Bend and Helical Twist Associated with a Symmetric Internal Loop from 5S Ribosomal RNA†

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ABSTRACT: We have used gel electrophoretic mobility measurements to investigate the conformation of the symmetric eubacterial loop E sequence of 5S rRNA (seven nucleotides in each strand). The loop strongly retarded the gel mobility of duplex RNAs containing it. In contrast, only asymmetric $A_5{\cdot}A_n$ or $U_5{\cdot}U_n$ internal loops ($n \neq 5$) strongly affected duplex RNA gel mobility. A phasing experiment, in which an A_2 bulge and loop E were placed in the same duplex RNA and the number of base pairs between them varied, showed that loop E has a permanent bend and is torsionally stiff. A second phasing experiment substituting loop E for duplex sequences between two A_2 bulges measured the helical twist associated with loop E; it is about 30° (±15°) overwound compared to a duplex RNA of the same number of bases. Ribosomal protein L25 specifically recognizes loop E but had little or no effect on the twist of the loop. These results suggest that loop E adopts a specific, roughly helical structure.

Internal loops frequently interrupt helices in the conserved secondary structures of most RNAs. They may have the same number of nucleotides on each strand (symmetric loops) or different numbers (asymmetric loops) and are usually rich in purines. The conformations of internal loops potentially differ from standard A form RNA helix in terms of flexibility, helical twist, and curvature of the axis; these differences will in turn influence the tertiary contacts that can be made within an RNA and the overall three-dimensional conformation of the RNA. Little information is presently available concerning the magnitudes of internal loop bend, twist, and flexibility.

The loop E of 5S ribosomal RNA is perhaps the best studied internal loop. The sequence is conserved, and among the eubacteria it is always a symmetric loop with seven bases on each strand (Specht *et al.*, 1990). Nuclease and chemical probe experiments show that the nucleotides are ordered, and specific structures for the loop E sequence from spinach chloroplast and *Escherichia coli* based on chemical reactivities and computer modeling have been proposed (Romby *et al.*, 1988; Brunel *et al.*, 1991). NMR studies also indicate that the bases are ordered and have ruled out standard Watson–Crick or G·U wobble pairs (Zhang & Moore, 1989). A more recent NMR study of a eukaryotic loop E sequence, which is smaller than the eubacterial loop and asymmetric, found that the loop adopts an approximately A form helix geometry with the extra G nucleotide bulged into the major groove (Wimberly *et al.*, 1993).

While NMR experiments are very powerful for determining local details of RNA conformation, they provide only short-range distance constraints that do not effectively define larger scale properties of the duplex such as curvature or twist. Simple gel mobility experiments have been used to detect the bend and twist associated with bulge loops interrupting a long RNA duplex (Tang & Draper, 1994). Here we use these same techniques to detect bending in the eubacterial loop E sequence and to show that the loop is slightly overwound compared to a standard RNA helix. We also show that ribosomal protein L25, which specifically recognizes the loop E, has little effect on the overall helical twist of the loop.

MATERIALS AND METHODS

Preparation and Electrophoresis of RNA Duplexes. Plasmids containing T7 and T3 RNA polymerase promoters facing each other and having appropriate sequences between them were prepared and transcribed, and the RNA transcripts were annealed and trimmed with T_1 RNase to give duplex RNA as described in detail elsewhere (Tang & Draper, 1990, 1994). The internal loops and their sequence contexts are shown in Figure 1. Electrophoresis was carried out in 10 or 12% polyacrylamide gels (0.4 mm thick by 40 cm long) in 75 mM disodium phosphate buffer (adjusted to pH 7.0 with phosphoric acid) with or without 2 mM $MgCl_2$. Runs were at 150 V/cm in the cold or at room temperature. The actual temperatures of the gels measured by a surface thermometer were 8 or 30 °C, respectively. One strand of each RNA was ^{32}P labeled during transcription, and the RNAs were detected by autoradiography. RNAs having the same number of nucleotides but canonically paired were run as controls in adjacent lanes, and the relative gel mobilities reported are the ratios of the mobilities of loop-containing and control RNAs.

