Single base bulges in small RNA hairpins enhance ethidium binding and promote an allosteric transition

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Received January 30, 1987; Revised April 14, 1987; Accepted April 27, 1987

ABSTRACT

A set of four RNA hairpin helices has been prepared by *in vitro* transcription with T7 RNA polymerase. The hairpins all contain the same nine base pair helix, but with an extra A, C, or U residue forming a bulge at one position; the fourth hairpin is a perfect helix with no bulge. The helix with a bulged A duplicates six base pairs of a helix in the 16S rRNA known to have an unusually high affinity for ethidium bromide [J. M. Kean, S. A. White, and D. E. Draper, *Biochemistry* **24**, 5062 (1985)]. Binding and chemical cleavage studies with ethidium or the reagent methidiumpropyl-EDTA-Fe(II) [MPE-Fe(II)] showed that i) the sequence CpG is a preferred intercalation site whether or not a bulge is present; ii) all three bulged bases enhance intercalation at the CpG sequence by an order of magnitude; and iii) intercalation in a bulged helix results in a concerted conformational change involving the entire helix backbone, again dependent on the presence of a bulge but independent of the particular base. These results suggest that an extra sugar-phosphate residue in an RNA helix backbone has a dramatic effect on the ability of the RNA to adopt new conformations. This effect could be an important reason for the conservation of bulges at certain positions in ribosomal and other RNAs.

INTRODUCTION

In double helical RNA and DNA a single base on one strand may be unpaired, forming a "single base bulge". In DNA, stabilization of such bulge structures by intercalating dyes has been suggested as a mutagenesis mechanism (1), and direct measurements of ethidium binding to synthetic DNAs support this conclusion (2). The possible functions of single base bulges in RNA are not known, though phylogenetic definition of conserved secondary structures in different RNAs clearly shows that single base bulges are a frequent structural motif (3). In at least two cases bulges are implicated as protein recognition features (4,5), and the formation of lariat structures in group II introns apparently requires a single base bulge (6). We have therefore thought it worthwhile to initiate a systematic study of the conformations of single base bulges in small RNAs; the information gained may be helpful in understanding the function of bulges in larger RNAs.

The starting point for our studies is our previous observation that a helix in the 16S rRNA containing a bulged A has an unusually high affinity for ethidium bromide (7). Since the helix has other unusual features (e.g. two potential G-A pairs) and is part of a larger structure probably containing significant tertiary interactions, it was not clear whether the single base bulge was responsible for the unusual intercalation site. To see if bulges in RNA can affect intercalator

binding, we have prepared a set of three homologous RNA hairpins containing A, C, or U bulged at the same position; a fourth hairpin has no bulged base. Six of the nine base pairs in the hairpin helix duplicate part of the 16S rRNA helix containing the ethidium binding site. As anticipated, all three bulges have a dramatic effect on the intercalation of ethidium in the helix. We also unexpectedly find that inclusion of a bulge in a helix has a profound effect on the conformational possibilities of the entire helix; this effect may be an important function of single base bulges.

MATERIALS & METHODS

Chemicals and reagents

All buffers were made up using water run through a Millipore "MilliQ" deionizer and filter. Solutions were autoclaved or filter sterilized to prevent nuclease contamination. Nucleoside triphosphates, poly(A)-poly(U), and V_1 nuclease were purchased from Pharmacia-P-L, ethidium bromide and diethylpyrocarbonate (DEP) from Sigma, and [^{32}P]cytidine bisphosphate from Amersham. MethidiumpropylEDTA (MPE) was a generous gift from Peter Dervan.

