Low Energy CD of RNA Hairpin Unveils a Loop Conformation Required for λN Antitermination Activity*

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N protein coded by phage λ is a transcription factor that stimulates the antitermination activity of Escherichia coli RNA polymerase by binding specifically to the nascent RNA transcript at a stem-loop structure called boxB. We use a new biophysical technique, involving the monitoring of the low energy circular dichroism spectra of 2-aminopurine residues site-specifically placed in the boxB RNA loop, to investigate this binding interaction. The low energy CD spectra of these 2-aminopurine probes reflect specific asymmetric interactions with adjacent nucleotide bases. Consequently, these CD spectra provide detailed and specific conformational information about the RNA chain at these chromophores that cannot be obtained from changes in the related fluorescence signals of these probes. CD changes were observed on binding the N peptide to boxB RNA that correspond to structural changes that had been previously seen by NMR, thus validating our experimental approach. The low energy CD method was then used to quantify the ordered and disordered states of the free hairpin loop and to show that a significant fraction of the boxB loop assumes a product-like structure in the absence of protein. A boxB derivative with an intact stem and a reduced concentration of ordered loop was identified and used to show that the extent of the reaction between protein and boxB depends on the concentration of structured loop in the RNA reactant population. This result has general implications for the conformational specificity of RNA-protein interactions.

Protein N, which is coded by bacteriophage λ, regulates the transcription of phage genes during the lytic phase of growth by inducing an antitermination phenotype in the elongating transcription complex (1, 2). In this process, N causes transcription of the delayed early genes of phage λ by stimulating read-through of intrinsic and Rho-dependent terminators located within a few kilobases downstream of the λ pl and pR promoters. The specificity of the N effect on these genes depends on the transcription of a λ sequence called nut (N-utilization), which codes for two RNA regulatory elements, designated boxA and boxB (3, 4). The N protein binds specifically to a stem-loop structure formed by the boxB element of nut (5–7) and acts by contacting the elongating RNA polymerase located at downstream terminators by means of a cis RNA looping mechanism (8, 9). Such looping-dependent antitermination should decrease at terminators located far from the nut site (10), and we have recently quantified the relationship between the nut-to-terminator distance and antitermination activity.3

N peptide, the amino-terminal 22 amino acid residues of the λN protein, comprises a basic (arginine-rich) RNA binding domain. This peptide binds to boxB RNA with an affinity (Kd) of ~10−9 M, which is close to the Kf measured for this interaction with the intact protein (7, 11, 12). As a consequence of the binding interaction with boxB RNA, the N peptide, which is largely unfolded in solution, assumes a bent α-helical conformation. BoxB, the specific target for λN binding on the RNA transcript, is an RNA hairpin that is 15 nucleotides in length with a stem that contains 5 stacked bp and a five-membered loop. NMR studies have shown that the peptide binds to the 5′ surface of the boxB hairpin and causes the loop to assume a structure identical to that of the GNRA tetraloop (13–16). Formation of structured boxB RNA as a consequence of N binding appears to involve several conformational changes in the loop domain (Fig. 1). These include (i) increased stacking between bases at positions 7 and 8 of the loop, (ii) extrusion of the base at position 9 from the loop, which then does not participate in base stacking, and (iii) the induction of base stacking between nonadjacent residues at boxB positions 8 and 10.

We have recently demonstrated that the low energy CD spectrum (i.e. the spectrum above 300 nm) of 2-aminopurine (2-AP)4 can be used to observe conformational changes in the vicinity of a single mono- or dinucleotide residue within DNA (17). 2-AP is a structural isomer of adenine in which the amino group has been moved from position 6 to positions 8 and 10.