For the phasing experiments, plots of relative gel mobility as a function of base pairs between bend sites were fit to an equation describing the end-to-end distance of the RNA, as previously described (Tang & Draper, 1994).

Ribosomal Protein L25. L25 protein purifed from *E. coli* ribosomes was a generous gift from Peter Moore (Yale University). It was dialyzed into 0.1 M KCl and 10 mM Tris, pH 7.6, to remove urea, and it showed a single band in SDS gels. For gel shift assays, protein (~10 μM) was incubated on ice for 30 min with labeled RNA before electrophoresis, and the gels were run at 30 °C in sodium phosphate buffer.

RESULTS

Gel Mobility of RNAs Containing A and U Internal Loops. Duplex RNAs containing either $A_5{\cdot}A_n$ or $U_5{\cdot}U_n$ (n between 1 and 9) internal loops were synthesized and are shown in Figure 1; their mobilities relative to those of completely

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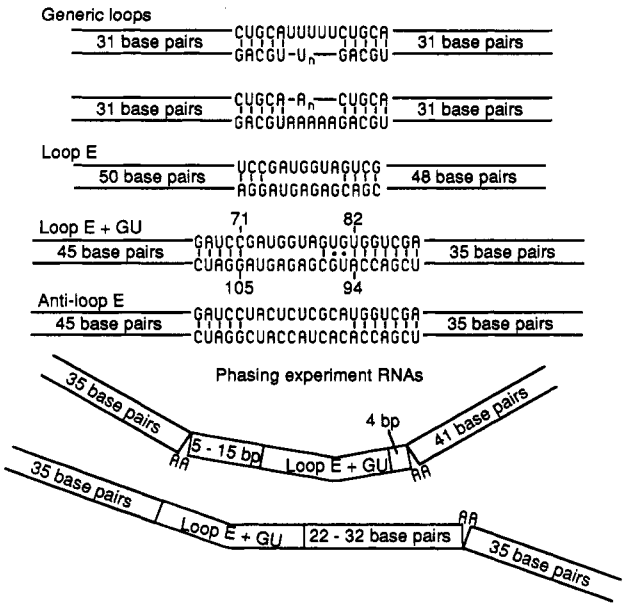

FIGURE 1: RNA internal loops used in this study. Shown are the loop sequences with surrounding duplex RNA and the total length of the duplex RNA used for gel mobility experiments in Figures 2 and 3. The phasing experiment RNAs show the duplexes used in Figure 4. The box labeled Loop E is nucleotides 71–82 and 93–104 of the *E. coli* 5S rRNA, as shown in the loop E + GU RNA, and the positions of A_2 bulges and segments varied in length are shown. The bending directions are drawn arbitrarily and will of course depend on the number of base pairs in the varied segments.

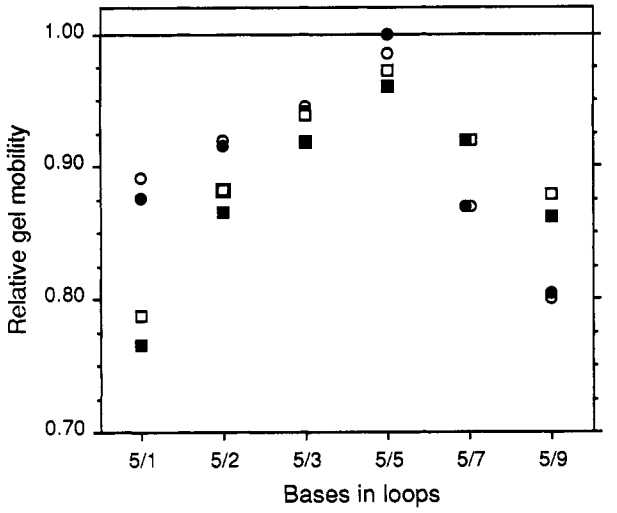

FIGURE 2: Relative gel mobilities of $A_5{\cdot}A_n$ (●, ○) or $U_5{\cdot}U_n$ (■, □) internal loops. Gels were run at 30 °C in sodium phosphate buffer as described in Materials and Methods. Open and closed symbols are results of two independent experiments.

