Correction

BIOPHYSICS. For the article “H/ACA small nucleolar RNA pseudouridylation pockets bind substrate RNA to form three-way junctions that position the target U for modification,” by Haihong Wu and Juli Feigon, which appeared in issue 16, April 17, 2007, of Proc Natl Acad Sci USA (104:6655–6660; first published April 5, 2007; 10.1073/pnas.0701534104), the authors note that in Fig. 1A, the sequence of the 5′ side of the 5′ pseudouridylation pocket of the U65 H/ACA snoRNA, as well as a portion of the lower stem of the 3′ hairpin, were drawn incorrectly. Additionally, in the Fig. 1 legend and elsewhere in the text, the numbering of the substrate RNA, which was based on Bortolin et al. [Bortolin M-L, Ganot P, Kiss T (1999) EMBO J 18:457–469], differs by one nucleotide from that found in the genomic sequence (refer to the snoRNA database, snoRNA-LMBE-db, at www-snorna.biotoul.fr). The correct numbers for the pseudouridylation sites are 4427 and 4373 for the 5′ and 3′ pseudouridylation pockets, respectively. The corrected figure and legend appear below. These errors do not affect the conclusions of the article.

Fig. 1. Human U65 H/ACA RNA and substrate. (A) Sequence and secondary structure of U65 H/ACA snoRNA (9). (B) Sequences and secondary structures of the model U65p pocket (U65hp), the substrate rRNA (S14), and the U65hp/S14 complex. S14 contains the sequence of human 28S rRNA (4422–4434), including U4427 (underlined), which is targeted for pseudouridylation, and an extra U (lowercase) at the 5′ end.

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H/ACA small nucleolar RNA pseudouridylation pockets bind substrate RNA to form three-way junctions that position the target U for modification

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During the biogenesis of eukaryotic ribosomal RNA (rRNA) and spliceosomal small nuclear RNA (snRNA), uridines at specific sites are converted to pseudouridines by H/ACA ribonucleoprotein particles (RNPs). Each H/ACA RNP contains a substrate-specific H/ACA RNA and four common proteins, the pseudouridine synthase Cbf5, Nop10, Gar1, and Nhp2. The H/ACA RNA contains at least one pseudouridylation (ψ) pocket, which is complementary to the sequences flanking the target uridine. In this article, we show structural evidence that the ψ pocket can form the predicted base pairs with substrate RNA in the absence of protein components. We report the solution structure of the complex between an RNA hairpin derived from the 3′ ψ pocket of human U65 H/ACA small nucleolar RNA (snRNA) and the substrate rRNA. The snoRNA–rRNA substrate complex has a unique structure with two offset parallel pairs of stacked helices and two unusual intermolecular three-way junctions, which together organize the substrate for docking into the active site of Cbf5. The substrate RNA interacts on one face of the snoRNA in the complex, forming a structure that easily could be accommodated in the H/ACA RNP, and explains how successive substrate RNAs could be loaded onto and unloaded from the H/ACA RNA in the RNP.

NMR | structure | ribonucleoprotein particle | pseudouridine

Pseudouridine (Ψ) is the most frequently occurring posttranscriptionally modified ribonucleotide and is found in almost all tRNAs, rRNAs, and small nuclear RNAs (snRNAs) (1, 2). In human rRNA, there are ~95 Ψs, clustered in functionally important regions of 5.8S, 18S, and 28S rRNAs (3–5), such as the peptidyl transferase region of the 23S rRNA (6, 7). Conversion of uridine (U) to Ψ results in transfer of the glycosidic bond from the N1 position of U to the C5 position and is catalyzed by a variety of specific pseudouridine synthases (8). Although conversion of U to Ψ in tRNA and rRNA in prokaryotes generally is catalyzed by single-polypeptide enzymes, in eukaryotes and archaea the modification of rRNA and most spliceosomal RNAs requires H/ACA small nucleolar (sno) ribonucleoprotein particles (SNPs) and small Cajal body-specific (sca) RNPs, respectively (9–12).