Preparation of RNA

RNA was synthesized by *in vitro* transcription of linear plasmid DNA, using T7 RNA polymerase purified from the overproducing strain BL21(pAR1219) (8). Preparation of the plasmids carrying the T7 promoter and transcription sequences has been described in detail elsewhere, along with the conditions used for T7 transcription (9). Both 5' and 3' [^{32}P]-end labeled RNA fragments were sequenced by standard techniques (10) to make sure that the expected sequence had been transcribed. Transcription reactions were carried out in 0.5 ml using 0.5 mg linear plasmid DNA, and the reactions were injected directly onto a Superose 12 gel filtration column (1.6 x 50 cm; Pharmacia). The peak of RNA transcript was ethanol precipitated and used for labeling reactions. 3' end labeling of RNA with [^{32}P]cytidine bisphosphate and T4 RNA ligase, and purification of the labeled material from denaturing electrophoresis gels, were carried out according to standard protocols (7, 10).

RNA cleavage reactions

All reactions were carried out at pH 7.6 and 37° in a buffer containing 0.1 M NaCl. DEP reactions were carried out approximately as described by Peattie & Gilbert (11), using 10% DEP reacting for 10 min in 20 mM MOPS buffer, followed by aniline cleavage. MPE-Fe(II) reactions were carried out as previously described (7), using 5 or 10 mM Tris as buffer; 2 mM dithiothreitol was added to initiate the cleavage reaction. V_1 nuclease required 1 mM MgCl_2 for activity, in 2 mM Tris buffer. Photochemical cleavage of ethidium-RNA complexes was carried out in 10 μL 0.1 M NaCl, 20 mM Tris, in the bottom of a 5 mm siliconized glass tube. The tube was placed in the beam of an argon ion laser, adjusted to give ≈ 2 watts of light at 514 nm in a ≈ 1 mm beam. The amount of cleavage obtained was sensitive to the exact placement of the tube in the beam. In all cases, an equal volume of 10 M urea, 20 mM Tris (pH 7.6) and xylene cyanol tracking dye was added at the end of the reaction, the mixture placed on ice, and loaded onto a

50% urea, 20% acrylamide sequencing gel soon thereafter. The gel was run at 50 watts for 1.5 hr, and exposed to pre-flashed film at -70° . A Joyce-Loebl Chromoscan 3 densitometer was used to scan the autoradiographs for quantitative measurements.

All four cleavage reactions used leave a 5' phosphate after reaction, while alkaline hydrolysis and T_1 ribonuclease (G specific), which were used as standards on the gels, leave a 5' hydroxyl. As a control to identify the positions of the bands relative to the standards, DEP cleavage (A specific) and S_1 nuclease digests under denaturing conditions (also leaving a 5' phosphate) were compared with alkaline hydrolysates and T_1 ribonuclease digests on the same gel.

Calculation of equilibrium binding constants was carried out as described previously (7). Note that the concentration of RNA in the reactions is between 10^{-9} and 10^{-8} M, so the concentration of RNA binding sites can be neglected in calculating the free dye concentration (10^{-7} to 10^{-5} M). Working at low concentrations also insures that the RNA is present as a monomeric hairpin and not as a dimeric, partially self-complementary helix. Gel filtration of the helices at concentrations in the micromolar range did not detect any dimerization, therefore the RNA should be a hairpin in our gel experiments.

RESULTS

Strategy for synthesizing small RNA hairpin helices

To synthesize RNA hairpin helices, we used T7 RNA polymerase and an appropriate DNA template. The set of hairpins discussed here was made by cloning synthetic DNA into a plasmid containing a T7 promoter immediately followed by a restriction site. After cleaving the plasmids with Sma I (CCCGGG), run-off transcription gave the RNAs shown in Figure 1. A fourth hairpin containing C as the bulged base has also been prepared; initial studies showed it to have the same properties as the A or U bulge hairpins, so it has not been studied in as much detail.

Figure 1. RNA hairpin sequences prepared by *in vitro* transcription of linear plasmid DNA by T7 RNA polymerase. The box in the bulge A hairpin indicates sequence and secondary structure identical to G656-G661 and C744-A749 in the *E. coli* 16S rRNA (3).

