacking in association with changes in the dimension of grooves. Are such features an epiphenomenon of recognition or of broader biological importance? In particular, does the diverse structural repertoire of RNA make possible novel mechanisms of gene regulation? To address these questions, we have investigated the RNA-based control of a viral developmental program.

Phage λ provides a model of antitermination in the positive control of transcription (Roberts 1969; Salstrom and Szybalski 1978; Franklin 1985ab). Expression of delayed-early genes requires that RNA polymerase read through Rho-dependent and intrinsic terminators (for re-

view, see Das 1993; Greenblatt et al. 1993). Antitermination is directed by the λ N protein in concert with host factors NusA, NusB, NusG, and S10 (Fig. 1A; Schauer et al. 1987; Whalen et al. 1988; Mason and Greenblatt 1991; Li et al. 1992; Mason et al. 1992; DeVito and Das 1994). N activity at physiological concentrations is directed by RNA enhancer elements (λ N-utilization sites; *nutL* and *nutR*) upstream in the nascent message (de Crombrughe et al. 1979; Olson et al. 1982; Barik et al. 1987; Whalen and Das 1990; Patterson et al. 1994). A *nut* site consists of a 5'-single-stranded RNA element (*boxA*), a short single-stranded linker, and a 3' hairpin (*boxB*). *boxA*, conserved among lambdoid phages λ , P22, and ϕ 21 (Friedman and Olson 1983; Olson et al. 1984; Nodwell and Greenblatt 1993), resembles antiterminator elements in the RNA (*rrn*) operons of *Escherichia coli* (Li et al. 1984; Morgan 1986; Albrechtsen et al. 1990). λ *boxA* is inactive as an antitermination signal in the absence of *boxB* (Salstrom and Szybalski 1978), the site of N binding (Barik et al. 1987; Whalen and Das 1990). Among lambdoid phages analogous *boxB* sites differ in sequence (Franklin 1985a), restricting the specificity of N-dependent antitermination. Phage-specific *boxB* recognition by the λ , P22, and ϕ 21 N proteins (Franklin 1985b) is mediated by analogous arginine-rich motifs (Lazinski et al. 1989; Tan and Frankel 1995).

λ *boxB* is a 15-base nonstandard RNA hairpin containing a purine-rich pentaloop (Fig. 1B). The importance of N-*boxB* recognition has been established by genetic and

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Figure 1. Overview of λ N regulatory system. (A) The N protein binds to an RNA enhancer element in the nascent message (*nut* site; asterisk indicates *boxB* RNA hairpin) and to host factors and RNAP to direct formation of a processive antitermination complex. Not shown: *nut boxA* RNA motif and interactions of the N-*nut* complex with host Nus elongation factors, including NusA. (B) Closing base pair (U5 and A11) and purine-rich pentaloop (bases 6–10; underlined) of 15-base *nut_L boxB* (5′-GCCCUGAAGAAGGGC-3′), with numbering scheme as shown. Red-outlined box (Trp-18) and nucleoside position (7) indicate site of indole–adenine stacking; blue nucleosides (7–10) exhibit a specific pattern of base-pairing (A10), -stacking (asterisk), and flipping (G9). Black rectangles indicate stacking between closing base pair (UA) and GA sheared base pair. Bidirectional arrows indicate NOEs between purines; base 9 is “flipped out.” Peptide–RNA contacts (such as A7–Trp-18) were identified by isotope-filtered NMR experiments designed to resolve NOEs between ^{13}C - or ^{15}N -attached protons in a labeled peptide and ^{12}C - or ^{14}N -attached protons in the unlabeled RNA (Su et al. 1997). (C) Effects of base substitutions on the biological activity of the N-*nut* system in vivo (Doelling and Franklin 1989); analogous results have been obtained by Chattopadhyay et al. (1995a). One hundred percent is defined as the activity exhibited by the GAAAA loop. Bars are color-coded by base: dark blue (G), red (A), green (U), and black (C). (D) Structure of sheared GA base pair. The 2-amino group of guanine is shown in red; the asterisk indicates guanine imino proton (not involved in hydrogen bonding).

biochemical studies (Salstrom and Szybalski 1978; Nodwell and Greenblatt 1991; Franklin 1993; Chattopadhyay et al. 1995a; Mogridge et al. 1995). Evidence for an RNA signal in vivo has been provided by genetic analysis (Friedman and Olson 1983; Olson et al. 1984; Warren and Das 1984; Zuber et al. 1987); evidence in vitro has been provided by kinetic analysis of reconstituted transcription systems (Whalen and Das 1990). The N-binding surface of *boxB* has been mapped by RNase protection and mutagenesis; a second functional surface is recognized by NusA in the assembly of a transcriptional antitermination complex (Chattopadhyay et al. 1995a; Mogridge et al. 1995). *boxB* thus provides a bipartite “RNA bridge” in a network of protein–protein and protein–RNA interactions.

In this paper we investigate a minimal model of the λ N antitermination complex. We demonstrate that asymmetric binding of an arginine-rich peptide to one face of a flexible RNA hairpin leads to restructuring of the other. The N peptide stabilizes a specific pattern of base-pairing, base-stacking, and base-flipping. This pattern resembles that of the classical GNRA tetraloop (Fig. 2A), an organizational motif of rRNA and catalytic RNA (Antao et al. 1991; Heus and Pardi 1991; Pley et al. 1994a,b; Wimberly 1994). A central feature of this motif is a sheared GA base pair (Fig. 1D). Such side-by-side pairing (shown red in Fig. 2A) reorients the RNA backbone relative to a Watson–Crick base pair (arrow in Fig. 2B). The pattern of stacking in *boxB* is extended by an aromatic side chain in the peptide: Tryptophan acts as a pseudo-

Figure 2. (A) Crystal structure of a GNRA (GAAA) tetraloop (Pley et al. 1994b; Brookhaven Databank accession no. 1HMH). The closing sheared GA base pair is shown in red; the structure is otherwise shown in cyan. (B) Solution structure of a non-GNRA (CUUG) tetraloop (F. Jucker and A. Pardi, in prep.; Brookhaven Databank accession no. 1RNG). The closing Watson-Crick GC base pair is shown in white; the structure is otherwise shown in green. The arrow indicates overall reorientation of the loop and redirection of CG base pair. (C) Comparison of tetraloop structures. The two structures are aligned according to the backbone atoms of the stem. The coloring scheme is as in A and B. The pairing scheme of the closing base pair (sheared GA vs. Watson-Crick CG) defines the orientation of the loop relative to the stem.

base at the N-binding surface. Extended peptide-RNA stacking is coupled to induction of a specific internal RNA architecture and is blocked by RNA mutations associated in vivo with loss of antitermination activity (Doelling and Franklin 1989; Chattopadhyay et al. 1995a). Comparison of active and inactive RNA variants demonstrates the role of an inducible RNA structure in a genetic switch.