The H/ACA snoRNPs contain a common set of four core proteins and a snoRNA that usually is specific to one or two substrate RNAs. The canonical H/ACA snoRNA has a bipartite structure containing conserved secondary structural elements in a “hairpin–hinge–hairpin–tail” arrangement (9, 10). The hinge connecting the two hairpins includes the consensus (H box) sequence 5’-ANANNA-3’ (where N is any nucleotide), whereas the 5’-ACA-3’ sequence in the tail always is located 3 nt from the 3′ end of the mature H/ACA snoRNAs. The two hairpins contain a large internal loop, called the pseudouridylation (ψ) pocket (Fig. 1). This ψ pocket is complementary in sequence to ~3–10 nt on either side of a UN in the substrate RNA, where U is the nucleotide to be modified and N is any nucleotide. The nucleotides on the 5′ and 3′ sides of the ψ pocket therefore are proposed to be “guides,” which form base pairs with substrate RNA, leaving the UN unpaired at the top of the pocket. Either one or both of the ψ pockets can be guides for pseudouridylation of specific USs. In budding yeast, the formation of all 44 ψs in rRNA is guided by 28 H/ACA snoRNAs (13).

In archaea, the H/ACA RNAs can have from one to three hairpins. The H/ACA RNAs also are found in the more recently identified scaRNPs (12), which are targeted to Cajal bodies, via a conserved Cajal body localization signal found in the hairpin terminal loop (14), and are involved primarily in pseudouridylation of spliceosomal snRNAs. There also are a few H/ACA RNPs that do not appear to be involved in pseudouridylation, including, e.g., vertebrate telomerase, which has a scaRNA domain at its 3′ end (15), and U17 (snR30 in yeast), which is required for ribosomal RNA processing (16).

The H/ACA RNP proteins in vertebrates and yeast are Cbf5 (17) (Dyskerin in humans) and the basic Nop10, Nhp2 (18), and Gar1 (19–21). Cbf5 is highly homologous to the pseudouridine synthase TruB (8), which is responsible for modification of the conserved Ψ55 found in the TΨC stem–loop (TSL) in almost all tRNAs. Biochemical studies have indicated that the pseudouridine synthase Cbf5 can assemble with Nop10 and either Gar1 or Nhp2 to form two different ternary complexes (22–26). In vitro, Cbf5 and Nop10 are the minimal proteins required with the H/ACA RNA to assemble a pseudouridylation-competent RNP, but this activity is facilitated by the addition of Gar1 (22).

Cocrystal structures of archaeal Cbf5–Nop10 (27, 28) and Cbf5–Nop10–Gar1 (29) complexes, and most recently an archaeal H/ACA RNP (30), have provided insight into the assembly, role of the proteins, and structure of the H/ACA RNP. In the protein complexes as well as the RNP, Nop10, which largely is unstructured in solution (27, 31), as well as Gar1 interact extensively with Cbf5 but not with each other. The archaeal H/ACA RNP for which the structure was determined contains a single-hairpin H/ACA RNA in complex with Pyrococcus furiosus Cbf5, Nop10, Gar1, and L7Ae, which in archaea replaces Nhp2 (30). Nop10 is sandwiched between Cbf5 and L7Ae, and the three proteins provide a linear platform for binding the H/ACA RNA, while Gar1 binds to one side and interacts only with Cbf5. Both Nop10 and Gar1 appear to play a role in forming or stabilizing the active site of Cbf5 (27–30). In the H/ACA RNP, there are extensive interactions between the bottom of the lower (P1) stem of the H/ACA RNA and the PUA domain of Cbf5 and between L7Ae and the top of the upper (P2) stem of the H/ACA RNA, but only a few, from Nop10 and Cbf5, to the bottom of P2 and the ψ pocket. The ψ pocket itself, where the substrate is proposed to bind, largely is dynamic and not well

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Abbreviations: sno, small nucleolar; RNP, ribonucleoprotein particle; sca, small Cajal body-specific; TSL, TΨC stem–loop; HPQC, heteronuclear single quantum correlation.