A

B

Figure 2. MPE-Fe(II) cleavage of RNA hairpins. **A**, cleavage of the bulge A and bulgeless helices with 1.0 μ M MPE-Fe(II). **B**, relative sensitivity of nucleotides in the bulge A and bulgeless hairpins to MPE-Fe(II) cleavage. Concentrations of MPE-Fe(II) used were bulge U and bulge A, 1 μ M; bulgeless, 10 μ M. Full length RNA is at the left of the densitometer scan.

Transcripts from strong T7 phage promoters all begin pppGGGAGA; at the time we prepared these plasmids it was not known how much these bases could be varied and still obtain good polymerase activity. The sequences we made, which all begin pppGGGUCU, generate a large quantity of short, abortive transcripts and only a small fraction of the triphosphates are incorporated into full length transcripts. While ample material was obtained for experiments using 32 P end-labelled RNA, obtaining even one A₂₆₀ unit of very pure material has been difficult. Subsequent work has shown that changing the sixth base in the transcript to a purine gives much

Figure 3. Double reciprocal plot of the variation in MPE-Fe(II)-induced cleavage with free MPE-Fe(II) concentration. ●, bulge A hairpin; ○, bulge U hairpin. The least squares best fit line gives a binding affinity of $3.0 \mu\text{M}^{-1}$; similar values are obtained from non-linear least squares fits to hyperbolic plots of the data.

more efficient transcription (9), and we have been able to prepare milligram quantities of other sequences for optical and other physical studies.

Single base bulges enhance MPE-Fe(II) intercalation at a CpG sequence

To see if the different hairpins shown in Figure 1 have different affinities for intercalators, we titrated ^{32}P end-labelled hairpins with MPE-Fe(II), a reagent containing the methidium intercalator linked to a moiety cleaving nucleic acids (12). The titrations were actually carried out by reacting the hairpins with a fixed MPE-Fe(II) concentration in the presence of increasing poly(A)-poly(U) competitor; the poly(A)-poly(U) improves the stability of the MPE reagent during the reaction (7). An autoradiograph comparing MPE-Fe(II) cleavage of the bulge A and bulgeless helices is shown in Figure 2A, and densitometer scans are shown in Figure 2B. It is clear from the results that about a 10 fold higher concentration of MPE-Fe(II) is required to obtain the same level of cleavage with the bulgeless helix as with the bulge A or bulge U helices. A quantitative treatment of the data, using band intensities from a densitometer tracing to construct binding curves, is shown in Figure 3. The bulge A and bulge U helices bind MPE-Fe(II) with nearly identical affinities of 4.3 ± 1.3 and $3.8 \pm 1.6 \mu\text{M}^{-1}$, respectively, while the bulgeless helix has an affinity of $0.3 \pm 0.03 \mu\text{M}^{-1}$ (values are averages of three or four independent determinations). Less extensive studies with a bulge C hairpin also gave MPE-Fe(II) binding constants in the same range as the other helices with bulges.

Figure 4. Cleavage of ethidium - hairpin complexes induced by 514 nm irradiation. The two different bulge helices were irradiated in the presence of 2.5 μM ethidium, while 10 μM ethidium was present with the bulgeless hairpin; irradiation was for 1, 2, or 5 min. Each hairpin was also irradiated for 2 min in the absence of ethidium (C). Other lanes on the gel are OH^- , alkaline hydrolysis, and T_1 , partial T_1 nuclease digestion under denaturing conditions. The hairpins are contaminated with a small amount of material one nucleotide shorter than full length, evident in the T_1 sequencing lanes. Therefore intense cuts are followed by a faint shadow band.

The site of the MPE-Fe(II) intercalation can be deduced from the cleavage intensity at each position along the helix backbone. From the known structure of an ethidium - RNA complex (13), it is expected that the EDTA-Fe(II) moiety of MPE-Fe(II) will be situated in the minor groove of the helix, and be closest to the sugar phosphate units to the 3' sides of the intercalation site (14). The most intense cleavages in the two bulge hairpins are at the sequence GpA on the 5' side of the helix and Gp(U or A)pA on the 3' side (Figure 2B). These intense cleavages are symmetrical about the CpG sequence, and imply that intercalation takes place between the two C-G base pairs.