Results

A model of the λ N antitermination complex is provided by a 15-base RNA hairpin (*nutL boxB* 5'-GC-CCUGAAGAAGGGC-3'; the underlined pentaloop is shown in Fig. 1B) and the amino-terminal 21 residues of the N protein without initiator methionine (designated λ_{P1} ; Lazinski et al. 1989; Tan and Frankel 1995). The peptide-RNA dissociation constant [K_d 6 nM at 4°C, as determined by gel retardation (Tan and Frankel 1995; Cilley and Williamson 1997)] is similar to that of the intact protein (1.3 nM; Chattopadhyay et al. 1995a; Van

Gilst et al. 1997). The strength of the binding of λ_{P1} to variant RNA sites (Table 1) correlates with efficiency of N-directed transcriptional antitermination (Doelling and Franklin 1989; Chattopadhyay et al. 1995a; Mogridge et al. 1995); λ_{P1} does not bind to segmental DNA analogs (Table 1 footnote). Circular dichroism (CD) spectra of λ_{P1} , *boxB* RNA, and their specific complex demonstrate that whereas the isolated peptide exhibits a largely random coil spectrum (Tan and Frankel 1995), the bound peptide exhibits an α -helix content of 80% (Table 2; Su et al. 1997). RNA-directed folding of the peptide thus recapitulates that of the intact N protein (Van Gilst et al. 1997). In each case, RNA-dependent quenching of the intrinsic fluorescence of Trp-18 enables weak dissociation constants to be measured (Table 1) and provides a structural probe of the peptide-RNA interface (Van Gilst et al. 1997).

A flexible RNA hairpin adopts a precise structure on peptide binding

The base and anomeric (ribose H_{1'}) ¹H and ¹³C nuclear magnetic resonance (NMR) spectra of *boxB* RNA have been assigned (Varani and Tinoco 1991) in the absence and presence of λ_{P1} peptide (Fig. 3). The free RNA consists of a stem and a flexible loop. Its imino ¹H-NMR spectrum contains three sharp resonances (12–14 ppm), assigned to the central CG base pairs of the stem (spectrum b in Fig. 3A). The resonances of base pairs 1 and 5 are broadened by exchange with water (due to “fraying” of the double helix). A broad imino resonance is also observed at 10.75 ppm and assigned below to a fraying GA base pair (G6-A10; see below). Stacking of G6 over U5 is indicated by a nuclear Overhauser enhancement (NOE) between G6-H₈ and U5-H₆ of intensity similar to that between G12-H₈ and G13-H₈ in the stem. Although no contacts are observed between base protons in the loop at 25°C in D₂O (observation is restricted in part by limited dispersion of chemical shifts), the presence of selected nonsequential base-ribose NOEs (A8-H₈/A10-H_{1'}) and absence of some sequential base-ribose NOEs (A7-H₈/A8-H_{1'} and A8-H₈/G9-H_{1'}) suggest that a nascent nonrandom structure is present but unstable. In contrast, the bound RNA is well-organized at 25°C. Base-pairing and -stacking are maintained in the stem and extend into the loop. The imino resonance of U5 is sharp in the complex (asterisk at 13.5 ppm in Fig. 3A, spectrum c), indicating that its pairing with A11 is stabilized on peptide binding. Retention of NOEs between successive base pairs in the stem indicates that the peptide does not intercalate or induce RNA base-flipping in this region.

Upon peptide binding, large changes in ¹H and ¹³C chemical shifts occur throughout the RNA (RNA “complexation” shifts, illustrated in Fig. 3A). No correlation is observed between sites of large or small RNA complexation shifts and the asymmetric RNase footprint of protein binding (5' eight bases, 5'-GCCCUGA-AGAAGGGC-3'; Chattopadhyay et al. 1995a). Such lack of correlation suggests that upon peptide binding the

Table 1. Relative N peptide- and protein-RNA affinities

Analog	Relative affinity				
	N peptide λ_{P1}	peptide 2 ^a	N peptide 3 ^b	N protein 1 ^c	N protein 2 ^d
Wild-type <i>boxB</i>	100%	100%	100%	100%	100%
<i>boxB</i> DNA ^e	N.D. ^f	— ^g	—	N.S. ^h	—
<i>boxB</i> RNA variants					
G6 → C (GCCCUC AAGAAGGGC)	N.S.	—	—	N.D.	N.D.
G6 → A (GCCCUA AAGAAGGGC)	N.S.	N.D.	<0.5%	N.S.	—
G6 → U (GCCCUI AAGAAGGGC)	—	—	—	N.D.	—
G6 → I (GCCCUI AAGAAGGGC)	N.S.	—	—	N.S.	—
A7 → G (GCCCUG GAGAAGGGC)	90%	6%*	—	100%	—
A7 → U (GCCCUG UAGAAGGGC)	4% ⁱ	—	—	100%	—
A7 → C (GCCCUG CAGAAGGGC)	2%–4%	—	—	100%	50%–100%
A8 → G (GCCCUG AGGAAGGGC)	40%	—	—	100%	—
A8 → C (GCCCUG AC GAAGGGC)	—	—	—	5%	<10%
A8 → U (GCCCUG AU GAAGGGC)	3% ⁱ	—	2–3%	5%	—
G9 → C (GCCCUG AACAAGGGC)	—	—	100%	100%	—
G9 → U (GCCCUG AU AAGGGC)	100%	—	—	100%	—
G9 → A (GCCCUG AA AAGGGC)	—	—	100%	100%	100%
A10 → C (GCCCUG AG ACAGGGC)	6% ⁱ	—	<1%	25%–100% ^j	N.D. ^k
A10 → G (GCCCUG AG AGAGGGC)	—	N.D.	—	100%	—
A10 → U (GCCCUG AG AUAGGGC)	—	—	—	12%–100% ^l	—

