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Unveiling substrate RNA binding to H/ACA RNPs: one *side* fits all

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Abstract

The H/ACA RNP pseudouridylation function on a large number of extraordinarily complex RNA substrates including pre-ribosomal and small nuclear RNAs. Recent structural data show that H/ACA RNPs capture their RNA substrates via a simple one-sided attachment model. However, the precise placement of each RNA substrate into the active site of the catalytic subunit relies on the essential functions of the RNP proteins. The specific roles of each H/ACA RNP protein are being elucidated by a combination of structural and biochemical studies.

An impressive list of noncoding RNAs is now known to guide chemical reactions on other functional RNAs^[1,2]. These RNAs, generally referred to as guide RNAs, secure substrate RNAs through Watson-Crick base pairings that help place the substrate RNA in the active site of the enzyme. The guide RNAs assemble with one or several partner proteins that are responsible for the actual catalytic processes. In guide-RNA mediated enzymatic processes, an intricate network of RNA-RNA, RNA-protein, and protein-protein interactions sustains the efficiency and specificity of the enzymes. Unlike stand alone enzymes, the presence of the guide RNA makes RNA-guided enzymes more versatile, although non-conventional, for selecting the site of chemical reactions.

A major class of noncoding RNAs in eukarya and archaea is the box H/ACA small nucleolar or small RNA (snoRNA or sRNA), which guides site-specific isomerization of uridine to pseudouridine (Ψ) in precursor ribosomal RNA (rRNA) and spliceosomal RNAs^[3–5]. Several unique members of this class also function in ribosomal rRNA processing in eukarya^[6] and telomerase maturation in vertebrates^[7]. As such, H/ACA RNA-mediated functions are essential to ribosome and spliceosome biogenesis^[8–10] and to chromatin maintenance^[7]. In addition to the collective effects of uridine isomerization on RNA stability and local environment^[11–13], the process of modification itself is believed to have chaperone effects on the complex process of ribosome folding and assembly^[14]. In H/ACA RNA-mediated ribosome biogenesis, H/ACA RNAs act on pre-rRNA following its transcription but dissociate prior to its folding and assembly with ribosomal proteins^[15]. Thus H/ACA RNAs must attune their functional cycles to the rate of ribosome production that in turn, sustains the needs of cell growth.

Each H/ACA RNA contains either one or two (or less frequently, three) hairpins interrupted by internal loops (Figure 1A). Common to these hairpin repeats is the presence of an H-box (ANANNA) or ACA-box (ACA) at the 3' tail (Figure 1A). In archaeal H/ACA RNAs, a kink-

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turn (K-turn) motif is also present at the base of the apical loop (Figure 1A). *In vitro* reconstitution of purified archaeal proteins and transcribed H/ACA RNAs demonstrates that a single hairpin unit of the H/ACA RNA is sufficient for both RNP assembly and pseudouridylation activities^[24,25]. The biochemical studies with the reconstituted RNPs further show that the internal loop, the 3' ACA tail, and the K-turn motif of the hairpin unit are all required for the *in vitro* functions. The internal loop sequences are complementary to the modification sites while the ACA tail and the K-turn motif are required for the association of H/ACA RNA with partner proteins^[24,25]. Unlike other known guide RNAs, H/ACA RNAs bind RNA substrates through base pairing between both strands of their internal loop and the substrate RNA, leading to a unique three-way junction constrained by a fourth helix underneath (Figure 1A).