8-AP residues interact with nucleic acids and proteins in the same way as do adenosine residues. In particular, the incorporation of 2-AP into DNA does not significantly disrupt the structure or stability of double-stranded DNA (dsDNA) (20, 21), and 2-AP nucleoside triphosphates can serve as polymerase substrates in vivo and in vitro (22–27). DNA molecules that contain 2-AP residues exhibit two general types of low energy CD signals (17). The first reflects exciton coupling between adjacent 2-AP bases stacked in a B-form right helical conformation, such as in dsDNA (28–30). This signal is characterized by a CD spectrum with a low energy positive peak located at a wavelength above the maximum UV absorbance of the 2-AP residue, no CD intensity at the absorbance maximum, and a negative band at lower wavelength. The negative band can be masked by the tail of the large CD signal of the oligonucleotides near 270 nm; thus, in practice, a positive band above 320 nm is often the most reliable indicator of exciton coupling between 2-AP residues. The second class of 2-AP-dependent low energy CD signals comprises a single band centered at the wavelength of maximum absorbance.
Nucleotide Conformations in RNA Hairpins

FIGURE 1. Box B structure based on NMR determination of the N-peptide-box B complex (see the Introduction). Large circles, sugar; small circles, phosphate; small rectangles, designated nucleic acid bases.

UV absorbance. This signal has been observed for 2-AP residues in DNA that are not adjacent and is characteristic of 2-AP dimers in conformations that do not permit exciton coupling.

In this paper, we report the CD spectroscopic properties of nutR boxB RNA sequences (16) containing site-specifically inserted 2-AP residues (TABLES ONE and TWO). Since low energy CD is a new way to observe conformational features of RNA, we validate the technique by showing that the local conformational changes identified in the loop of the N-boxB complex by NMR structural studies can also be observed by CD measurements of these 2-AP-containing RNA hairpins. We then use this method to study the conformation of the loop sequence of boxB in ssRNA and in the hairpin. Results show that, in the absence of protein, a significant fraction of the boxB RNA molecules assumes a conformation characteristic of the “product conformation” that is observed in the N peptide-boxB complex. Finally, we use boxB derivatives with an intact stem and different fractions of structured loop to show that specific binding with the N peptide depends on the quantity of structured loop in free boxB RNA. These results have general consequences for the binding mechanisms of specific RNA-protein interactions (see “Discussion”).

MATERIALS AND METHODS

Chemicals, Peptides, and Oligonucleotides—N peptide (NH$_2$-MDA QTR RRE RNA EKQ AQW KAA N-COOH) was purchased from Global Peptide Service (Fort Collins, CO), and peptide concentrations were determined by UV absorbance, using a molar absorptivity ($\varepsilon_{280}$) of 5600 M$^{-1}$ cm$^{-1}$. Oligonucleotides, synthesized and high pressure liquid chromatography-purified by Qiagen or TriLink Biotechnologies, were dissolved in buffer, heated to 70 °C, and slowly cooled. Concentrations were determined from the A$_{260}$ values of single-stranded oligonucleotides at 80 °C, using the recently reported molar absorbivity values of Cavaluzzi and Rorer (31) and a molar absorptivity of 1700 M$^{-1}$ cm$^{-1}$ for the 2-aminopurine monophosphate residue (19). The concentration units used for RNA are expressed in mol of oligonucleotide/liter. Unless otherwise stated, experiments were performed at 20 °C in 20 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA.

Measurement of Fluorescence and CD Spectra and Thermal Melting Experiments—Fluorescence spectra were measured using a Jobin-Yvon Fluorolog spectrometer. Samples in a 10 × 4-mm cuvette, with the long path length oriented in the direction of the incident light, were excited at 305 nm, and emission spectra between 310 and 450 nm were recorded; results are reported as counts/s at the emission maximum near 370 nm. CD spectra (3.5–8 µmol of oligonucleotide/liter) were taken at wavelengths between 450 and 200 nm on a Jasco model J-720 CD spectrometer equipped with a thermostated cell holder. The results are the average of 8–20 spectra and are presented as Δε, the difference in molar extinction coefficient of left and right circularly polarized light, per mol of residue. Low energy CD intensities at wavelengths greater than 300 nm are presented on a per mol of 2-AP residue present basis (units, liter/mol(2-AP)$^{-1}$ cm$^{-1}$). Thermal denaturation experiments were performed by increasing the temperature of the cuvette by 2 °C/min, and melting was monitored by UV absorbance at 260 nm or by CD at the indicated wavelength.