^aData were obtained from Tan and Frankel (1995) using peptide N amino acids 1–22, including initiator methionine.^bData obtained from Cilley and Williamson (1997) using peptide N amino acids 2–19.^cData obtained from Chattopadhyay *et al.* (1995a) and Van Gilst *et al.* (1997) using native N protein.^dData obtained from Mogridge *et al.* (1995) using native N protein.^eFour DNA analogs were tested in the present study: all DNA as in Van Gilst *et al.* (1997); and three RNA–DNA hybrids (5'-GCCCUGAAGAAGGGC, 5'-GCCCUGAAGAAGGGC, and 5'-GCCCUGAAGAAGGGC; underlined bases represent deoxyribonucleotides).^f(N.D.) No shifted band was detected.^g(—) Assay not performed.^h(N.S.) Binding was nonspecific (affinity 1000-fold less than specific binding; Van Gilst *et al.* 1997).ⁱValues measured by tryptophan fluorescence.^j100% binding was observed by GMSA, but only 25% relative affinity was obtained by competition with the native site (A. Das, pers. comm.).^kDiscrepancy between studies (c,d) may be attributable to differences in exact gel-shift conditions (including temperature of the gel as a function of electrophoresis current) and confounding effects of kinetic instability [Tan and Frankel (1995), column 2].^lValue of 100% was obtained by GMSA (Chattopadhyay *et al.* 1995a) and 12% by fluorescence (Van Gilst *et al.* 1997).

(*)We cannot account for the difference in peptide binding to the A7G RNA site between the present study (column 1) and that of Tan and Frankel (1995; column 2). In the present study near-native affinity is observed by both gel retardation and fluorescence assays.

RNA undergoes a global change in structure or dynamics. Of particular interest, G6 (the first base of the pentaloop) yields a narrow imino resonance at 11 ppm (asterisk in Fig. 3A, spectrum c). The importance of G6 is highlighted by the genetic selection of mutations at this position (Salstrom and Szybalski 1978) associated with loss of N-dependent antitermination (Fig. 1C; Doelling and Franklin 1989). Evidence for a G6–A10 base pair is provided by three observations. First, the chemical shifts of the exocyclic amino resonances of each purine are widely inequivalent, diagnostic in each case of hydrogen bonding. Second, NOEs demonstrate that G6 is stacked over U5, whereas A10 overlies A11; furthermore, cross-strand contacts are observed between A11–H₂ and G6–H₁. Third, indirect NOEs are observed between the G6 imino and A10 amino protons under experimental conditions in which spin diffusion is not observed between successive base pairs in the stem.

In conventional GA pairing schemes (characterized by

anti-anti and *anti-syn* glycosidic torsion angles) the guanine imino proton participates in hydrogen bonding. In the *boxB* complex the G6 imino resonance remains in the upfield region (11 ppm), uncharacteristic of such schemes. The G6 imino proton makes no direct contacts with either A10–H₂ (excluding formation of an *anti-anti* GA base pair) or A10–H₈ (excluding formation of either an *anti-syn* GA base pair or the side-by-side GA base pair of an RNA aptomer; Fan *et al.* 1996). These observations (providing evidence of absence rather than absence of evidence) suggest by elimination that G6 and A10 form a sheared GA pair (reverse Hoogsteen), a pairing scheme first observed in the GNRA tetraloop (Fig. 1D). Formation of a sheared GA base pair is corroborated by observation of a diagnostic spin-diffusion NOE spectroscopy (NOESY) cross peak (mixing time, 300 msec) from the G6 imino resonance to the A10–H₈ (but not to A10–H₂). The sheared geometry rationalizes the otherwise anomalous H₁ chemical shift of A11, whose upfield position

Table 2. Summary of CD and fluorescence results

RNA analog	$\Delta\theta^a$ -(222 nm)	Fluorescence quenching ^b
Wild type	-25,000	0.25
G6 → C	-12,000	0.85*
G6 → I	-6,000	0.82*
A7 → G	-24,000	0.31
A7 → U	-18,000	0.47*
A8 → G	-25,000	0.24
A8 → U	-5,000	0.53*
G9 → U	-25,000	0.26
A10 → C	-15,000	0.75*

^aValues shown represent the difference in mean residue ellipticity at 222 nm between the complex and the sum of the spectra of its constituents (free peptide and free RNA). Spectra were normalized with respect to the peptide concentration; values shown are given to nearest thousand. A helix content of 100% at 4°C corresponds to a $[\theta]_{222}$ value of -34,000.

^bFluorescence quenching is represented as the ratio of intensities at 350 nm of the bound and free peptides after correction of the latter for the inner filter effect. The excitation wavelength was 295 nm. Values <1 represent quenching of intrinsic tryptophan fluorescence. Asterisks indicate attenuation of RNA-induced blue shift as illustrated in Fig. 4A.

(4.99 ppm; Fig. 3B) is characteristic of the 3' nucleoside of the closing base pair of the GNRA tetraloop (Orita et al. 1993).

Whereas the sheared GA base pair in *boxB* is associated with sequential and nonsequential contacts in the

pentaloop, no base-base contacts are observed from G6 to A7, from A8 to G9, or from G9 to A10. The bound RNA thus assumes a specific pattern of base-pairing, base-stacking, and base-flipping as illustrated in Figure 1B. This structure is remarkable for successive stacking of A7, A8, and the sheared GA base pair. The core of the induced RNA structure is analogous to that of the GAAA tetraloop (Fig. 2A; Jucker et al. 1996). A pentaloop differs from a tetraloop in that it has an extra base. The odd base is G9, which exhibits no sequential NOEs to adjoining bases. The G9 ribose exhibits C2' *endo* pucker [as indicated by the anomalous intensity of cross peaks in double-quantum filtered correlation spectroscopy (DQF-COSY) and total correlation spectroscopy (TOCSY) spectra], and its glycosidic torsion angle is *syn* (as indicated by the anomalous intensity of the G9 H₈-H_{1'} NOE; Fig. 3B). Not essential for N binding (Table 1), the flipped base (G9) nevertheless contributes to biological activity (Fig. 1C; Doelling and Franklin 1989). [The term base-flipping (Roberts 1995) is meant in the broad sense of protein recognition of an exposed, unstacked, and unpaired base and is not meant to suggest that purine G9 is extruded from an RNA double helix on peptide binding.] The pattern of base-stacking is extended at the top of the structure by direct stacking between the purine ring of A7 and the indole ring of Trp-18 (Fig. 1B; Su et al. 1997). Such stacking rationalizes the quenching of tryptophan fluorescence on *boxB* binding (van Gilst et al. 1997) and a functional requirement for an aromatic amino acid at this position (Franklin 1993).