MPE-Fe(II) reaction with longer DNA molecules gives fairly uniform cleavage (15). However, the MPE-Fe(II) cleavage pattern of the bulgeless hairpin is very similar to that of the bulge hairpins, with the two GpA sequences most readily cut (Figure 2B). CpG must be a preferred intercalation site (among the six different dinucleotide sequences available in these hairpins), even in the absence of a single base bulge.

To confirm the site of ethidium intercalation, we irradiated ethidium-RNA complexes with the 514 nm line of an argon ion laser. This treatment is known to result in cleavage of the backbone sugar and release of free base, similar to what is seen with MPE-Fe(II) cleavage (M. Hogan, personal communication). Figure 4 shows that cleavage occurs almost exclusively at the four nucleotides forming the preferred CpG intercalation site, in hairpins with and without the bulge (higher ethidium concentrations were present with the bulgeless helix to compensate for the weaker binding). Although the symmetries of ethidium and the CpG intercalation site would predict that the pair of C nucleotides and the pair of G nucleotides should each be cleaved at the same rate, this is clearly not the case. Asymmetrical cleavage is particularly pronounced in the bulge A hairpin, where G19 is cleaved much more efficiently than G8.

Cleavage at the loop G residue is also seen in all three helices, as well as very weak cleavage of the G nearest the 3' terminus. Nucleotides adjacent to these Gs, which should be cleaved if intercalation is taking place, do not react. Neither of these sites is suggested as an intercalation site by the MPE-Fe(II) reactions, and a hairpin loop base is probably not a good site for ethidium binding. We therefore suspect that a photochemical reaction takes place very readily with G residues, and that cleavage of these Gs reflects weak, possibly non-intercalative binding.

Ethidium competes with MPE-Fe(II) for binding

MPE-Fe(II) binding to nucleic acid helices is presumably similar to ethidium binding, and we previously demonstrated that ethidium competes with MPE-Fe(II) for binding to high affinity sites in a 16S rRNA fragment with a three to five fold weaker binding constant. The same result holds for the small RNA hairpins (Figure 5): addition of ethidium decreases the MPE-Fe(II) cutting efficiency. There was considerable variability in the data, which is probably due to the instability of the MPE-Fe(II) during the 20 min reaction. Poly(A)-poly(U) helps to stabilize the

Figure 5. Competition between ethidium bromide and MPE-Fe(II) for binding to RNA hairpins. The decrease in MPE-Fe(II) induced cleavage of the bulgeless (▲), bulge U (●), or bulge A (■) hairpins is plotted as a function of the ratio of free ethidium to free MPE-Fe(II) concentrations. The total MPE-Fe(II) concentration in the reaction was 1.0 μ M with bulge hairpins, and 10 μ M with the bulgeless. The curve is drawn presuming that ethidium binds with 3 fold weaker affinity than MPE-Fe(II) (7). Intensity units are arbitrary.

reagent and more reproducible competition curves are obtained, but calculation of the free MPE-Fe(II) and ethidium concentrations becomes difficult (16). From several competition experiments we estimate that the ethidium affinity for any of the hairpins is two to four fold weaker than the MPE-Fe(II) affinity.

Two bulge positions are almost equally effective at enhancing ethidium binding

Diethylpyrocarbonate (DEP) carbethoxylates the N7 position of adenosine (17). The reaction takes place with adenosine in bulge or loop regions of an RNA, but adenosine in double helical RNA is unreactive (4, 11). In the bulge A hairpin, either of two As (A17 or A18 in the sequence) can be written as the bulged base with the other paired to U6. There is no known reason to prefer one possible pairing over the other. DEP reacts readily with both A17 or A18, but not with the two other As in the molecule (Figure 6). This suggests that both bulge conformations are found. Control experiments with the bulge U helix show that A18, when paired to U6 and adjacent to a bulged base, is not reactive with DEP.

We thought it likely that intercalation at the CpG sequence would be enhanced only if the bulge A hairpin were in the same configuration as the bulge U hairpin, i.e. with A17 bulged.