All members of the H/ACA RNAs function with a set of conserved proteins that contain the catalytic subunit for the isomerization reaction itself. Using archaeal naming conventions, these proteins are Cbf5 (Nap57 or dyskerin in mammals), L7Ae (Nhp2 in eukaryotes/mammals), Nop10 and Gar1, all of which are critical to the pseudouridylation process. Cbf5 has sequence homology to TruB, a stand alone bacterial pseudouridylyase responsible for isomerization of U55 in tRNA^[16]. Nhp2 belongs to the family of K-turn binding proteins but interestingly, only archaeal H/ACA RNAs are known to contain the K-turn motif. This is in accord with the fact that the archaeal homologue of Nhp2, L7Ae, is a specific K-turn binding protein previously shown in the ribosome^[17] and the archaeal small ribonucleoprotein particles^[18]. Because there are no known functional homologues, less is known about Nop10 and Gar1. Intense efforts are devoted to searching for the structural basis for how H/ACA RNPs function, especially for the roles of accessory proteins. Previous structural studies already revealed the mode of H/ACA RNP assembly in the absence of substrate RNA. More recently, new insights into the roles of H/ACA RNP proteins in the placement of substrate RNA are obtained. This short review summarizes these structural findings with emphasis on the topic of substrate RNA loading and the functional roles of the H/ACA RNP components in this process. For other extensive reviews on H/ACA RNPs, readers are referred to those on biological function and biogenesis^[1,19], on assembly^[20–23], and on telomerase function^[7].

The tripartite protein core

In archaea, Cbf5, Nop10, and Gar1 are known to form a stable complex independent of RNA while L7Ae associates stably only with the guide RNA^[24,25]. Shortly after their biochemical identification and characterization, crystal structures of the archaeal Cbf5-Nop10 binary^[26, 27] and the Cbf5-Nop10-Gar1 ternary^[28] complexes were obtained (Figure 1A). The structures of these Cbf5-Nop10 and Cbf5-Nop10-Gar1 complexes, together with two previously determined structures of TruB in complex with a tRNA T-stem helix^[29,30], provided an initial glimpse of H/ACA RNP architecture.

The Cbf5-Nop10 dimer is similar to the Cbf5-Nop10-Gar1 ternary complex, suggesting Nop10 and Gar1 act independently of each other in assembly. Cbf5 is the catalytic subunit of the H/ACA RNP pseudouridylyase and the principle organizer of the ternary protein complex (Figure 1A). Cbf5 contains a larger N-terminal domain and a smaller C-terminal domain; both domains are similar to those of TruB. The N-terminal domain is catalytic and contains the consensus motifs of the TruB family of the pseudouridine synthases, including the strictly conserved aspartate^[29,30]. The recent reports that archaeal Cbf5 is capable of modifying U55 of tRNA attests to the strong similarity between TruB and Cbf5 structures^[31,32]. The C-terminal domain belongs to a family of RNA binding domains called PseudoUridine synthase and Archaeosine transglycosylase (PUA) domain^[33] and is thus predicted to bind the guide RNA.

Nop10 and Gar1 bind separately to two orthogonal faces of the elongated Cbf5 catalytic domain. The two domains of Nop10, a small β -domain tethered to a single helix, straddle across the longer face while Gar1 affixes to the shorter face of Cbf5 using one side of its all- β -domain (Figure 1A). The large distances of the two proteins from the catalytic site immediately preclude their direct roles in catalysis itself. Rather, the essentiality of Nop10 and Gar1 to the H/ACA RNP likely resides in their ability to facilitate association with the guide RNA and possibly the substrate RNA molecules. Biochemical and structural evidence has already alluded to this possibility. NMR analysis of yeast Nop10 in isolation detected a weak but specific binding activity for a guide RNA derived from human U65 RNA^[34]. Mammalian Gar1 was previously shown to be cross-linked to a 4-thioU substituted target uridine of a substrate RNA *in vivo* ^[35], suggesting its close proximity to the substrate RNA at one point during assembly and modification. In addition, structural homology searches showed that Gar1 folds similarly to the tRNA binding domains of the elongation factors EF-Tu and EF-G, although the potential RNA binding surface of Gar1 equivalent to the elongation translation factors is engaged in contacting Cbf5^[28]. The catalytic domain is thus bordered by a tripartite set of RNA binding domains and subunits (Figure 1A).