complementary duplexes during gel electrophoresis are summarized in Figure 2. The symmetric loops (n = 5) have gel mobilities closest to that of the completely duplex RNA control, while greater degrees of asymmetry induce slower gel mobilities. Similar results for internal loops containing A nucleotides have been obtained by others (Riordan *et al.*, 1992). The gel mobility of duplex DNA containing a bend has been observed to decrease with bend angle (Wu & Crothers, 1984; Zinkel & Crothers, 1990). Flexibility at the joint may also cause the molecule to transiently adopt bent conformations with slow mobilities; drastically slower mobilities are seen in long helical DNAs after introduction of a few-nucleotide gap (Mills *et al.*, 1994). Whether the slower gel mobilities of the asymmetric internal loops are a consequence of an intrinsically bent conformation, more extreme flexibility, or both cannot

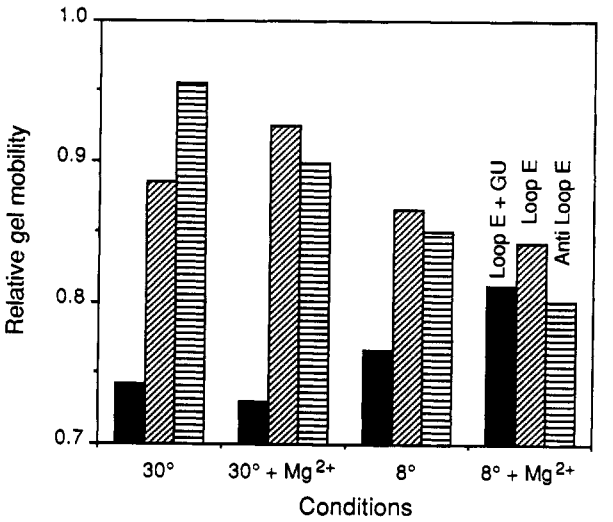

FIGURE 3: Relative gel mobilities of loop E related sequences. Solid bars, loop E + GU; bars with diagonal lines, loop E; bars with horizontal lines, anti-loop E. Gels were run at the indicated temperatures (°C) in sodium phosphate buffer with or without 2 mM $MgCl_2$.

be determined from these experiments alone; we also cannot rule out a small contribution of loop nucleotide interactions with the gel matrix. The symmetric internal loops are unlikely to introduce any static bend, and the small effect of the $A_5{\cdot}A_5$ loop is probably a consequence of flexibility. The $U_5{\cdot}U_5$ loop almost certainly contains wobble base pairs, as observed in a $U_2{\cdot}U_2$ loop (SantaLucia *et al.*, 1991), which explains its nearly identical gel mobility with the control double helix.

Gel Mobility of RNAs Containing the Internal Loop E. We next examined the gel mobilities of duplex RNAs containing three different symmetric internal loops with the sequences shown in Figure 1. Two contain loop E of *E. coli* 5S rRNA (Specht *et al.*, 1990) and differ only by the inclusion of two G·U pairs separated from the loop by one G·C pair; this is a conserved feature of the eubacterial 5S RNA helix containing loop E. Eleven of the 14 bases in the loop are purines, and NMR experiments do not detect any canonical base pairs within it (Zhang & Moore, 1989). The third loop, called "anti-loop E", is simply the Watson–Crick complement of loop E. It is thus composed mostly of pyrimidines and is not expected to have any specific structure. Surprisingly, the two loop E internal loops show significantly slower gel mobilities than the control duplexes (Figure 3), unlike the generic $A_5{\cdot}A_5$ and $U_5{\cdot}U_5$ symmetric internal loops.

Electrophoresis of the loop-containing RNAs was also carried out under conditions expected to stabilize RNA structure, i.e., low temperature to enhance base stacking and added Mg^{2+} to reduce electrostatic free energy. These results are also shown in Figure 3. Stabilizing conditions increase the mobility of loop E + GU but tend to decrease the mobility of loop E and anti-loop E. Stabilization of weak base pairing, or of conformations in which nucleotides are bulged, conceivably contributes to the complex behavior.