Data deposition: Coordinates and restraints for the 20 lowest-energy structures have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2PB9).

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defined in the crystal structure. Although substrate binding has been modeled (30), an outstanding question is how the substrate RNA interacts with the RNA in the snoRNP.

U65 is a human H/ACA snoRNA that guides the modification of human 28S rRNA at positions U4374 and U4428 (10, 32, 33) (Fig. 1). We previously determined the solution structure of the 3′ ψ pocket of U65 snoRNA (31). In the absence of rRNA substrate and proteins, the ψ pocket is partially (mismatch) base-paired to form an irregular helix flanked by A-form P1 and P2 stems. Here, we report the solution structure of the human U65 H/ACA snoRNA 3′ ψ pocket in complex with an oligonucleotide corresponding to its 28S rRNA substrate RNA sequence. The complex forms in the absence of proteins, and the substrate RNA binds on only one face of the pocket. The snoRNA/rRNA substrate complex has a unique structure with two offset parallel pairs of stacked helices and two unusual three-way junctions. Analyzed together with the structures of the archaeal H/ACA RNP (30) and TruB RNA (34, 35) complexes, the structure of the complex reveals that the H/ACA RNA functions to not only recruit its specific RNA substrate complex, the structure of the complex reveals that the H/ACA RNA substrate for docking into the active site.

Results

The 3′-Pseudouridylation Pocket of U65 H/ACA snoRNA Binds the rRNA Target in the Absence of Proteins. To study the interactions between H/ACA snoRNA and rRNA substrate, we used an RNA hairpin (U65hp) derived from the 3′ ψ pocket of human U65 H/ACA snoRNA and a 14-nt oligonucleotide (S14) derived from the rRNA substrate sequence as our model system (Fig. 1). U65hp comprises the 3′ ψ pocket and flanking stems P1 and P2. Additional non-wild-type sequence base pairs were added to the end of P1 for in vitro transcription efficiency by T7 RNA polymerase, and a UUUCG tetraloop was added to P2 for stability. S14 corresponds to the sequence flanking U4428 of human 28S rRNA that is predicted to interact with the 3′ ψ pocket (Fig. 1B). An additional U was added to the 5′ end of S14 in place of G4428 in wild-type rRNA.

To determine whether the rRNA substrate can bind U65hp in the absence of proteins, we monitored the titration of rRNA substrate (S14) with U65hp by using NMR spectroscopy (Fig. 2). The free S14 is in a single-stranded conformation with no observable imino resonances. Inmino proton spectra of free U65hp show resonances corresponding to the upper stem and UUUCG tetraloop, the lower stem, and a G9–C26 and mismatch A11–G24 bp in the ψ pocket (Fig. 2) (31). Free U65hp forms an irregular helix with A-form lower and upper stems and partial pairing and stacking in the internal loop. Thus, in the absence of substrate, this ψ pocket (internal loop) is in a “closed” conformation. Upon addition of S14 to U65hp, new imino resonances emerged, whereas imino resonances corresponding to nucleotides in the ψ pocket decreased in intensity, indicating formation of the complex in slow exchange with the free U65hp. Substrate binding can be monitored easily by the increasing intensity of a low-field shifted resonance corresponding to U5 in the complex [Fig. 2 and supporting information (SI) Fig. 6]. Imino resonances of nucleotides in P1 and P2 showed little chemical shift change, except for U5 and U23, which are located at the top and bottom of P1 and P2, respectively (Fig. 1). The new peaks were assigned to imino protons of nucleotides in two newly formed helices (Figs. 1 and 2) by using 2D NOESY and 15N-heteronuclear single quantum correlation (HSQC) experiments. These results indicate that the substrate RNA can bind the ψ pocket in the absence of protein cofactors. Furthermore, the substrate must displace the mismatch base pairs in the ψ pocket to bind.

Although the complex is in slow exchange with free RNA on the NMR time scale, binding is weak ($K_D \approx 200–300 \mu M$), and complex formation was observed by NMR only at millimolar concentrations of RNA. Addition of Mg$^{2+}$ and/or a higher NaCl concentration slightly stabilized the complex but did not result in significant spectral changes (data not shown).