The previously determined TruB-RNA complex structures from the Ferré-D'Amaré and Stroud's laboratories^[29,30] provide valuable guidance regarding the substrate RNA placement within the core of H/ACA proteins. Based on the assumption that the uridine targeted by both H/ACA RNP and TruB bind similarly near the conserved aspartate residue, a model of the H/ACA RNP enzyme bound with the substrate RNA could be constructed^[28]. In this model, the catalytic domain of Cbf5 anchors the central pseudouridine pocket of the guide RNA and the base paired substrate RNA while the PUA domain and Nop10 assist with placement of the lower and upper stem, respectively (Figure 1A). This RNP architecture places L7Ae at the top of the upper stem where the K-turn resides and near where Nop10 binds. In spite of no known K-turn motif in eukaryotic H/ACA RNAs, Nhp2 has already been shown to associate directly with Nop10 in a Cbf5-dependent fashion^[35], thereby arguing for an evolutionarily conserved model of assembly. Although there is still no structure of a fully assembled holoenzyme to date, many features of this model are now confirmed or updated in the recent studies of H/ACA RNA-protein complexes discussed below.

Dual anchoring of guide RNA

The seminal work by Li and Ye in determination of a guide RNA bound with all four proteins but free of substrate RNA provided a remarkable mechanism of guide RNA-protein interactions ^[36]. Indeed, the lower and upper stems are orientated to interact with the PUA domain and the Nop10-L7Ae-Cbf5 cluster, while the pseudouridine pocket is positioned at the center of the Cbf5 catalytic domain (Figure 1B). Due to the absence of a substrate RNA, the Ψ pocket in this structure is disordered or engaged in crystal packing interactions^[36]. This structure explains qualitatively the dominant activity of Cbf5 and the interdependence between its catalytic and PUA domains in binding the guide RNA^[24,27].

The most striking features of the RNA-protein interactions occur at the two ends of the guide RNA. The K-turn motif of the upper stem is bound to L7Ae, which in turn interacts with a composite protein surface formed between Nop10 and Cbf5. As a result, the upper stem wraps tightly around the catalytic domain of Cbf5 and passes over the interdomain loop of Nop10 (Figure 1A & 1B). Here, the upper stem establishes sequence independent interactions involving its phosphate backbone and minor groove with conserved polar and charged residues of Nop10. At the opposing end, the 3' ACA tail is engaged in sequence specific interactions with the PUA domain of Cbf5. The two conserved adenosine residues confer binding specificity for the PUA domain by being buried in a RNA-protein hybrid pocket and by formation of adenine-specific hydrogen bonds. This is the second structure of the PUA RNA binding domain

bound with a trinucleotide. The first is of the PUA domain in the tRNA modification enzyme, archaeosine tRNA-guanine transglycosylase, which has been shown to interact specifically with the 3' CCA of tRNA at the same relative location by a similar mechanism^[37,38].

Single-sided attachment of substrate RNA

At the secondary structure level, the guide RNA appears to secure the substrate RNA by a complex base pairing arrangement. The sequences flanking the target uridine base pair with the two strands of the pseudouridine pocket, leading to a three way junction around the target uridine. Unique among all other known three way junction RNAs, the pseudouridine pocket-substrate RNA complex is further restrained by a fourth helix that fastens together two of the three arms of the junction. The four helices could, in principle, intertwine in multiple fashions whether the proteins are present or not. Two structures of the pseudouridine pocket bound to a substrate RNA and accompanied by biochemical characterization were produced almost simultaneously by the Feigon and Moore laboratories^[39,40] (Figure 2). Both RNA complexes are derived from the human U65 snoRNA^[41] but represent the two separate H/ACA units. These structural studies revealed a simple single-sided attachment topology that allows successive docking of the substrate RNA.