Figure 3. (A) 1D ¹H-NMR spectra in H₂O at 400 Mhz and 25°C: (a) Free λ_{P1} with unique tryptophan (Trp-18) indole NH resonance; (b) free *boxB* RNA hairpin with imino resonances assigned as indicated. G1 and U5 exhibit partial broadening at ends of stem. The arrow indicates additional unassigned broad imino resonance of guanine (10.75 ppm), presumably representing a fraying GA base pair. (c) Spectrum of specific complex with imino resonances assigned as indicated. Asterisks indicate sharp downfield imino resonance of U5 and sharp but upfield imino resonance of G6, proposed to participate in a sheared GA base pair (Fig. 1D). The indole NH resonance of Trp-18 exhibits a 0.9 ppm upfield complexation shift (broken line; see Fig. 5A). The peptide and RNA were each 2 mM. (B) Sequential assignment of RNA in the λ_{P1} complex at 25°C and 750 MHz. Connectivities in stem and loop are shown in red and blue, respectively. Positions of adenine H₂ resonances are indicated at top. The broken line indicates only a single NOE from the H₂ of A7 in this region (cross peak g, A7-H₂/A8-H_{1'}). The asterisk indicates NOE between G9-H₈ and G9-H_{3'}, the latter at an anomalous chemical shift. Assignment of cross peaks a, A10-H₂/A11-H_{1'}; c, A11-H₂/G6-H_{1'} (immediately below is the larger A11-H₂/U5-H_{1'}); d, A8-H₂/A10-H_{1'}; and e, A10-H₈/A8-H_{1'}. Boxes b and f indicate missing NOEs between nucleosides 8-9 and 9-10, respectively, reflecting flipping out of G9 (see Fig. 1B).

The GA base pair is essential for peptide-induced fit and biological activity

G6 and A10 are each essential for biological activity (Fig. 1C; Doelling and Franklin 1989). Although the functional requirement for adenine is absolute (Fig. 1C; Doelling and Franklin 1989), C10, U10, and G10 may in principle pair with G6 and provide alternative bridges across the RNA loop. The ^1H -NMR spectrum of the free A10C RNA (Fig. 4C) demonstrates that the variant site contains the expected GC base pair (arrow) and is more stably folded than the unbound native RNA: In the absence of peptide U5–A11 and non-native G6–C10 base pairs exhibit (in contrast to native *boxB*) little or no broadening due to fraying. λ_{P1} binds with decreased affinity and reduced kinetic stability to this structure (Table 1; Cilley and Williamson 1997). Although the A10C peptide dissociation constant is estimated to be 100 nM at 4°C by fluorescence (Table 1), no binding is detectable at this concentration by the standard gel mobility-shift assay (GMSA). The induced fit of the peptide (Su et al. 1997), as monitored by CD (signal a in Fig. 4B), is disrupted (estimated α -helix content <50%; Table 2). Likewise, the CD signature of induced fit in the RNA is absent (signal b in Fig. 4B). Quenching of Trp-18 fluorescence in the A10C complex is incomplete (Fig. 4A; Table 2). The ^1H -NMR spectrum of the A10C variant

complex (Fig. 4D) reveals two modes of binding in slow exchange on the ^1H -NMR chemical-shift time scale (>10 msec). G6 substitutions are likewise associated with attenuation of peptide-induced fit and inefficient quenching of tryptophan fluorescence (Table 2). G6 $\rightarrow$ C and G6 $\rightarrow$ I (inosine) variants form weak equimolar peptide complexes (K_d 0.5 μM ; Table 1), a value similar to the non-specific RNA-binding affinity of the intact N protein (1.5 μM ; Van Gilst et al. 1997).

Stacking of tryptophan with a pyrimidine is incomplete and unstable

Direct stacking between the purine ring of A7 and the indole ring of Trp-18 in the peptide (Su et al. 1997) is associated with a functional requirement for an aromatic amino acid at this position of the protein (tryptophan or tyrosine; Franklin 1993) and a purine at RNA position 7 (Fig. 1C; Doelling and Franklin 1989). The peptide binds with essentially native affinity to an A7G RNA analog but with 20-fold decreased affinity to an A7U analog (Table 1). The extent of shifted complex as observed by gel electrophoresis (Fig. 5E) is less than would be predicted by the equilibrium constant, suggesting that the variant complex partially dissociates on the time scale of electrophoresis (Cilley and Williamson 1997). Native

Figure 4. (A) Tryptophan fluorescence spectra of the free peptide (open boxes), native complex (solid line), and variant complex C10 complex (thick dashed line). Peptide and RNA concentrations were 5 μM . A control for the inner filter effect is shown in the two upper control spectra: free peptide (---) and an equimolar mixture of free peptide and free RNA (---) in 2 M KCl (pH 7.4). (B) CD spectra of the free C10 RNA (open boxes), free peptide (---), and variant complex (thick dashed line). A reference spectrum of the native complex is also shown (wt; solid line). Deconvolution of the wild-type difference spectrum suggests that 16 residues are helical in the bound state. The arrow (a) indicates an attenuated signal at the helix-sensitive wavelength, 222 nm. The asterisk indicates RNA-specific perturbation in the native complex; its attenuation in the variant complex is labeled at arrow b. This perturbation, similar to that of a Rev-RRE complex (Tan and Frankel 1994), is not amenable to detailed interpretation. Analysis of difference spectra reveals attenuation of induced α -helix content (see Table 2 and footnote). Peptide and RNA concentrations were 25 μM . (C) 400-MHz

^1H -NMR spectrum of amino protons in the C10 RNA variant demonstrate stabilization of the U5–A11 base pair (asterisk) and extension of the stem to G6–C10. Assignments are as indicated; the arrow indicates the new G6 imino resonance of the GC base pair. (D) 600 MHz ^1H -NMR spectra of 1:1 peptide–A10C RNA complex at 5°C, 15°C, and 25°C. Corresponding resonances in major and minor states are outlined; the asterisk indicates upfield indole NH resonance of Trp-18 in the minor state. In the major mode the RNA chemical shifts are similar to those of the free RNA; the chemical shifts of Trp-18 (but not those of amino acids such as Thr-5) are near those of the free peptide. In the minor mode these chemical shifts resemble those of the native complex, including the indole ring's large upfield complexation shift. The ratio of major to minor populations increases with increasing temperature. Spectra were obtained at a complex concentration of 1 mM in 50 mM NaCl and 10 mM sodium phosphate (pH 6.0).