Overview of the Solution Structure of U65hp/S14 Complex. The U65hp/S14 complex is in equilibrium with free components even at stoichiometric ratios. Therefore, most of the assignments and distance restraints were obtained from NMR spectra of samples prepared with one $^{13}C$,$^{15}N$ base-selectively labeled RNA and 1.5-fold excess of the other RNA unlabeled. Resonance assignments and structure calculations are described in Materials and Methods. A total of 768 nonredundant NOE restraints (for an average of 16 per nucleotide) and 288 experimental dihedral angle restraints were used in the structure calculation. The ensemble of the 20 lowest
Two Three-Way Junctions Are Formed upon Substrate Binding. Binding of substrate RNA to the ψ pocket results in the formation of unique intermolecular three-way and pseudo-three-way junctions (3H) at the upper (J2) and lower (J1) stems of the ψ pocket, respectively. The 3H at the top of the ψ pocket consists of three helices P2, P2S, and P1S, and a 2-nt loop (U41C42) connecting P1S and P2S [3HS2, according to International Union of Pure and Applied Chemistry (IUPAC) nomenclature]. The P1S helix is parallel to P2S, which is stacked coaxially with P2. At the lower part of the pocket, P1S, P2S, and P1 form the other intermolecular 3H (J1), where P1 and P1S are stacked coaxially on each other, and P2S is parallel to P1S. We call J1 a pseudo-3H because there is no covalent linkage between P1S and P2S at the junction.

Like all 3H, both J2 and J1 in the U65 snoRNA/rRNA complex have two helices that are stacked on each other to form a continuous helix, and there are no (or few, in the general case) nucleotides linking the two stacked helices. RNA 3Hs have been classified by Lescout and Westhof into three families, A, B, and C, based on the number of nucleotides in the loops connecting the three helices (36). The J2 (as well as J1) 3H is most similar to family C in the arrangement of helices. However, the junction is unusual among RNA 3H structures reported to date in the short lengths of the loops connecting P2 and P2S (0 nt) and P1S and P2S (2 nt), the absence of interactions of the longer loop with the minor groove of helix P2, and the absence of interhelical interactions (36). In family C, the longer loop is 3–9 nt, is structured like a hairpin, and interacts extensively in the minor groove of the adjacent helix. Of the two loop nucleotides, C42 is stacked partially on the top G24–C40 base pair of the P1S helix, whereas the base of the target U41 (U4428 in the free U65hp structure, are opened up to form two new base pairs with A48 and U35 of the substrate, respectively. To explain this change, we propose that the two helices PS, i.e., NOE cross-peaks were observed between H1 and H9 of U43 (Fig. 4 A and B) and between H1', H2', and H3' to U23 to H5 and H6 of U43 (Fig. 4 A and C). The helices are unwound slightly at both the P2/P2S (twist ≈ 43°) and P1/P1S (twist ≈ 38°) helical junctions, consistent with weaker sequential H6–H2' NOE cross-peaks (Fig. 4 A).

The sugar-phosphate backbone of the U65hp has two kinks, between U5 and U6 and between U23 and G24 (Fig. 3 C). These kinks result from an almost 90° rotation of U6 and G24 so that they can form base pairs with substrate nucleotides A48 and C40, respectively, at the bottom of P2S and top of P1S. The P1S and P2S helices are aligned parallel to each other, but there are no helical packing interactions between them; rather, there is a narrow gap (Fig. 3 D). The closest phosphate-to-phosphate distance between the adjacent helices is ≈ 7 Å.
A helicase would not necessarily be required for substrate RNA color-coded by location in helices as in Fig. 3 showing observed NOEs between U65hp U23 and S14 U43. Nucleotides are complex only (Figs. 3 and 5 stabilizing the complex. Significantly, the structure shows that the H/ACA RNP protein interactions may be important for components of the H/ACA snoRNP, although the weak binding suggests recognition does not require the presence of any protein component that targets rRNA sequence flanking the U to be modified. This human 28S rRNA) is exposed to solvent (Fig. 4). The backbone turns between U41 and C42. The unique arrangement of the J2 3H largely is because of the geometric restraints imposed by the existence of the P1 helix of the H/ACA RNA. Family C 3Hs often have apical loops that cap the two parallel helices, which interact with each other or with the neighboring helix. Here, the P1 helix functions in a similar manner to restrain the relative positions of the P2S and P1S helices. Likely because of the physical constraints imposed by formation of a 3H within an internal loop, J2 (as well as J1) also is unusual in that it folds in the absence of divalent cations or protein (37, 38).