RESULTS

ssRNA and dsRNA—Since the low energy CD method had not previously been used to study RNA, we first characterized the CD properties of ssRNA and dsRNA oligonucleotides labeled with 2-AP at specific sites. The ssRNA molecules that we have designated ssR0, ssR9, ssR89, and ssR810 (see TABLE TWO for the nomenclature of the oligonucleotides used) have the same sequence as boxB RNA, except for permutations in the stem sequence that disrupt base pairing. Equimolar concentrations of the complementary RNA oligonucleotide ssRC were added to form the corresponding dsRNA molecules, dsR0, dsR9, dsR89, and dsR810.
dsR810. Thermal melting increased the absorbance at 260 nm of these dsRNA complexes by 25% and showed a cooperative transition with a melting temperature (Tm) of 74 °C for all of the molecules examined. These results demonstrate that 2-AP residues do not significantly disrupt the stability of dsRNA, as also previously reported for dsDNA (20).

As expected, incorporation of single-stranded oligonucleotides containing 2-AP residues into dsRNA molecules resulted in significant quenching of the 2-AP fluorescence that occurs as a consequence of the formation of double-stranded complexes or after binding to N peptide. Fl, fluorescence.

![Figure 2](image2.png)  
**FIGURE 2. Low energy CD spectra of RNA molecules containing 2-AP residues.** A and C, ssRNA. B and D, boxB hairpin.

![Figure 3](image3.png)  
**FIGURE 3. Low energy CD spectra of dsRNA and dsDNA.** A, dsRNA. Inset, difference spectra in which the CD spectrum of dsR0 (black trace) has been subtracted from the spectrum of dsR0 with a fluorescent counter (light trace) and dsDNA (dark trace) containing 2-AP residues.

spectra of adjacent 2-AP residues in dsRNA and dsDNA may reflect the A-form and the B-form oligonucleotide conformations, respectively. Consistent with this conclusion, we have previously shown that adjacent 2-AP residues in the DNA strand of an A-form RNA-DNA hybrid oligonucleotide also did not exhibit exciton coupling (17). However, we have found more recently that the presence or absence of exciton coupling in A-form RNA-DNA hybrid duplexes may be sensitive to the local sequence context of the 2-AP probes.5 This issue is currently being explored further with relevant RNA-DNA hybrid sequences.

The BoxB Hairpin—Oligonucleotides with complementary sequences at the 5'- and 3'-extremities (TABLE ONE) form hairpin structures. The formation of a stem-loop structure can change the fluorescence of the included 2-AP residues by a factor of 2–3. The sign and magnitude of this fluorescence intensity change varies with the position of the 2-AP residue(s) in the loop (TABLE THREE).

The low energy CD spectra of boxB hairpins containing 2-AP residues are remarkably different from the CD spectra of the corresponding ssRNA with 2-AP at the same position (Fig. 2). The spectrum of a boxB hairpin containing a 2-AP residue located at position 7 (AP7) showed a small trough at 312 nm and could not be distinguished from the CD signal of the stem-loop without 2-AP residues. The CD spectra of AP8, AP9, and AP10 showed peaks at 320 nm, with intensities of 1.7, 2.4, and 0.6 liter (mol of 2-AP)−1 cm−1, respectively. No exciton coupling is expected for these molecules, and the CD has a maximum intensity at the same wavelength as the UV absorbance.

5 K. Datta, unpublished results.

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**TABLE THREE**

Fluorescence intensity of polynucleotides containing AP (counts/s per µM AP × 104)

Table showing the fluorescence intensity of polynucleotides containing 2-AP residues. The table includes columns for the oligonucleotide, fluorescence intensity (FI) alone, FI for the complex, and FI for the formation of double-stranded complexes.