Figure 6. DEP reactivity of A residues in the bulge A hairpin. Shown are densitometer scans of the reaction with the indicated ethidium concentration.

If this were the case, the ethidium - bulge A hairpin complex should react with DEP predominantly at A17. In titrations with ethidium, we tend to see a slight increase in the ratio of A17 to A18 reactivity (Figure 6), though the change is not large enough to obtain a good titration curve. At most, the equilibrium constant for switching from one bulge to the other changes by a factor of 1.5 when ethidium binds. Therefore, about the same enhancement of ethidium binding is found for either A17 or A18 bulged.

Ethidium intercalation affects the structure of the entire helix when a bulge base is present

As an attempt to detect structural changes occurring in the vicinity of the CpG sequence when ethidium binds, we digested the hairpin - ethidium complexes with V_1 nuclease. This nuclease requires an approximately helical RNA backbone for a substrate; the hydrolysis rate is sensitive to the exact conformation present (18). We expected that the V_1 nuclease digestion rate would decrease at the CpG site of intercalation, as ethidium distorts the backbone. The bulge A helix cleavage pattern in the presence of increasing ethidium bromide is shown in Figure 7. The CpG phosphodiester is digested weakly, and show a 2.5 to 3 fold decrease in cutting rate. However, major changes in the backbone reactivity also occur along the entire helix as ethidium binds, and include significant increases in the cutting rate (G3, U4, G10, and G19) as well as decreases (U6 - A9 and C15- A18). In general, residues near the middle of the helix cut less well in the presence of ethidium, and residues near the ends are cut more efficiently (Figure 8). Two observations indicate that V_1 is detecting a concerted change in helix conformation as the result of ethidium intercalation at the single, high affinity site. First, all the changes in phosphodiester sensitivity occur in parallel as ethidium is added, at sites of both enhanced and depressed digestion

Figure 7. V₁ nuclease digestion of RNA hairpins at increasing ethidium concentrations. **A**, bulge A helix. **B**, bulgeless helix. Ethidium concentrations are indicated, and the other lanes are marked as in Figure 4. Cleavage has occurred to the 3' side of the indicated base.

Figure 8. Histograms of the V_1 nuclease cutting rates with and without ethidium in the bulge A (A) and bulgeless (B) helices. Shaded bars represent cutting in the presence of 5 μM (A) or 24 μM (B) ethidium; hatched bars indicate cutting in the absence of ethidium.

rate. Second, the apparent ethidium binding constant deduced from changes in cutting intensity with ethidium concentration is $1.3 \pm 0.4 \mu\text{M}^{-1}$ (Figure 9), which agrees well with the binding constant determined by competition between ethidium and MPE-Fe(II) ($0.5 - 2 \mu\text{M}^{-1}$).

Both the bulge A and bulge U helices behave identically in this experiment (Figure 9), with the minor exception that U4 and G19 in the bulge U hairpin are less sensitive to V_1 nuclease

Figure 9. Relative V_1 nuclease cutting rate at G19 in the bulge A or U hairpins as a function of ethidium concentration. The curve is a single site binding isotherm with $K = 1.3 \mu\text{M}^{-1}$. ●, bulge A hairpin; ○, bulge U hairpin.

in the absence of ethidium. The bulgeless helix is also cut by V_1 nuclease, though with a different pattern of cleavage rates than the bulge helices. However, no significant changes in V_1 nuclease reactivity are found with ethidium, even at the higher concentrations needed to saturate the CpG sequence (Figure 8). Slight decreases in cutting rate were sometimes seen at very high ethidium concentrations ($>50 \mu\text{M}$), probably due to ethidium binding at sites other than CpG. (The CpG bonds, which might be expected to show some effect, are cut extremely weakly). Apparently the switch to a new helix conformation requires the presence of a bulge.