In space, the four helices are arranged into two parallel pseudo-continuous helices. The lower stem, P1, of the guide RNA coaxially stacks with the 3' substrate binding helix, SH2 (PS2 in ref. ³⁶, SBH2 in ref. ³⁹, or P1S in ref. ⁴⁰), and the upper stems, P2, of the guide RNA stacks similarly to the 5' substrate binding helix, SH1 (PS1 in ref. ³⁶, SBH1 in ref. ³⁹, or P2S in ref. ⁴⁰) (Figure 2A). The transitions from P1 to SH1 (junction J1) and from SH1 to SH2 (junction J2) are facilitated by large rotations of the guide RNA nucleotides at the two junctions (Figure 2B). Owing partly to the differences in secondary structure, the overall shapes of the substrate strands are different between the two structures. This is not surprising and likely reflects a diverse distribution of RNA structures in isolation. When bound to proteins, however, the substrate strand is expected to conform to the enzyme pocket in order to correctly position the target uridine. Jin et al. coined the name “ Ω -motif” based on the Ω -shape of the U65 5' guide-substrate complex^[39]. Both structural studies clearly establish that the substrate RNA, which can be thousands of nucleotide long *in vivo*, access the pseudouridine pocket from one side of the guide molecule (Figure 2A). As noted by Wu and Feigon and Jin et al.^[39,40], the one-sided binding is significant, not only because most RNA substrates are large and complex but also because the RNP enzyme would allow successive binding of the substrate without the requirement for helix unwinding factors such as a helicase. Interestingly, this RNA motif may exist in other biological contexts as well. A viral RNA was found to use this motif between the polyA tail and a polyU pocket to escape the host polyA-dependent degradation^[42].

Roles of proteins on substrate RNA placement

A second recent advancement in understanding substrate RNA binding came from the crystal structure of a substrate-bound archaeal subcomplex containing three of the four essential proteins^[43]. In this structure, the protein L7Ae and its corresponding RNA binding site are not present and will thus be addressed as the L7Ae-minus structure (Figure 2). Strikingly, the target uridine in the subcomplex is about 11 Å away from the catalytic site and is unable to be modified. Structural analysis and the complementary fluorescence studies summarized below provide a molecular basis for the essential roles of the H/ACA proteins in the placement of the substrate RNA.

Like those observed in isolation, the guide-substrate RNA complex in the presence of proteins also forms two pseudo-continuous helices (Figure 2A). However, the two pseudo-continuous helices are no longer in parallel but are rather inclined at a $\sim 120^\circ$ angle. The restructuring is due primarily to a curved RNA binding surface of Cbf5 and as a result of a small rigid swing

around the junctions J1 and J2 (Figure 2B). The direction of the rotation positions the target RNA in a favorable orientation for its delivery into the active site.

In the L7Ae-minus structure, the protein-RNA contacts occur between Cbf5 alone and the RNA (Figure 2B). In addition to the nearly identical interactions between Cbf5 and the lower stem and the 3' ACA tail as those in the substrate-free H/ACA RNP structure^[36], additional contacts are observed between Cbf5 and the minor groove of the substrate binding helix, SH2. Consistent with the requirement for Cbf5 to bind and to modify substrate RNAs of varying sequences, the protein-RNA interactions in this region are predominantly sequence-independent (Figure 2B). These include positive residues contacting phosphoribose backbone and imidazole stacking on nucleobases (Figure 2B).

The most revealing finding about L7Ae in substrate RNA placement comes from comparing the L7Ae-minus structure^[43] with the structure of substrate-free complex containing all four proteins (L7Ae-plus structure)^[36]. In the two structures, the lower stem P1 binds analogously to the PUA domain of Cbf5 but the upper stem P2 exhibits a large orientational difference, implicating a key role of L7Ae in anchoring the upper stem (Figure 3A). Biochemical evidence is already available that supports a critical role of L7Ae^[24,25]. More directly demonstrated in this study, by monitoring 2-aminopurine fluorescence signals originated from the nucleotide immediately 3' to the target uridine, L7Ae is able to completely move the substrate RNA in the H/ACA RNP from an undocked to a fully docked state^[43]. Strikingly, this process requires the catalytic aspartate, suggesting an involvement of the catalytic residue in the placement of the substrate RNA. The molecular basis for the L7Ae-induced conformational change is reasonably modeled by superimposing only the upper stem of the L7Ae-minus structure to that of the L7Ae-plus structure^[36] while maintaining the coaxial stacking of the substrate binding helix, SH1. In the transformed guide-substrate RNA complex, the target uridine can be readily placed at the position near the catalytic residue with adjustment only in the junction regions (Figure 3A).