| Oligonucleotide | FI (RNA alone) | Oligonucleotide complex | FI (after formation of double-stranded complexes) |
|-----------------|----------------|-------------------------|-----------------------------------------------|
| ssR9            | 15             | dsR09                   | 1.0                                           |
| ssR89           | 3.2            | dsR9                    | 0.58                                          |
| ssR810          | 4.9            | dsR810                  | 1.5                                           |
| AP7             | 13             | AP7-N                   | 6.3                                           |
| AP8             | 20             | AP8-N                   | 7.9                                           |
| AP9             | 23             | AP9-N                   | 50                                            |
| AP10            | 19             | AP10-N                  | 14                                            |
| AP78            | 0.85           | AP78-N                  | 0.07                                          |
| AP89            | 1.5            | AP89-N                  | 1.2                                           |
| AP910           | 1.4            | AP910-N                 | 0.66                                          |

* Fluorescence of the RNA alone.
* The same fluorescence as in column 2 after formation of double-stranded complexes (results for ssR9, ssR89, and ssR810) or after titration with N (the remaining results).
The CD spectra of AP89 and AP810 contained peaks at 317 nm with intensities of 1.9 and 0.95 liter/(mol of 2-AP) cm⁻¹, respectively (Fig. 2D). Hence the exciton coupling that was observed in the CD spectrum of ssR89 (Fig. 2C) disappeared in AP89. In contrast, the spectrum of AP78 showed a peak at 325 nm and a trough at 317 nm, indicating base stacking (17). Differences between the CD signals obtained with ssRNA and an RNA stem-loop can be explained by the conformational changes that occur when the boxB sequence forms a stem-loop (see "Discussion").

The N-BoxB Complex—In order to determine that binding of the N peptide to the various boxB derivatives was complete under our reaction conditions, we titrated oligonucleotides with N peptide and monitored the fluorescence quenching of the 2-AP residues of the various boxB constructs (Fig. 4). Binding N peptide increases base stacking in the loop and can also be followed by UV hyperchromism (not shown). Peptide binding decreased the fluorescence intensities of all of the derivatives tested except AP9 (TABLE THREE). The intensity of the AP9 fluorescence was increased by N binding as a result of a decrease in the quenching of 2-AP residues caused by interactions with neighboring nucleotide residues (32). Fluorescence titration curves show that 3.7 μM AP7, AP8, and AP9 bind N peptide with a 1:1 stoichiometry. Similar binding curves were observed for AP78 and AP89 (data not shown). However, well defined equivalence points were not observed with this concentration of AP10 or AP810. Rather both of these oligonucleotides bound N peptide with a reduced apparent K_d value of ~5 μM. These results permitted us to establish the concentration conditions under which the N peptide binds quantitatively to various 2-AP-modified boxB RNA constructs.

The binding of N peptide changed the intensities of the low energy CD spectra of boxB derivatives containing a single 2-AP residue differently, depending on the position of the 2-AP residue within the construct (Fig. 5). Thus, complex formation increased the intensity of the CD spectra of AP7 and AP8 by a factor of 2–3. The low energy CD of AP10 remained small, even in the presence of a 2-fold excess of N peptide, although under these conditions binding is expected to occur (Fig. 4). N peptide decreased the CD of AP9, and no CD intensity above 310 nm was observed for AP9 in the presence of a 1.4-fold excess of N peptide (data not shown).

The addition of equimolar N peptide to a boxB hairpin containing two adjacent 2-AP residues did not change the shapes of the low energy CD spectra (Fig. 6, A and B). The CD band at 325 nm that was observed for AP78 is also present in the CD spectrum of the complex, although the intensity of the spectrum for the complex was increased by a factor of 2.5. In contrast, peptide binding increased the CD peak of AP89 at 317 nm by only 20%.

Comparison of the low energy CD spectra of AP810 in the presence and absence of N peptide shows that N binding increased the intensity of the CD spectrum above 320 nm and decreased the intensity at lower wavelengths (Fig. 6C). It is possible that the N peptide may not bind AP810 to saturation at these oligonucleotide concentrations (Fig. 4). We therefore measured the CD spectra of AP810 in the presence of a 2-fold excess of N peptide. Excess peptide increased the intensity of the peak at 325 nm from 1.1 to 1.2 liter/(mol of 2-AP) cm⁻¹ and decreased the trough at 320 nm from 0.6 to 0.4 liter/(mol of 2-AP) cm⁻¹. These results indicate the presence of base stacking between 2-AP residues at positions 8 and 10 in the N-boxB complex, consistent with NMR results showing that N peptide binding induces a GNRA tetraloop conformation in boxB (14–16).