Phasing of an A_2 Bulge with Loop E. The above experiments cannot distinguish unequivocally RNAs with static bend from those with a flexible joint that averages over time to an unbent conformation. To detect static bend, a phasing experiment was carried out. We have previously shown that the electrophoretic mobility of an RNA duplex containing two A_2 bulges varies periodically as the number of base pairs between the bulges is increased (Tang & Draper, 1990, 1994). This "phasing" behavior detects the helical twist of the duplex

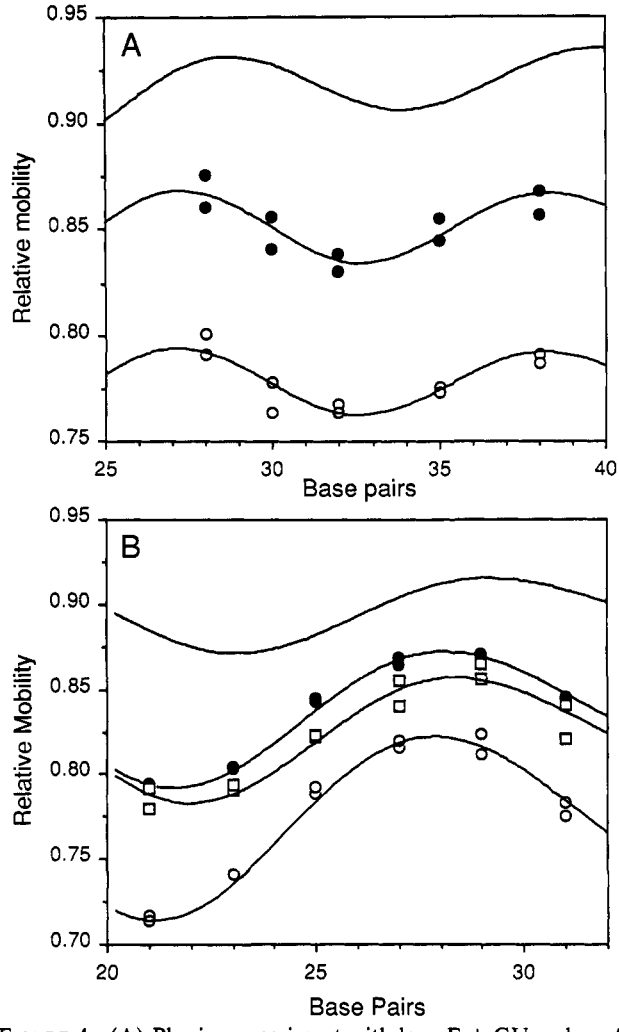

FIGURE 4: (A) Phasing experiment with loop E + GU and an A_2 bulge in the same RNA: ●, gels run at room temperature in phosphate buffer; ○, gels run at 4 °C in phosphate buffer. (B) Phasing experiment with loop E + GU between two A_2 bulges: circle symbols are the same as in panel A; □, phasing experiment repeated in the presence of L25 protein. Solid lines in each panel are least-squares fits to the data sets; the top line in each panel is from a phasing experiment with two A_2 bulges at room temperature in sodium phosphate buffer (Tang & Draper, 1994).

RNA as the molecule varies from *cis* to *trans* conformation, and it is possible only if the bulge introduces a static and torsionally stiff bend (Zinkel & Crothers, 1987). We can ask whether the same is true of loop E by substituting it for one of the two bulges in the set of "phased" RNAs (Figure 1). The phasing of an A_2 bulge with loop E + GU is shown in Figure 4A. A minimum is seen in the relative gel mobility under all the different conditions examined, and the magnitude of the gel mobility variation is about the same as seen with the two A_2 bulges. We therefore conclude that loop E + GU creates a permanent bend in the helix that is torsionally stiff to the degree required to maintain phasing.

It is interesting to note that the A_2–loop E + GU phasing curve (Figure 4A) shows very little variation in the position of the maximum (range 31.9–32.6 bp) or in the amplitude of the curve among the four conditions tried (8 or 30 °C, ±2 mM $MgCl_2$), in contrast to the large variation in relative gel mobility for the RNAs containing only the internal loop (Figure 3). A possible interpretion of this is that the average bend angle and bend-associated twist of the loop are relatively insensitive to environmental conditions, while hinge-like flexibility is strongly affected. Changes in flexibility may also account for the shifts of the phasing curves along the *y*-axis of Figure 4A.