Figure 5. ¹H-NMR analysis of A7U variant complex. (A, B) Imino and nonexchangeable aromatic resonances of the native (b) and variant (a) complexes. Large changes in Trp-18-specific resonances are shown as solid vertical lines (A); constancy of A10-H₂ and A8-H₂ resonances are shown as broken vertical lines. Assignment of imino resonances is as indicated. H₂O and D₂O spectra were collected at 400 and 600 MHz, respectively, at 25°C. (C) TOCSY spectrum of A7U complex (mixing time 55 msec at 25°C at 600 MHz) reveals motional narrowing of U7 base protons relative to cytosine and uridine resonances in the stem. The chemical shifts of these major groove pyrimidine resonances are not significantly changed by the A7U substitution. (D) NOESY spectrum in D₂O (mixing time 300 msec at 25°C and 600 MHz) shows weak contact between Trp-18 indole ring (H₅) and U7-H₅ (arrow) but no other U7-base contacts. (box) The A7U complex was made 1 mM in NMR buffer. (E) GMSA showing weak binding of the A7U RNA (a–k) at concentrations 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 nM. A wild-type control is provided in the first two lanes. The dissociation constant as measured by fluorescence quenching is ~125 nM (Table 1); the disproportionately weak gel shift is attributable in part to kinetic instability of the complex during the course of electrophoresis.

and A7U variant complexes exhibit a marked difference in the extent of the quenching of Trp-18 fluorescence (Table 2); furthermore, the variant complex (unlike the wild type) exhibits no blue shift of its fluorescence emission maximum (asterisk in Table 2). These observations demonstrate that the decrement in peptide–RNA affinity is associated with a qualitative change in local structure: incomplete stacking of Trp-18 on the smaller pyrimidine

ring. This, in turn, is associated with a small abridgement of RNA-dependent folding of the peptide (reduction in negative ellipticity at 222 nm; Table 2). In contrast, fluorescence and CD spectra of an A7G complex are similar to those of the wild-type complex (Table 2). ¹H-NMR studies of the A7U complex demonstrate that the induced RNA structure is similar to that of the native complex. Stem-specific chemical shifts of imino (Fig. 5A) and major groove pyrimidine (Fig. 5C) resonances are essentially identical in the native and variant complexes. This correspondence indicates that the substitution in the RNA loop does not perturb the docking of the peptide in the RNA stem. Likewise unchanged are chemical shifts of structure-sensitive H₂ protons of internal adenines in the loop (A8 and A10; broken vertical lines in Fig. 5B). As expected, chemical shifts of Trp-18 aromatic protons are in each case downfield of their frequencies in the wild-type complex (solid vertical lines in Fig. 5, A and B), representing an attenuation of the up-field complexation shifts characteristic of the wild-type complex (Fig. 5A; Su et al. 1997). This attenuation is presumably due (at least in part) to the smaller aromatic ring current of a pyrimidine relative to that of a purine. The aromatic resonances of U7 (unlike those of A7) exhibit relative motional narrowing (as seen in the intensity of and resolved coupling within the U7 TOCSY cross peak; Fig. 5C). Such resonance narrowing indicates that the pyrimidine ring has enhanced mobility on a time scale more rapid than that of overall tumbling of the complex (<2 nsec), that is, the U7–Trp-18 contact is unstable at the peptide–RNA interface. No NOEs are observed between U7 and A8 analogous to those between A7 and A8 in the native complex (Table 2). Despite these dynamic perturbations, close spatial proximity of Trp-18 and U7 is on average maintained, as shown by a weak intermolecular NOE (arrow in Fig. 5D); non-specific spin diffusion is excluded by the absence of contacts between U7 and other bases in the pentaloop (empty rectangle in Fig. 5D).

A specific internal RNA architecture is required for tryptophan stacking at one RNA surface and for NusA binding at the other

boxB RNA exhibits an analogous purine requirement at position 8 (Fig. 1C). ¹H-NMR, CD, and fluorescence spectra of the active A8G variant complex exhibit native features. The substitution A8U is associated with essentially complete loss of activity (Doelling and Franklin 1989). The variant RNA peptide dissociation constant is reduced (*K_d* 150 nM; Table 1). Fluorescence spectra of native and A8U variant complexes reveal marked attenuation of the quenching of Trp-18 (Table 2). This perturbation is indirect, as Trp-18 and A8 are not in contact. These observations suggest that external stacking of Trp-18 and A7 requires a purine-specific internal RNA architecture. The U8-, but not G8-, variant complex also exhibits a dramatic abridgement of the α-helical transition induced in the native peptide–RNA complex (Table 2). The block to peptide folding in the U8 complex is greater

than that observed in the U7 complex. This perturbation, presumably transmitted through the RNA (Fig. 1B), recapitulates the folding requirements of a GNRA tetraloop (Jucker et al. 1996). These results demonstrate a physical coupling between an induced RNA architecture and peptide folding. This coupling correlates in turn with antitermination activity in vivo (Fig. 1C; Doelling and Franklin 1989).

Although the flipped base G9 does not participate in the internal RNA architecture, its substitution by a pyrimidine causes a reduction in the efficiency of N-directed antitermination (Fig. 1C; Doelling and Franklin 1989). Such substitutions do not affect the affinity of *boxB* for N (Chattopadhyay et al. 1995a; Mogridge et al. 1995) or N peptides (Table 1; Cilley and Williamson 1997). Reduction in biological activity correlates instead with decreased affinity of the variant N-RNA complex for NusA and thus inefficient recruitment to the antitermination complex (Chattopadhyay et al. 1995a; Mogridge et al. 1995). As expected from the model (Fig. 1B), no NOEs are observed between G9 and the peptide in the NOESY spectrum of the native λ_{P1} complex. Native and G9U complexes exhibits identical fluorescence and CD spectra (Table 2).

Discussion

The λ N protein contains an arginine-rich motif characteristic of a diverse family of prokaryotic and eukaryotic RNA-binding proteins (Lazinski et al. 1989). Well-characterized examples are the Tat and Rev proteins of mammalian immunodeficiency viruses (Tan et al. 1993; Battiste et al. 1994, 1996; Ye et al. 1995, 1996). A general feature of their RNA complexes is costabilization of peptide and RNA structures (Tan and Frankel 1995). The present study has focused on the organization of RNA within a *boxB*-N peptide complex (Lazinski et al. 1989; Tan and Frankel 1995). The bound RNA has been demonstrated to exhibit a specific pattern of base-pairing, base-stacking, and base-flipping (Fig. 1B). Comparison of active and inactive RNA variants strongly suggests that this pattern is required for biological activity.