DISCUSSION

The preference of ethidium for CpG sequences

The MPE-Fe(II) cleavage data with the bulgeless helix demonstrates that methidium intercalates at CpG in preference to the other available sites, and this is confirmed by the photochemical cleavage of the hairpin - ethidium complex. Some sequence preference is expected, since ethidium binding to synthetic polymers shows two orders of magnitude variation with sequence (19), and pyrimidine-purine ribodinucleotides form intercalation complexes with ethidium much more readily than purine-pyrimidine dinucleotides (20). Since CpG is the only pyrimidine-purine sequence in the hairpins we have examined, ethidium binding to these RNAs may reflect a simple preference for pyrimidine-purine sequences over purine-pyrimidine dinucleotides.

Comparison of crystal structures of DNA in the A form (21, 22) with the crystal structure of ethidium bromide intercalated with two Watson-Crick base paired CpG dinucleotides (13) suggests two reasons why CpG may be a preferred intercalation site. First, the stacking of guanine bases with ethidium appears to be the main interaction stabilizing the complex. In a CpG sequence the two guanines on opposite strands overlap and trace out a figure already closely corresponding to the dimensions of the phenanthroline ring. Other sequences (such as GpC) would require a much larger distortion of the helix to achieve the same ethidium-guanine stacking. Second, intercalation requires the helix to unwind, and probably is accompanied by an opening of the base pairs towards the minor groove (23). CpG sequences have an unusually large propeller twist of the bases (24), which leads to 'cross chain clash' of the guanines in the minor groove (25). The clash is relieved (in A form DNA) by the same distortions required for ethidium intercalation: the helical twist is reduced and there is a major opening of the bases towards the minor groove (24). The opening produces the same sugar pucker in A form dCpdG (C3' *endo* for dC, C5' *endo* for dG) as seen in the ethidium - CpG crystal structure (13, 22). Thus, the distortions already introduced into an A form helix by a CpG sequence are very similar to the distortions required for ethidium intercalation, and the thermodynamic cost of intercalation may be correspondingly lower.

It is of course possible that larger sequence contexts influence the ethidium affinity for a particular dinucleotide intercalation site. Further experiments with defined sequence RNAs will be required to determine the generality of the CpG sequence preference observed here.

How do single base bulges affect helix structure?

Before carrying out these studies, we had thought that helices with A, C or U bulges would behave differently. The bulge conformations of two small DNA helices have been determined in NMR studies (26, 27): a bulged adenine is stacked into the helix ("intrahelical") while a bulged cytosine is exposed to solvent without interrupting base pair stacking ("extrahelical"). It seemed to us that the way the bulged base is accommodated by the helix would have a major effect on the intercalation of ethidium. However, our findings clearly show that the identity of the bulged base is irrelevant to ethidium binding: the binding enhancement is the same for each bulge, and a switch to the same altered helix conformation occurs after intercalation in each case. Also significant is the finding that a bulge at either position 17 or 18 in the helix leads to the same enhancement of ethidium binding. These observations suggest that the most important aspect of single base bulges is the introduction of an extra sugar-phosphate unit somewhere into the helix, and that the conformations of the different bulged bases are either identical or differ by only a small free energy, so that all bulges are able to have the same effect on ethidium binding and helix conformation. If we presume that a factor of two difference in MPE-Fe(II) or ethidium binding constant would have been detected, then free energy differences between conformations available to A or U bulges must be less than ≈ 0.4 kcal.

How an extra sugar-phosphate unit in the backbone is able to enhance ethidium binding

is suggested by our finding that the helices containing a bulge are able to adopt a new backbone conformation when ethidium intercalates. The helix distortions required to accommodate ethidium probably create "strain" (less favorable bond angles or base stacking) near the intercalation site. The extra sugar-phosphate unit may be able to relieve this strain by allowing a different conformation elsewhere in the helix; the two changes in conformation could be coupled by the sugar phosphate backbone and/or base stacking interactions. In the absence of a bulge there may not be enough flexibility in the helix to accommodate new conformations. We noted above that the C-G base pairs in a CpG sequence have a large propeller twist in A form helices; in contrast, the ethidium - CpG complex has nearly planar bases (22). If the propeller twist is reduced in the hairpin, base stacking with adjacent base pairs would be affected and this might have a "domino effect" on base pairs some distance from the intercalation site. Calladine & Drew (28) have argued that base stacking geometry and backbone conformations are tightly coupled; therefore a bulge, by allowing a different backbone conformation, may also allow a new base stacking arrangement.