Tetraloop Formation in the Absence of Peptide—These latter results indicate that CD changes due to base stacking between AP residues located at loop positions 8 and 10 can be used to monitor tetraloop formation. Such base stacking may contribute to the shape of the low energy CD spectrum of hairpin construct AP810 (Fig. 6C). An apparent CD band is observed in the spectrum of the hairpin, with a small positive intensity (~+0.2 liter/(mol of 2-AP) cm⁻¹) at 325 nm and a negative signal (~−0.17 liter/(mol of 2-AP) cm⁻¹) at 312 nm, compared with the ssRNA control. These results suggest that formation of a stem-loop...
structure within an ssRNA molecule that carries the boxB sequence results in the formation of a measurable population of tetraloop, even in the absence of N peptide.

If this hypothesis is correct, destabilizing the loop would be expected to disrupt the putative tetraloop conformation. We therefore decreased the stability of the hairpin by introducing mismatches into the stem and measured the CD. The AP810p and AP810d constructs are derivatives of AP810, with one proximal and two distal mismatches in the stem sequence, respectively (TABLE TWO, second section). The effect of the mismatch on the stem structure was determined from the CD spectra below 300 nm, which reflect the presence of single-stranded DNA or dsDNA. AP810 and AP810p have identical CD bands at 267 nm (Fig. 7A). The intensity of this peak decreases by half and undergoes a 4-nm red shift in the CD spectrum of AP810d, resulting in a signal that corresponds to the spectrum of the ssRNA oligonucleotide, ssR810. These results suggest that under our reaction conditions, the oligonucleotides AP810 and AP810p have hairpin structures with an intact stem. In contrast, the two distal mismatches of AP810d destabilize the stem, and, judging from the CD spectrum, this oligonucleotide assumes the unstructured conformation for the equivalent ssRNA.

Based on the intensities of the low energy CD spectrum (Fig. 6D), AP810d (which has no stem-loop structure) showed no base stacking between AP residues. AP810p, with one proximal mismatch, displayed base stacking that was intermediate between that shown by AP810 and AP810d. These results suggest that the tetraloop structure in the AP810p construct is reduced by ~50% compared with AP810 by a mismatch near the loop that does not destroy the hairpin stem.

**DISCUSSION**

Low energy CD measurements of oligonucleotides containing 2-AP residues can probe the secondary structure of DNA at the mono- and dinucleotide levels (17). Here we have used this method to study conformational changes of the loop of the boxB RNA hairpin that play a role in the regulation of the transcription of phage λ (1, 2).

**Using the Low Energy CD Spectrum of 2-AP to Measure Local RNA Conformational Changes**—NMR experiments have shown that the loop of the boxB hairpin assumes a GNRA tetraloop structure in the N-boxB complex (15, 16). Formation of this tetraloop from the initial pentaloop structure within an ssRNA molecule that carries the boxB sequence results in the formation of a measurable population of tetraloop, even in the absence of N peptide.

In addition, a base on the 3′ surface of the boxB stem is “flipped out” of the loop as a consequence of the binding of the N peptide to the 5′ surface (Fig. 1). This extruded base does not interact with the peptide and appears to be highly mobile in the NMR structures. The CD spectrum of AP9 that was observed at wavelengths above 310 nm in the absence of peptide completely disappeared in the N-AP9 complex (Fig. 5; see “Results”). This observation is consistent with NMR results showing that the base at position 9 flips out of the N-boxB complex and assumes a large range of conformations. As a result, the optical activity of 2-AP in this position resembles that of a planar molecule free in solution. In addition, peptide binding increased the CD peak of AP89 at 317 nm by only a small amount (Fig. 6B), an observation that is also consistent with the suggestion that the 2-AP residue at position 9 is unstructured in the peptide complex.