RNA Containing Loop E + GU between Two A_2 Bulges. The helical twist of an internal loop may be distorted from that of a standard helix. To compare the twist of loop E + GU with that of a canonical RNA helix, we placed the loop E + GU sequence between two A_2 bulges and varied the number of base pairs between one of the bulges and the loop (see Figure 1). The bend in loop E + GU means that its position in relation to the nonrotating end segment (on the right in Figure 1) has to be considered. In a phasing experiment, the three helical segments are expected to be coplanar in the *cis* conformation when the relative gel mobility (and the end-to-end distance) is a minimum (Tang & Draper, 1990). If the middle segment of the molecule is substituted with a bent sequence, then the axes of all of the segments may no longer be coplanar in the *cis* or *trans* conformations, and the minimum end-to-end distance will not occur when the RNA conformation is *cis*. To find the *cis* conformation from gel mobilities, the middle segments and one of the end segments must be held planar while the other end segment is rotated. Then the minimum end-to-end distance will occur when the entire molecule lies in a plane and the torsion angle between the end segments is zero (i.e., the *cis* conformation). The torsion angle in this situation is a sum of the helical twists of the middle segments and the twists associated with the three bends. The effects of curved or bent RNA on phasing experiments are discussed in more detail by Tang and Draper (1994).

The phasing experiment of Figure 4A gives us the number of base pairs to insert between the A_2 bulge and the internal loop to obtain all three helix segments in the same plane. (The data in both panels of Figure 4 have been plotted assuming that the bend is between G76 and U77 of the internal loop. This is arbitrary; the interpretation of the experiment does not depend on the assumed bend location or whether the bend is sharp or gradual.) A minimum in the gel mobility is reached at 32.4 bp (averaged over all four conditions tested; the range is 31.9–32.6) between the bulge and the loop; this number has been shortened to 10 bp in constructing the phased molecules, which should maintain the helix segments in a plane if the RNA helical repeat is 11 bp/turn (Figure 1). The results of the phasing experiment are plotted in Figure 4B. There is a maximum in the gel mobility under all the conditions tried. Only small shifts in the maxima are seen relative to RNAs containing only canonical helix; the differences were an average of 0.8 base pair and showed no significant variation with temperature and buffer conditions (Table 1). If the helical repeat of RNA is taken to be 11 bp/turn, then loop E + GU is overwound compared to a standard helix by ~27°.

The major uncertainty in this measurement is whether the loop E + GU bend is correctly phased with respect to the bulges; the RNA helical repeat may not be precisely 11 bp/turn. If the average repeat is in the range 11 ± 0.3 bp/turn, we estimate that the maximum error in estimating the overwinding is ±15°. Calculations of potential error if the loop-associated bend is not in the same plane as the fixed bulge are in Tang and Draper (1994). Curvature of the helical segments themselves could also bias the measurements, but to a smaller degree than the uncertainties in the helical repeat.

We also have to consider the possibility that the helix is not overwound by 27°, but underwound by 333°. If such severe underwinding were confined to the 14 bases of the internal loop, a net 104° left-handed twist would be produced. Although underwound models of loop E have been considered

Table 1: Positions of Maximum Gel Mobility in Phasing Experiments[a]

conditions	maximum (bp)	difference from duplex (bp)	overwinding angle (deg)
30 °C	27.91	−1.01	33
30 °C + Mg^{2+}	27.86	−0.63	21
8 °C	27.72	−0.72	24
8 °C + Mg^{2+}	27.34	−0.89	29
+L25	28.18	−0.74	24

[a] The numbers of inserted base pairs giving maximum gel mobility, as calculated from least-squares fits of phasing curves to data as in Figure 4B, are listed for the A_2–loop E + GU–A_2 RNAs shown in Figure 1. The differences between the maximum mobility and those found for similar RNAs having only two A_2 bulges (Tang & Draper, 1990) are also listed and converted into degrees of overwinding, assuming that the RNA helical repeat is an average of 11.0 bp. From duplicate experiments, the error in determining the maximum is estimated as ±0.1 base pair; an extra significant figure is shown, as it was carried through in the calculation of the winding angle. The overall error in the determination is about ±8°; potentially larger systematic errors are discussed in the text.