Discussion

The Pseudouridylation Pockets of the H/ACA snoRNAs Are the Loading Docks for rRNA Target Sites. Our structural results show that the H/ACA snoRNA $\psi$ pocket can form the predicted base pairs with the target rRNA sequence flanking the U to be modified. This recognition does not require the presence of any protein components of the H/ACA snoRNP, although the weak binding suggests that H/ACA RNP protein interactions may be important for stabilizing the complex. Significantly, the structure shows that the substrate RNA interacts with the snoRNA on one face of the complex only (Figs. 3 and 5D). This geometry of binding means that a helicase would not necessarily be required for substrate RNA loading. Because the substrate RNA binds on only one face of the complex, and does not thread through, there would be no topological problem with binding substrates to both the 5′ and the 3′ $\psi$ pockets simultaneously.

In the crystal structure of the archaeal H/ACA RNP (30), the H/ACA RNA is tied down at each end by extensive interactions between the PUA domain of Cbf5 and the ACA tail and bottom 4 bp and between the archaeal H/ACA RNP-specific L7Ae and a kink–turn (39, 40) in the RNA at the top of the helix and 3′ side of the terminal loop (Fig. 5D). There are only a few protein interactions with the rest of the H/ACA RNA. The $\psi$ pocket is largely disordered, with 2 nt being too mobile to be modeled and 4 nt stabilized by crystal-packing forces. Thus, the $\psi$ pockets in the H/ACA RNP likely do not differ significantly from the free RNA (Fig. 5D) and are not prearranged for binding of the substrate RNA. The H/ACA RNA is oriented on the protein with the 5′ side away and the 3′ side proximal to Cbf5, such that the face that the substrate RNA binds to in our complex is exposed and ready for loading (and unloading) of the substrate RNA. Together, the structures of the U65hp/S14 complex and the archaeal H/ACA RNP illustrate how this could occur. The H/ACA RNA is believed to remain stably associated with the snoRNP for multiple rounds of pseudouridylation (24), although a very recent study indicates that only Cbf5 is irreversibly associated with the H/ACA RNA (41).

Coaxial Stacking of P1S on P1 Provides a Constant Distance from the ACA Tail to the Substrate Binding Pocket. The number of nucleotides from the top of the $\psi$ pocket to the ACA (or H box) always is 14–16 nt, but the loop size on the 3′ side varies from 3 to 10 nt. However, because P1 and P1S are stacked coaxially on each other to form a “continuous” helix, the distance between the ACA tail (or H box) and the substrate U will be approximately constant for all H/ACA substrate–RNA complexes. Thus, binding of the substrate RNA to the H/ACA RNA defines the distance from the ACA tail to the target U to be 14–16 bp for all H/ACA RNPs, as previously proposed based on modeling the substrate RNA on the crystal structure of an archaeal H/ACA RNP (30).