Finally, NMR studies have revealed stacking between the nonadjacent bases at positions 8 and 10 in the structure of the N-boxB complex. The low energy CD spectrum of the complex of N peptide and AP810 exhibited a peak at 325 nm and a trough at 310 nm, relative to the spectrum of the same RNA in the absence of N peptide (Fig. 6C). This CD change is consistent with base stacking between the RNA bases at positions 8 and 10 in the loop.

These results show good agreement between known structural changes that occur when boxB binds to its cognate N peptide and changes in the low energy CD spectra of 2-AP residues placed at specific sites in boxB RNA. This agreement demonstrates that low energy CD of 2-AP can be used to study local RNA conformations. Likewise, pyrroloctosine, a chromophore recently used to probe local DNA conformations at GC sequences by low energy CD (33), should be useful to study RNA conformation.

**Comparison of Low Energy CD and Fluorescence Quenching Results**—Optical activity reflects the asymmetric environment of the chromophore, and, as might therefore be expected, low energy CD of 2-AP provides information that is not available from fluorescence quenching experiments (compare Fig. 8 and TABLE THREE). For example, base stacking causes fluorescence quenching between adjacent 2-AP residues in both duplex RNA and DNA. The low energy CD spectra of dsDNA and dsRNA, however, are remarkably different (Fig. 3), reflecting particular stacking interactions and transition dipole orientations in A-form and B-form conformations. In our studies of boxB RNA conformations, the fluorescence intensities of AP89 and AP810 were the same (TABLE THREE); however, low energy CD measurements showed that the 2-AP residues were unstacked in AP89 and stacked in AP810 compared with the corresponding ssRNA molecules (Fig. 6).

As a further example of the increased conformational “information content” of the CD versus the fluorescent spectra, binding of N peptide to AP10 and AP810 produced 25 and 50% fluorescence quenching, respectively (Fig. 4, TABLE THREE). In contrast, a small low energy CD signal was observed for AP10 that did not greatly change upon peptide binding (Fig. 5). This small signal probably comes from an average of several chiral interactions with opposite signs. However, and perhaps as a result of the low CD intensity of AP10, the peptide-induced base stacking of 2-AP residues at positions 8 and 10 in this complex could be observed (see above). The adenosine residue at position 10 (Fig. 1) is known to have an unusual conformational environment in the N-boxB complex, compared with the local environments of the other A residues in the loop. This includes the formation of a reverse Hoogsteen base pair between the A residue at position 10 and the G residue at position 6 and atypical associations with neighboring bases (14–16). These results illustrate that in favorable cases the low energy CD spectra of site-spe-
cifically inserted 2-AP can distinguish interactions between particular residues in a complex involving many nucleotide bases.

Taken together, these results demonstrate the capacity of low energy CD spectra to provide unique information about local nucleotide conformations, compared with the fluorescence of 2-AP. In particular, exciton coupling between 2-AP residues is an unambiguous and readily determined signature of base stacking in B-form duplex conformations.

Different Local Conformations of ssRNA and BoxB Hairpin—Low energy CD of 2-AP is sensitive to local conformational changes that occur when ssRNA forms a boxB RNA hairpin (Fig. 8). It seems reasonable to assume that the CD spectrum of ssR9 is representative of the spectrum of a single 2-AP residue in an A(2-AP)A sequence of ssRNA. If this is indeed the case, low energy CD measurements show unique conformations at each position of the loop in a stem-loop structure that differ from those seen in the equivalent free ssRNA (Fig. 2, A and B). However, we note that we cannot at present provide a unique conformational interpretation of the changes responsible for these particular CD signals.

In contrast, a clear relationship does exist between the observation of exciton bands in the low energy CD spectra of two site-specifically placed 2-AP residues and their base stacking interactions (see above) (28, 29). Thus, more base stacking occurs between bases at positions 7 and 8 in the hairpin than in the reference unstructured ssRNA, as shown by the increased intensity of the low energy exciton band of AP78 relative to the low energy CD spectrum of adjacent 2-AP residues in ssR89 (Fig. 2, C and D). We can also see that stem-loop formation disrupts the stacking interactions observed between bases at positions 8 and 9 in the initial ssRNA, since the small exciton coupling observed in the CD spectrum of the ssR89 construct is not present in the spectrum of the AP89 hairpin (Fig. 2, C and D). Judging from the peptide binding results, this probably reflects the extrusion of the base at position 9 of the hairpin loop. Finally, hairpin formation stacks bases at positions 8 and 10 to a small extent, as indicated by the presence of exciton bands in the low energy CD spectrum of AP810 (Fig. 6, C and D).