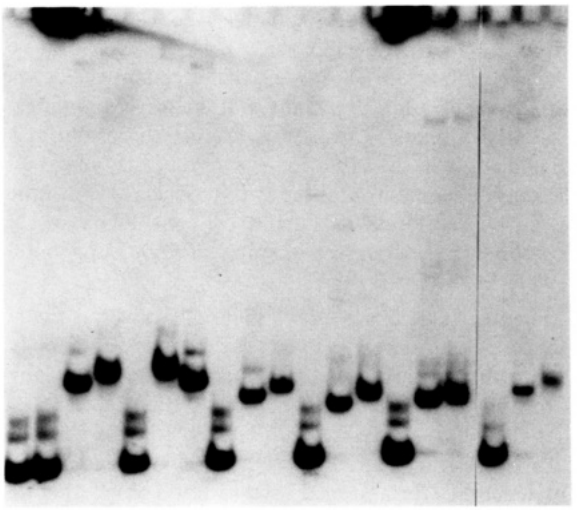

FIGURE 5: Autoradiogram of a phasing experiment with and without protein L25 present. The RNAs containing loop E + GU between two A_2 bulges were electrophoresed at 30 °C in sodium phosphate buffer. The number of base pairs between the bulges is indicated below each lane on the gel; underlined numbers are control RNAs lacking both the bulges and loop E but having the same total number of bases. L25 protein has been included in the samples as indicated.

(Romby *et al.*, 1988; also see Discussion), this degree of unwinding seems unlikely. The formal possibility of 333° underwinding cannot be ruled out by gel mobility experiments alone, but NMR studies of loop E should be able to easily distinguish overwound and underwound conformations.

Effect of L25 Binding on the Helical Twist of Loop E + GU. L25 binds tightly to a 5S rRNA fragment containing nucleotides 69–87 and 90–110 (Douthwaite *et al.*, 1979), while our loop E + GU RNAs duplicate nucleotides 71–82 and 94–105. Addition of the protein retards the mobility of our loop E + GU RNA, but not that of other RNAs containing canonical helices (Figure 5). Upon repeating the phasing experiment in the presence of L25, we find that the shifted RNAs still show a maximum gel mobility at about the same number of base pairs. The estimated change in the helical twist is an 8° (±8°) unwinding.

DISCUSSION

Bend of Loop E. On the basis of the behavior of A_5·A_5 and U_5·U_5 internal loops, it was unexpected that the symmetric loop E should have a static bend. An obvious possibility is that the loop forms some noncanonically paired structure with one or more nucleotides excluded from the helix, making a "kink" in the backbone. Neither chemical protection data (Brunel *et al.*, 1991) nor NMR evidence (Zhang & Moore, 1989) provides any support for this idea; both studies agree that all bases are stacked within each strand. If this is the case, then loop E is qualitatively similar to A-tract DNA, which induces bending within the context of a standard helix (Wu & Crothers, 1984).

While the gel phasing experiments unequivocally show that loop E is bent, a good estimate of the associated bend angle is not obtainable from gel mobility experiments. In the first place, we have no calibration of bend angle versus relative gel mobility for RNAs with stiff bends. Second, the sensitivity of the gel mobility of loop E containing sequences to temperature and Mg^{2+} may mean that hinge-like flexibility of the loop is substantial. (The insensitivity of the twist angle to the environment suggests that there has not been significant rearrangement of the structure.) Comparisons with the gel mobilities of other bent RNA sequences with unknown degrees of flexibility (e.g., A_2 bulges) might therefore be misleading.