The Three-Way Junctions Position the Target U in the Active Site. Cbf5 is highly homologous to TruB, the pseudouridine synthase that modifies the conserved $\Psi$ found in the TSL in almost all tRNAs. Because there is no bound substrate in the archaeal H/ACA RNP, we first compared the U65hp/S14 structure with the TSL in complex with TruB (34). Superposition of P1S and the UC loop (J2) of U65hp/S14 on the upper stem–loop of TSL showed that binding of the substrate RNA to the $\psi$ pocket results in an RNA backbone conformation that mimics that of the TSL loop in the active site (Fig. 5A). The target U sticks out of the loop in the solution structure, but its position is not well defined. In the modeled complex based on the superposition, both the C and U nucleotides just need to be rotated to move into the active site positions equivalent to those in the TruB complex (Fig. 5A and B). A similar rotation, facilitated by a conserved histidine, is observed in the structures of free versus bound TSL (34, 35) (Fig. 5B). All H/ACA RNA–substrate RNA complexes are predicted to have two unpaired nucleotides in the substrate, including the target U, at the top of the pocket. This 2-nt loop, which is unique among RNA class C 3H structures reported to date, results in an absence of loop–helix interactions (although they might occur for some sequences) and therefore assures that the only sequence requirements are for complementarity between the 5′ and 3′ guides of the $\psi$ pocket and 3–10 nt on either side of the UN loop. Thus, all H/ACA RNA–substrate RNA complexes will form the same structure at J2, where the target U is located.

In the TruB–TSL complexes, the RNA loop and upper stem are bound in a deep cleft between the body of the protein and a protruding “thumb” (34). In our model of U65hp/S14 docked onto TruB, the thumb would sterically clash with major groove of P2S (Fig. 5A and SI Fig. 8). This thumb, which binds in the major groove of the TSL loop and helps clamp the RNA onto the protein, largely is deleted in Cbf5 (and Dyskerin). To model U65hp/S14 on the archaeal H/ACA RNP, we next replaced TruB–TSL with the archaeal H/ACA RNP by superimposing homologous regions of their respective pseudouridine synthases. The shorter thumb loop in Cbf5 largely is disordered in the H/ACA RNP (30) (Fig. 5C), as is the case for apoTruB (35, 42) as well as Cbf5–Nop10 (27–29). It has
been proposed that this loop would become ordered when substrate binds, interacting with the major groove of PS1 (30), as illustrated in Fig. 5C. The interaction between the shorter thumb of Cbf5 and the H/ACA RNA–substrate RNA complex would be expected to be weaker than the interaction of TruB with the TSL. We propose that the loss of the thumb clamp is compensated for by formation of P2S, which would help fix the RNA complex in place on Cbf5–Nop10.

Thus, H/ACA RNP requires the formation of both the intermolecular RNA helices 5' and 3' of the target U for the recognition and positioning of the target U for modification (Fig. 5D).

In the archaeal H/ACA RNP, the binding of the H/ACA RNA creates a 50° bend toward the protein in the helical axis of P1 and P2 (Fig. 5D). If P1 interacts with the H/ACA RNP proteins in the same way once the substrate is bound, there would need to be some rearrangement of the P1 helix relative to its position in the free complex for the ACA tail to interact with the PUA domain (Fig. 5). This interaction could be accommodated easily by some unwinding or bending at J1. Specific structures of three- and four-way junctions often are required for recognition by their target proteins (38) even though they rearrange on binding (37). Alternatively, the C-terminal PUA domain could move. A large domain movement of the TruB PUA domain, which is connected to the rest of the protein by a flexible hinge, is observed in the apoTruB versus RNA-bound TruB (35). It also is worth noting that deletion of the ACA tail does not abolish catalysis of an archaeal snoRNP (22). At the other end of the ψ pocket, the P2 helix would need to be reoriented only slightly to form the same interactions with Nop10 observed in the snoRNP. Pseudouridylation-competent snoRNPs can be reconstituted with aChf5, aNop10, and H/ACA snoRNA alone (22). Thus, the additional interactions between L7Ac and the kink-turn (39, 40) in archaeal H/ACA RNA are not required to tie down the RNA. Eukaryotic H/ACA snoRNAs do not have a conserved kink-turn sequence in the P2 stem-loop, and binding to Nhp2 is likely to differ somewhat (22).