Interpretation of these CD changes is facilitated by the observation that many of these effects also occur with greater magnitude in the spectra of the N-boxB complexes (Fig. 8). Taken together, these results indicate that, in the absence of protein, a fraction of the boxB RNA population assumes a tetraloop structure similar to the product RNA conformation.

The presence of a partial GNRA tetraloop structure in the boxB hairpin in the absence of peptide has also been suggested from NMR results (14, 34). Low energy CD provides new information about this structure, as demonstrated by the conformational changes in free RNA molecules detected (see above) and by the quantitative potential of the technique. For example, the low energy CD intensity of the AP78 spectrum increased by a factor of 2.5 in the presence of N peptide (Fig. 6A), reflecting increased stacking of the bases at positions 7 and 8 in the complex. Assuming (i) that the unstructured to structured transition within the free ssRNA molecule is two-state and (ii) that the intensity of the low energy CD band for the unstructured state is represented by the spectrum of ssR89, we can estimate that ~30% of the AP78 RNA population is in the product conformation in the absence of N peptide. This estimate is consistent with the amount of stacking observed in AP810, relative to unstructured ssR810 and fully structured AP810-N (Fig. 6C).

This quantitative aspect of the CD measurement also allows us to show that a single mismatch in the stem, proximal to the loop, reduces the structured fraction of the loop population without disrupting the overall stem (Figs. 6D and 7A). In particular, the CD signal for the structured loop of AP810p had about 50% intensity of the signal for AP10. The fraction of tetraloop in a free RNA population should be
independent of the total oligonucleotide concentration, because stem-loop formation is an intramolecular reaction.

The Role of the Hairpin Loop Conformation in the Specific Binding of N Peptide to BoxB RNA—We could therefore ask if the reaction of boxB with N peptide is sensitive to the amount of structure in the loop by investigating the reaction between N peptide and various boxB derivatives. The reaction was followed by fluorescence (Fig. 7B). Low concentrations of oligonucleotides (about one-tenth of \( K_d \) for the reaction of AP810 with N peptide (Fig. 4)) were used in order to study the reaction under equilibrium conditions in which most of the RNA is free.

Under these conditions, we observed that AP810 bound to N peptide, whereas AP810d did not. Hence, we conclude that the presence of a stem is required for N peptide binding, at least at the ligand concentrations tested. However, the AP810p RNA molecule, which forms a hairpin with an intact stem and reduced tetraloop structure, also did not react with N peptide under these conditions. This result shows that specific binding of N protein to the boxB regulatory element of the phage \( \Lambda \) RNA transcript is favored by the existence, within the free RNA, of a significant population of the loop conformation that characterizes the RNA product when it is bound to the N peptide.

Intramolecular RNA interactions appear to provide the initial free energy to "pay for" the entropic costs associated with putting a fraction of the boxB population into this conformation. As the N peptide binds to the ordered fraction of the boxB population, it effectively removes of the boxB population into this conformation. As the N peptide binds to the ordered fraction of the boxB population, it effectively removes this "product fraction" from the pool of free RNA stem-loop structures. The conformations of the remaining free and "unstructured" stem-loop molecules reequilibrate to produce more structured product, which is then "trapped" by the structured N-peptide molecules in turn.

We note that an equivalent conformational shift of free N-peptide into the bent \( \alpha \)-helical binding conformation is observed as binding proceeds. CD measurements show that N peptide, which is reported to contain about 35% \( \alpha \)-helical structure at 4 °C in the absence of boxB RNA, increases its \( \alpha \)-helical content in the N-boxB complex (7, 11). Hence, binding would be expected to perturb the conformational equilibrium of both free boxB and peptide.