boxB provides a structural switch in transcriptional antitermination

boxB provides an RNA tether that enhances the local concentration of N near its site of action. Does the RNA also act as a structural switch? This question was first motivated by characterization of base substitutions that reduce antitermination activity disproportionately to destabilization of N binding (Chattopadhyay et al. 1995a; Mogridge et al. 1995). Such mutations interfere with binding of the N-*boxB* complex to NusA and its subsequent assembly within the RNA polymerase (RNAP)-antitermination machinery. Because *boxB* does not bind NusA in the absence of N, an N-induced change in RNA structure was predicted to define a novel recognition surface (Chattopadhyay et al. 1995a; Mogridge et al. 1995). This hypothesis is supported indirectly by the low effi-

ciency of antitermination achieved by “swap” of the λ N arginine-rich motif and *boxB* by analogous HIV-1 peptide and RNA motifs (TAT and *tar*; Harada et al. 1996). Here, we have tested a central feature of this hypothesis by investigation of the structure of *boxB* in the absence and presence of an arginine-rich N peptide (Lazinski et al. 1989; Tan and Frankel 1995). The structure of the unbound *boxB* contains a stem and flexible loop. Stable base-pairing and base-stacking are maintained in the stem, including the pairing of U5 and A11 and stacking of G6 over U5. We imagine that binding of the N peptide stabilizes a loop conformation, accessible at least in part to the free RNA. Such binding is associated with large changes in ^1H and ^{13}C chemical shifts throughout the stem and loop.

The bound RNA mimics a GNRA tetraloop

We propose an analogy between the induced structure of *boxB* and the GNRA tetraloop. This analogy is motivated by similarities in both function and structure. Each RNA presents a surface for higher-order assembly: Whereas the tetraloop mediates RNA-RNA assembly (Pley et al. 1994b; Wimberly 1994; Cate et al. 1996), *boxB* mediates protein-RNA-protein assembly (Chattopadhyay et al. 1995a; Mogridge et al. 1995). The RNA structures are in each case defined by patterns of base-pairing (including non Watson-Crick base pairs), base-stacking, and base-flipping (Fig. 2A; Heus and Pardi 1991; Pley et al. 1994a,b). The 5'-G and 3'-A form a sheared (reverse Hoogsteen) base pair over which is stacked a purine at position 3 (R). The second base (N) overlies the third and can be stably stacked (purine) or displaced (pyrimidine). Each contains a requisite purine at the third position of the loop, which is stacked over the GA base pair. Unlike the GNRA tetraloop, however, *boxB* is “incomplete” as a motif of RNA structure: Its folding requires peptide binding with indole-base stacking at the protein-RNA interface (Su et al. 1997). Such mimicry of a motif of RNA-RNA assembly by a protein-RNA complex may represent an early event in the transition between the proposed RNA and protein worlds.

The sheared GA base pair is required for biological activity and orients the RNA loop

The sheared GA base pair in *boxB* provides a physical bridge between opposing RNA surfaces. The orientation of this base pair (Fig. 1D) is distinct from that of other purine-purine base pairs, including those in Tat and Rev complexes (Puglisi et al. 1995; Ye et al. 1995; Battiste et al. 1996). In the GNRA tetraloop the sheared base pair specifies the orientation of the loop relative to the stem (Fig. 2A); its substitution by a Watson-Crick base pair is associated with a global realignment of the loop's position (arrows in Fig. 2, B and C). In *boxB* any base substitution of G6 or A10 causes complete loss of biological activity (Fig. 1C; Doelling and Franklin 1989). Replacement of the GA base pair in *boxB* by a Watson-Crick GC would likewise be expected to reposition the pentaloop.

Such an RNA variant, shown above to be in part preorganized, nevertheless forms a complex with reduced thermodynamic and kinetic instability (Mogridge et al. 1995; Cilley and Williamson 1997).

The present study demonstrates that a Watson–Crick bridge abridges peptide- and RNA-induced fit; the variant complex exhibits two distinct modes of binding. Based on the structures of variant tetraloops (Fig. 2), we propose that GA and GC *boxB* hairpins present distinct surfaces for peptide recognition: Global repositioning of the loop in the Watson–Crick analog precludes spatial complementarity to an induced peptide structure on one side and to NusA and the NusA–RNAP antitermination complex on the other. Unlike substitution of A10 by C, an A10U variant induces in the N protein a native α -helical transition with native fluorescence quenching (Van Gilst et al. 1997). We speculate that breakage of a GU base pair occurs in the complex and allows native positioning of the pentaloop, including stacking of A7 and Trp-18. The variant complex forms with high affinity but is without biological activity (Doelling and Franklin 1989; Chattopadhyay et al. 1995a).

Transcriptional antitermination requires a specific pattern of base-stacking and base-flipping

Efficient N-directed antitermination requires purines at positions 7, 8, and 9 (Fig. 1C). The requirement at position 8 is enforced by stacking of the purine over the sheared GA base pair. Substitution by U abridges induced fit of the peptide and the RNA. Purines at positions 7 and 9—in contrast, not integral to the induced RNA structure—provide respective landmarks for recognition by N and the core antitermination complex. Substitution of A7 by U destabilizes its stacking with Trp-18 (on one side) and A8 (on the other), whereas the overall RNA structure is preserved. Although the U7 RNA site exhibits a 12-fold loss of peptide affinity (Table 1), binding of the intact N protein to longer RNA fragments is not affected by pyrimidine substitutions (Chattopadhyay et al. 1995a; Mogridge et al. 1995). The discrepancy between affinities of the peptide and protein [also observed by Cilley and Williamson (1997)] is likely to reflect additional protein–RNA contacts not present in the peptide–*boxB* model. Given that the binding properties of the intact protein are likely to be more relevant to activity, what enforces a functional requirement for a purine at position 7 (Fig. 1C)? We propose that A7 and Trp-18 together define a recognition element for higher-order interaction with the NusA–RNAP complex and that dynamic instability of the joint Trp-18–U7 surface interferes with such recognition. This proposal is supported by the observation that a C7 *boxB*–N complex is not readily bound by NusA nor recruited into higher-order RNAP complexes (Mogridge et al. 1995). A G9U variant exhibits native peptide binding and structure but is associated with partial loss of antitermination activity (Fig. 1C; Doelling and Franklin 1989). This loss correlates with impaired binding to a NusA–RNAP complex (Chattopadhyay et al. 1995a; Mogridge et al. 1995). The

relative biological activities of variants at position 9 (G > A > U > C; Fig. 1C) are likely to reflect structural features of a binding pocket in NusA or the NusA–RNAP complex.