Helical Twist Associated with Internal Loops. A model for loop E from chloroplast ribosomes, based on chemical reactivity data, shows a ladder-like structure with very little helical twist and all three pyrimidines excluded from stacking with the purines (Romby *et al.*, 1988). In a similar study of the *E. coli* 5S rRNA, the loop E pyrimidines are protected from chemical modification and the loop was modeled as a roughly helical structure with no bulged bases (Brunel *et al.*, 1991). Our results clearly support the latter model. The *E. coli* sequence is nearly palindromic, with a $\frac{CGA}{GAU}$ sequence closing each end (C·G is the only Watson–Crick pair). This sequence is found at only one end of the eukaryotic loop E (with the G·C pair reversed), and it appears in other conserved loops such as the large subunit α-sarcin loop (Szewczak *et al.*, 1993). A cross link between the G and the U is readily induced by UV light in the eukaryotic sequence, and this is rationalized in the recent NMR-derived structure of the eukaryotic loop E by an unusually large 60° twist angle between noncanonical G·A and A·U pairs (Wimberly *et al.*, 1993). If this sequence adopts the same conformation in the eubacterial loop E, then its two occurrences should increase the total helical twist by ~50° over that of standard A form RNA. It is quite possible that this sequence is the source of the overwinding we observe. The sequence $\frac{TG}{GT}$ also has a high helical twist in A form DNA (44°), but this is compensated by low twists at the flanking steps to give an average close to 33° (Rabinovich *et al.*, 1988).

The two $\frac{CGA}{GAU}$ sequences in the *E. coli* loop E are oriented in opposite directions, and the central G·A pairs are separated by approximately one-half helical turn (6 base pair steps); any bending through the sequence will therefore be reinforced in the complete loop. It is tempting to speculate that this sequence is responsible for the observed bend, and this is a hypothesis which can be tested by distributing several of the sequences in phase throughout a long RNA.

There are now two structures of RNA internal loops available from NMR and X-ray crystallography, and in both of them the RNA backbone adopts a helical conformation. The structure of the eukaryotic loop E (9 nucleotides) has already been mentioned. The other loop studied was selected inadvertently, as the dimer of a hairpin structure (Holbrook *et al.*, 1991), and contains two U·C and G·U pairs. Our results indicate that the longer eubacterial loop E (14 nucleotides)

also adopts a conformation with nearly the A form average helical twist. It will be interesting to see whether this develops as a general principle of RNA internal loops. The gel phasing experiments described here provide an overall measure of helical twist that is complementary to the local conformational details revealed by NMR studies.

The effects of the two conserved G·U pairs on the behavior of loop E are surprising. The rather substantial differences in gel mobility between RNAs containing loop E and loop E + GU suggest that the overall bend and flexibility of the two sequences are quite different, though the difference under more stabilizing conditions (low temperature and added Mg^{2+}) is small. We have also seen that loop E alone is much more sensitive than loop E + GU to nicking by T_1 RNase at 37 °C in the absence of Mg^{2+} (data not shown), as if the G·U pairs help to stabilize the loop. How the G·U pairs might promote a particular loop E conformation is not obvious: the NMR data indicate that they are normal wobble pairs and suggest no direct interactions with the loop bases (Zhang & Moore, 1989). For an explanation one is left with effects propagated by base stacking through the single Watson–Crick G·C pair separating the G·U pairs and the loop. There is some evidence that single-base bulges may affect details of backbone conformation several base pairs removed (White & Draper, 1987) and that the anticodon stem sequences of initiator tRNAs affect the anticodon conformation (Seong & Rajbhandary, 1987), but substantial communication between base pairs at more than nearest neighbor distances is unexpected and merits further investigation.

L25 Binding to Loop E. The binding of ribosomal protein L25 to the internal loop E results in no major alteration in the overall helical twist of the loop. NMR results indicate that the binding of L25 changes some imino proton chemical shifts, but that the protein does not drastically disrupt the loop E structure (Kime & Moore, 1983). Intercalation of aromatic side chains into the RNA, which would be expected to unwind the loop structure, has been ruled out by NMR evidence (Kime & Moore, 1984). Extensive protein contacts in the deep major groove might be expected to unwind the RNA substantially. It is more likely that the protein hydrogen bonds to base features in the irregular minor groove of the loop, as is the case for specific synthetase recognition of a G·U pair in a tRNA stem (Musier-Forsyth *et al.*, 1991). Contacts of the protein with the RNA backbone, which are usually thought of as nonspecific, could also contribute to the specificity of binding by discriminating in favor of a unusually irregular or bent conformation of the backbone through the loop.

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