Reaction 1 summarizes the coupled equilibria that are involved.

\[
U_R \Leftrightarrow F_R \Leftrightarrow F_R F_P \Leftrightarrow F_P \Leftrightarrow U_P
\]

**REACTION 1**

Clearly, complex formation is favored by increasing the fraction of free RNA (and of free peptide) that is in the product (folded) conformation (\( F_R \) and \( F_P \)) at the expense of the unfolded free RNA and peptide (\( U_R \) and \( U_P \)). We note that if the intramolecular rates of formation of either the folded RNA or the peptide from their respective unfolded forms are slow, the binding interaction itself may not be rate-limiting.

We conclude that a significant concentration of a "product-like" RNA conformation in the free boxB RNA population participates in the binding competition that is responsible for specificity, as stated in Reaction 1. Preequilibration of RNA (or protein) populations into the folded product conformation will drive a specific RNA-protein binding reaction as a consequence of these coupled equilibrium considerations by application of Le Chatelier's principle. Clearly, this process can play a role in both the overall equilibrium and in the kinetics of the recognition of intrinsically unstable RNA structures by protein ligands. Thus, a small concentration of RNA folded in a particular conformation could favor a particular reaction pathway that is in kinetic competition with others by reducing its activation free energy (35). This notion can be used to develop quantitative molecular mechanisms that permit binding proteins to "trap" RNA-protein complexes in the specific conformations that may be required for biological activity.

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Low Energy CD of RNA Hairpin Unveils a Loop Conformation Required for λN Antitermination Activity

Neil P. Johnson, Walter A. Baase and Peter H. von Hippel

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Yih-Chern Horng, Scot C. Leary, Paul A. Cobine, Fiona B. J. Young, Graham N. George, Eric A. Shoubridge, and Dennis R. Winge

Pages 34119–34120:

In Fig. 6, A and B, and Fig. 7, A and B, the mutant listed as D239A on the far right of each panel should be H239A.

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Low energy CD of RNA hairpin unveils a loop conformation required for λN antitermination activity.

Neil P. Johnson, Walter A. Baase, and Peter H. von Hippel

PAGES 32179 –32182:

In the labeling of the $y$ axes on Figs. 2, 3, 5, 6, 7, and 8, the Greek lowercase epsilon was transformed into an exclamation point. The corrected figures are shown below.
Increased expression of Mcl-1 is required for protection against serum starvation in phosphatase and tensin homologue on chromosome 10 null mouse embryonic fibroblasts, but repression of Bim is favored in human glioblastomas.

Mark Austin and Simon J. Cook

Under "Experimental Procedures," subheading "Real-time RT-PCR," the human Bim and β-actin primers described are wrong. The published primers actually amplify murine Bim and β-actin transcript rather than the stated human transcript. The real-time RT-PCR data presented in Fig. 8C still remains factually correct, as it was derived using the human Bim and β-actin primers. The correct primers used in this study are described as follows:

Human Bim forward, 5'-TGC AGA CAT TTT GCT TGT TCA A-3', and reverse, 5'-GAA CCG CTG GCT GCA TAA TAA-3'; human β-actin forward, 5'-CTC CTC TCT GCA AGC AGT ACT C-3', and reverse, 5'-CGG ACT CGT CAT AGT CCT GCT T-3'.

Chemokine-glycosaminoglycan binding. SPECIFICITY FOR CCR2 LIGAND BINDING TO HIGHLY SULFATED OLIGOSACCHARIDES USING FTICR MASS SPECTROMETRY.

Yonghao Yu, Matthew D. Sweeney, Ola M. Saad, Susan E. Crown, Andro R. Hsu, Tracy M. Handel, and Julie A. Leary

Dr. Andro R. Hsu was inadvertently omitted from the author list. His affiliation is: Department of Molecular and Cell Biology, University of California, Berkeley, California 94720. The correct author list is shown above.

Proprotein convertases are responsible for proteolysis and inactivation of endothelial lipase.

Weijun Jin, Illa V. Fuki, Nabil G. Seidah, Suzanne Benjannet, Jane M. Glick, and Daniel J. Rader

"Convertases" in the title was printed as "Covertases." The correct title is listed above.