A puzzling result of genetic analysis (Franklin 1993) is the reported absence of mutations in λ N capable of suppressing specific loss-of-function associated with base substitutions in the pentaloop of *boxB*. This negative result is remarkable in light of the efficiency with which the protein’s “sequence space” was apparently sampled in that study by random cassette mutagenesis. Absence of specific suppression (and, hence, of N domains with altered specificity) stands in contrast to the efficiency with which such mutations have been found in Rev–*RRE* (Rev response element) variants (Jain and Belasco 1996). Because, these systems otherwise exhibit marked similarities [including costabilization of peptide and RNA structures (Tan and Frankel 1994)], why can base specificity be reprogrammed in one protein (Rev) but not in the other (λ N)? The present results suggest a structural explanation. We consider each position of the *boxB* pentaloop in turn. (1) *Position 7*. This base stacks with the indole ring of Trp-18. We speculate that such stacking requires an aromatic amino acid regardless of whether the base is a purine or pyrimidine. In contrast, sites of altered specificity in the Rev–*RRE* complex involve hydrogen bonds and van der Waal interactions between side chains and the edges of base pairs (Jain and Belasco 1996). (2) *Position 9*. The flipped base is proposed to contact the transcriptional machinery. Second-site mutations would thus map outside of N (e.g., in NusA). (3) *Positions 6 and 10*. Substitutions of the sheared GA base pair are likely to cause global changes in the orientation or stability of the loop. Such changes may require a switch in overall strategy of recognition, unlikely to be associated with a single amino acid substitution. (4) *Position 8*. Substitution of the central purine by a pyrimidine is likewise predicted to redefine the overall structure or stability of the loop.

The boxB complex does not deliver a specific “go” signal to RNAP

RNA-directed assembly of an N–*boxB*–NusA complex within the core transcriptional machinery can serve either or both of two biochemical functions. First, contacts between N-bound *boxB* and NusA may extend unrelated protein–protein interactions between the carboxy-terminal domain of N and NusA or RNAP (as in *nut*-independent antitermination; Rees et al. 1996). Such multidentate recognition would provide a mechanism of cooperativity in the higher-order assembly of a ribonucleoprotein complex (Mogridge et al. 1995). Second, the precise contacts by *boxB* within the antitermination complex could provide a specific antitermination signal, that is, an RNA-directed go switch may be thrown in RNAP (King et al. 1996). The latter mechanism is unlikely. Studies of the Nun transcriptional termination protein of phage HK022 (Chattopadhyay et al. 1995b; Hung and Gottesman 1995) have defined an analogous

arginine-rich motif (Fig. 6A) specific for λ *nut box B*. Although Nun and N have opposite functions (termination and antitermination; Hung and Gottesman 1995), the λ N arginine-rich motif in the intact N protein may be replaced by the Nun arginine-rich motif without change in RNA-binding specificity or function (Henthorn and Friedman 1996). Swap of RNA-binding domains is thus not associated with an interchange of function. The N and Nun arginine-rich motifs each contain aromatic side chains (boxed in Fig. 5A) and may exhibit similar mechanisms of RNA binding (Chattopadhyay et al. 1995b; M. Gottesman, pers. comm.). If their respective *boxB* complexes also interact similarly with the transcriptional machinery, these observations exclude the existence of a functionally specific (stop or go) binding site in the NusA-RNAP complex.

Structural determinants of N-nut specificities among lambdoid phages P22 and ϕ 21

Bacteriophages λ , P22, and ϕ 21 exhibit similar genomic organizations, including phage-specific N proteins and *nut* sites (Franklin 1985a,b). Although the structures of P22 and ϕ 21 *boxB* sites have not been characterized, in

principle, each can form a nonstandard hairpin (Fig. 6C,D). Will respective N-*boxB* complexes exhibit analogous modes of recognition? It is possible that the P22 complex will also contain sheared GA (boxed in Fig. 6C) and closing UA base pairs. Furthermore, position 7 of P22 *boxB* sites is adenine, as in λ *nut* sites. However, P22 *nut* sites contain a pyrimidine at position 8 (circled in Fig. 6C). This substitution—incompatible in λ *boxB* with stable stacking over the GA base pair (asterisk in Fig. 6C)—presumably blocks formation of a heterologous λ N-P22 *boxB* complex. This proposal is supported by the >20-fold enhancement in binding of a λ N peptide to a variant P22 *boxB* in which P22 positions 8 and 9 (5'-CA) are replaced by the corresponding λ *boxB* bases (5'-AG; Tan and Frankel 1995). Together, these observations suggest that the P22 pentaloop adopts a structure distinct from that in λ *boxB* and is recognized by a distinct mechanism. It is intriguing that the P22 N arginine-rich motif also lacks an aromatic residue corresponding to λ N Trp-18 (Fig. 6A, line 2; Franklin 1985b).

Inspection of putative ϕ 21 *boxB* hairpins (Fig. 6D) reveals more marked difference in predicted loop size (six nucleosides), sequence (pyrimidine-rich), and possible base-pairing (an absence of a GA base pair; asterisks in

Figure 6. (A) Alignment of arginine-rich N sequences in phages λ , P22, and ϕ 21 (Franklin 1985b; Chattopadhyay et al. 1995b) and termination factor Nun of phage ϕ HK022 (Hung and Gottesman 1995). Alanine conserved among N proteins is underlined. Dashes to the left of P22 and ϕ 21 sequences indicate the presence of amino-terminal additional residues not in λ N. Essential side chains (as inferred from genetic analysis; Franklin 1993) are indicated by dark blue squares (Ala-3, three of five arginines; and Trp-18); these have the most restricted patterns of allowed substitutions. Other contributing residues are indicated by red squares (solid > open), including two of five arginines. Substitution of proline at position 12 (P) confers native biological activity (Franklin 1993). (B–D) Comparison of *boxB* sites in lambdoid phages. (B) Consensus *boxB* hairpin in phage λ showing specific pattern of base-pairing and base-flipping (purines 7 and 9). Three sites of peptide-base contact (Su et al. 1997) are as indicated. The open box indicates a sheared GA base pair; the black box highlights the position of contact with Ala-3 in the major groove. The RNase footprint of the N protein (Chattopadhyay et al. 1995a) is shown at left; the proposed allosteric surface involved in binding to the core antitermination complex is shown at right. (C) A functional preference or requirement for purine (Fig. 1C; Doelling and Franklin 1989). The proposed interaction of the flipped base (R9) with NusA in an antitermination complex is indicated. (D) Putative hairpin structures of *boxB* sites in phages P22 and ϕ 21. P22 sites maintain possible GA base pair but lack a purine at position 8 (circle and asterisk); substitution of a purine enhances heterospecific binding of λ N peptide (Tan and Frankel 1995). The putative P22 stem also lacks a corresponding CC element (black square). ϕ 21 sites lack a possible GA base pair (oval and asterisk) and CC elements (black squares). Possible 5'-CU and 5'-UU recognition elements are highlighted. The red arrow in D indicates the absence of a purine at the site corresponding to A7-Trp-18 stacking in the λ *boxB*-peptide complex.