In conclusion, the structure of the U65hp/S14 complex reveals how the RNA substrate could bind directly to the H/ACA snoRNA on the H/ACA snoRNP, adopting a structure that has a conserved distance from the ACA tail bound to the PUA domain of Chf5 to the target U and an RNA backbone conformation at the target U that can be easily modeled into the active site of Chf5. Thus, formation of both of the unusual 3H is required for proper interaction of the substrate RNA with the H/ACA snoRNP. Based on the structure of the complex and comparison to TruB–TSL interactions and the archaeal H/ACA RNP, we propose that substrate RNA binding to the H/ACA RNA induces a conformation of the H/ACA RNA–substrate RNA complex that is recognized by initial rigid docking followed by induced fit. The H/ACA RNP proteins are not required for positioning of the substrate RNA on the H/ACA RNA ψ pocket. The absence of topological constraints means that substrates for one or both ψ pockets in an H/ACA RNA can be loaded and unloaded successively for pseudouridylation without dissociation of the RNA from the RNP.

Materials and Methods

RNA Synthesis and Purification. Unlabeled, uniformly 13C,15N-labeled, and A, U, G, and C specifically 13C,15N-labeled RNA were prepared by in vitro transcription with appropriate unlabeled (Amersham, Uppsala, Sweden) and/or 13C,15N-labeled (Silantes, München, Germany) nucleoside triphosphates (NTPs) by using purified His6-tagged T7 RNA polymerase with synthetic double-stranded DNA templates and purified as described (43, 44). The RNA construct of human H/ACA snoRNA U65 3' ψ pocket, U65hp, was designed as described in ref. 31 (Fig. 1B). The substrate RNA, S14 (5'-UUCGGCUCUUCCUA-3'), which contains the target site sequence of rRNA (4423–4435) except for the G4422U...
substitution, initially was synthesized enzymatically as a 22-nt RNA (5’GGCCUUAAUUCGCGCCUCCUA-3’) with the additional 8 nt at its 5’ end. The RNA subsequently was cleaved at the A8pU9 step by a specifically engineered 10–23 DNAzyme that produces RNA fragments with 5’-OH and 3’ phosphate as described in ref. 45. The cleavage reaction (0.5 mM RNA/50 μM DNAAzyme/50 mM Tris Cl, pH 8.0/100 mM NaCl/60 mM MgCl2) was allowed to proceed at 37°C for 4 h and then quenched by the addition of EDTA to a final concentration of 100 mM. All purified RNAs were desalted and exchanged extensively into 5 mM sodium phosphate buffer (pH 6.0) and concentrated by using an Amicon filtration system. The U65hp/S14 complexes were prepared by titrating 0.5–1 mM U65hp into 1–1.5 mM S14 (A, U, G, or C selectively 13C,15N-labeled U65hp/unlabeled S14 and A + U + G, or C selectively 13C,15N-labeled S14/unlabeled U65hp).

NMR Spectroscopy and Structure Calculations. All NMR spectra were recorded at 288 K on Bruker (Billerica, MA) DRX 500-, 600-, and 800-MHz spectrometers. Spectra were processed and analyzed by using Bruker XWINNMR and Sparky 3.113 (T. D. Goddard and W. Glieder, University of California, San Francisco, CA). Resonances assignments were obtained from analysis of 2D NOESY, 1H-13C and 1H-15N HSQC, 3D HCCH-TOCSY, 2D DQF-COSY experiments (47) on the individual samples at 300 K. Final structures were calculated with no experimental restraint violations (distance angle restraints, following standard X-PLOR protocols. Structures with no experimental restraint violations (distance >0.2 Å and dihedral angles >5°) from the initial 200 calculated structures were subjected to refinement against 32 residual dipolar coupling data as described (53, 54). The grid search for the optimal values of the magnitude and asymmetry of the alignment tensor produced an optimal value of 〈Dz〉 = −45.0 Hz and R = 0.55, respectively. The force constants for the back-calculated residual dipolar coupling couplings gradually were increased from 0.001 to 0.2 kcal/mole. Experimental restraints and structural statistics for the 20 lowest-energy structures are included in SI Table 1. All structures were viewed and analyzed with MOLMOL (55), and figures were generated by using MOLMOL and Corel Draw.

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