It might be argued that the bulgeless helix shows no conformational switch with ethidium binding simply because intercalation at the CpG sequence occurs at higher ethidium concentrations, where ethidium binding to other sites might prevent the transition. We do not think this is likely. The MPE-Fe(II) cleavages and the direct irradiation of the ethidium - hairpin complex both suggest that ethidium intercalation still occurs with a high preference at the CpG site in the bulgeless helix. If a conformational switch similar to that seen in the bulge hairpins were occurring, we might expect to see an enhancement of V_1 nuclease activity at lower ethidium concentrations, followed by a decrease at higher concentrations. However, the only changes we see in V_1 reactivity in the bulgeless hairpin are small decreases at high ethidium concentration.

The concerted transition of the bulge hairpins to a new conformation upon ethidium binding fits the definition of an allosteric transition as a "discrete reversible alteration of the molecular structure" with the binding of an effector (ethidium) (29). It might be possible to include two CpG sequences a number of base pairs apart in a helix with a bulge, and observe cooperative ethidium binding in a true allosteric mechanism. In fact, studies of drug binding to synthetic helical polynucleotides have detected unusual binding isotherms that suggest an allosteric switch to a higher affinity conformation when the drug saturation reaches a critical level (18, 30). Physical mechanisms for these phenomena have been difficult to deduce. Our results suggest that allosteric transitions may be studied in small, defined sequence RNAs amenable to NMR and crystallographic techniques.

Functional possibilities of single base bulges

Peattie et al (4) suggested that single base bulges might function as protein recognition sites. Our results suggest that bulges could direct intercalation of aromatic amino acids to specific sites in RNAs. Bulges could also be important for obtaining precise changes in RNA conformation potentially required for the function of ribosomal RNAs, RNA enzymes, and for

mRNA splicing. Relatively small changes in helix twist, for instance, could have a dramatic effect on tertiary interactions in a large RNA.

The bulge A hairpin duplicates a sequence in the 16S rRNA thought to be part of the S15 binding site (31). This bulge is highly conserved among eubacteria and archaebacteria (32), though with interesting variations: the position of the bulge in the helix can vary by one or two bases, and the bulge can be A, U, or G. A pyrimidine - purine sequence is also conserved in the helix 5' to the bulge. Selection has thus acted in the way we would expect if enhanced intercalation at a pyrimidine-purine sequence were being selected. This is a weak, but intriguing argument that the bulge helix properties we observe are functionally significant for protein recognition in the ribosome.

Implications for "footprinting" experiments

The detection of ethidium binding by a change in V_1 nuclease reactivity is exactly analogous to "footprinting" experiments being carried out with RNA or DNA binding proteins (or drugs). Using different enzyme or chemical reagents, these experiments look for protection conferred by a binding protein on particular nucleotides, and are used to define the nucleic acid sequences or structures recognized by the protein. The results obtained with ethidium binding to the bulge helices suggest that a good deal of caution is needed in interpreting footprinting results: changes in reactivity can occur either as the result of a direct interaction shielding a nucleotide from the reagent, or as the result of a conformational change induced by protein binding elsewhere in the molecule. RNA molecules, with more complex tertiary structures than DNA, may be more susceptible to major conformational changes when proteins bind.

ACKNOWLEDGEMENTS

We thank Dr. M. Hogan for suggesting the laser-induced RNA cleavage by ethidium, and Drs. C. Friedhoff and K. Bowen for help in carrying out the laser irradiation. This research was supported by NIH grant GM37005. S.A.W. is supported by a fellowship from the AAUW Educational Foundation, and D.E.D. is the recipient of an NIH Research Career Development Award (CA01081).

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