Chattopadhyay et al. 1995a) is shown at left; the proposed allosteric surface involved in binding to the core antitermination complex is shown at right. (C) A functional preference or requirement for purine (Fig. 1C; Doelling and Franklin 1989). The proposed interaction of the flipped base (R9) with NusA in an antitermination complex is indicated. (D) Putative hairpin structures of *boxB* sites in phages P22 and ϕ 21. P22 sites maintain possible GA base pair but lack a purine at position 8 (circle and asterisk); substitution of a purine enhances heterospecific binding of λ N peptide (Tan and Frankel 1995). The putative P22 stem also lacks a corresponding CC element (black square). ϕ 21 sites lack a possible GA base pair (oval and asterisk) and CC elements (black squares). Possible 5'-CU and 5'-UU recognition elements are highlighted. The red arrow in D indicates the absence of a purine at the site corresponding to A7-Trp-18 stacking in the λ *boxB*-peptide complex.

Fig. 6D). Although two purines are predicted in the loop, we speculate that in the absence of a specific GA-associated geometry a stable Trp-18-adenine interaction would not be possible. The $\phi 21$ N protein also lacks a corresponding aromatic amino acid in its arginine-rich motif (Fig. 6A, line 3; Franklin 1985b). Whether P22 or $\phi 21$ N-*boxB* complexes define analogous NusA-RNAP-binding surfaces in the assembly of a processive antitermination complex is not known. Future comparison of these structures and analysis of their interactions within the elongation machinery promises to reveal both general principles and divergent strategies of RNA-mediated transcriptional antitermination.

Molecular mimicry underlies the design of a genetic switch

The present study has focused on an RNA-based mechanism of transcriptional regulation in phage λ . N-directed antitermination requires a specific network of interactions between the nascent message and host proteins, including RNAP and the Nus elongation factors (Mason and Greenblatt 1991; Li et al. 1992; Mason et al. 1992; DeVito and Das 1994; Mogridge et al. 1995). The present dissection of one component of this network—induction of a novel *boxB* structure by an N peptide—demonstrates costabilization of a novel RNA structure. The distinct structural role of each base in the λ *boxB* pentaloop rationalizes its genetic analysis (Salstrom and Szybalski 1978; Doelling and Franklin 1989; Chatopadhyay et al. 1995a). The active RNA structure recapitulates features of the classical GNRA tetraloop and provides a novel surface for recognition by NusA (Chatopadhyay et al. 1995a; Mogridge et al. 1995). An induced pattern of RNA base-pairing, -stacking, and -flipping thus mediates successive steps of RNA-protein assembly in transcriptional antitermination. The diverse structural repertoire of RNA underlies the design of a genetic switch.

Materials and methods

RNA synthesis

Oligonucleotides were prepared by solid-phase synthesis with β -cyanoethyl-phosphoramidite reagents (Davis 1995). For binding studies the crude product was purified from denaturing PAGE and desalted with Sephadex G-25. Preparative purification for spectroscopic study was accomplished using ion-exchange high-performance liquid chromatography (HPLC). Purity (>98%) was assessed by HPLC and gel electrophoresis with ^{32}P autoradiography.

Peptide synthesis

Peptides were prepared by solid-phase synthesis using Fmoc chemistry and contain carboxy-terminal amide groups. Following deprotection and cleavage from the resin, the crude product was lyophilized, desalted using Sephadex G-25 Superfine, and purified by reverse-phase HPLC. Fidelity of synthesis was verified by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. Peptide concentration were

measured by OD at 280 or 210 nm. Purity (>98%) was assessed by HPLC and mass spectrometry [Peptide numbers refer to the codon number including the initiator methionine (Franklin et al. 1985b); the amino-terminal residue of the peptide is hence designated Asp2, the second residue Ala3, etc.].

GMSAs

Complexes were incubated at 4°C in 20 mM Tris-acetate and 50 mM potassium acetate at pH 7.9 (as measured at room temperature). Free and bound species were resolved at 4°C using a 10% gel (20:1 acrylamide/bis-acrylamide) in 10 mM Tris-HCl, 9 mM boric acid, 0.1 mM EDTA (pH 7.3) (at room temperature). Dissociation constants of weak complexes were obtained by fluorescence (Van Gilst et al. 1997).

CD

CD spectra were obtained at 4°C using an Aviv spectropolarimeter with a path length of 1 mm. Binding buffer consisted of 10 mM potassium phosphate (pH 7.4), 100 mM KCl and 0.1 mM EDTA.

Fluorescence spectroscopy

Spectra were obtained using a SPEX steady-state fluorimeter with an excitation wavelength of 295 nm to minimize the inner filter effect of peptide-RNA solutions. Correction of the inner filter effect was based on control experiments in 2 M KCl (Fig. 4A), in which no binding is presumed. Dissociation constants of weak complexes were estimated from the concentration-dependent quenching of an equimolar RNA-peptide solution (Van Gilst et al. 1997). A path length of 1 cm was used at a peptide concentration of 0.06–5 μM at 4°C. The buffers were as described for CD studies.

NMR spectroscopy

^1H -NMR spectra were obtained at 400, 500, 600, and 750 MHz. RNA resonance assignments using ^1H and natural abundance ^{13}C NMR methods were obtained as described (Verani and Tinoco 1991). NOESY mixing times of 40, 50, 60, 70, 150, 300, and 500 msec were employed; TOCSY mixing times of 55, 80, and 110 msec were employed. Direct NOEs were calibrated at low mixing times (30–70 msec at 25°C) in reference to standard distances constrained by covalent structure (pyrimidine H_5 – H_6); spin diffusion was assessed in reference to NOEs within the indole ring of Trp-18 ($\text{H}_4 \rightarrow \text{H}_5 \rightarrow \text{H}_6 \rightarrow \text{H}_7$). Spectra were obtained at 4°C and 25°C. Resonance assignments in the pentaloop were obtained by comparison of spectra of the native, A7U and A8G complexes. NMR buffer consists of 10 mM sodium phosphate (pH 6.0) and 50 mM NaCl. Spectra at 400, 500, and 600 MHz were obtained at the Biological NMR Facility at The University of Chicago (IL); spectra at 750 MHz were obtained at the National Institutes of Health (NIH)-supported NMR Facility at the University of Wisconsin at Madison (NMR-FAM).

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