Conclusion and Outlook

Recent structural studies on H/ACA RNPs including the substrate RNA underscore the important roles in the placement of the substrate RNA by both guide RNA and proteins. A remarkable revelation arises from comparison of H/ACA RNP structures in the absence and presence of L7Ae (Figure 3B). Supported by both structural and biochemical evidence, L7Ae critically anchors the upper stem of the guide RNA, which sets the stage for the placement of the target uridine at the active site of Cbf5. The short history of H/ACA RNP studies demonstrates that structures representing possible assembly intermediates are necessary in order to disclose the twists and turns possible by RNA and protein molecules in preparation for their destined functions. These “molecular movies,” together with complementary biochemical studies, will provide powerful tools to dissect the mechanism of RNA guided processes.

H/ACA RNP substrate RNA binding and unloading is an integral part of the ribosome maturation process. If each ribosomal RNA transcript is assumed to transit through the phase of snoRNA binding prior to its folding and assembly with ribosomal proteins, then the overall rate of RNA modification and processing may be the rate-limiting step of ribosome production. Contrary to its central importance, structural studies of substrate RNA association with snoRNAs and with assembled RNPs are only at its beginning stage.

The structural and biochemical characterizations of H/ACA RNPs raise additional mechanistic questions with regard to substrate loading and unloading in this RNA-guided process. First, what is the role of the accessory protein Gar1, if any, in the process of substrate binding? Although a previous study has placed Gar1 close to the substrate RNA during assembly and

catalysis, no structural or additional biochemical data is currently available that can explain this observation. Intriguingly, in eukaryotes, a number of assembly factors assist the assembly of early H/ACA RNP subcomplexes in a cell compartment-specific manner^[44]. One of these factors, Naf2, contains a domain homologous to Gar1 and is believed to compete with Gar1 for the same Cbf5 interface outside nucleolus or Cajal bodies^[45,46]. Does the compartment-specific binding of Gar1 have a role in temporal and spatial regulation of substrate binding? A second interesting mechanistic question is directed to substrate specificity. Although recruited through the similar Ω -like motif to the guide RNA, are all target sites surrounded by different sequences modified with the same efficiency, and if so, what is the molecular basis responsible for the functional convergence? The three observed guide-substrate RNA structures already exhibit a diverse range of structures near the target uridine, although none of these structures represents a fully assembled H/ACA RNP. Finally, once the substrate is loaded into the active site, how is the remarkable isomerization reaction instigated and how is the product released? These questions await a high resolution structure of the fully assembled H/ACA RNP, including substrate RNA and the complementary biochemical studies.

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Figure 1.

A. Secondary structures of H/ACA RNA and substrate RNA complex and the arrangement of the four proteins. Inset illustrates detailed features of the pseudouridine pocket (Ψ pocket) and its interaction with the substrate RNA. “K-turn” stands for kink-turn RNA motif (see ref. ¹⁷). “N” denotes a RNA nucleotide of any sequence and “ Ψ ” denotes the uridine targeted for modification. Two sets of the Cbf5-Nop10-Gar1 complex (Ref. 28, PDB ID: 2EY4) and L7Ae are believed to be bound to the two hairpin units of the H/ACA RNA. **B.** The tertiary structure of a single hairpin unit H/ACA RNA (gray and orange) bound with all four proteins in the absence of the substrate RNA (reference 36, PDB ID: 2HVY). Note the linear RNA-protein interface bypassing the catalytic aspartate.

A

B

C

Figure 2.

Three structures of H/ACA RNP involving substrate RNA reveal a one-sided attachment model. **A.** Overall view of the three substrate-containing structures. Both U65 5' (PDB ID: 2PCW, Ref. 39) and 3' (PDB ID: 2P89, Ref. 40) guide-substrate complexes are determined by NMR and are represented by one of the ensemble of models. Substrate RNA is colored in red and the target uridine is shown in yellow spheres. Note the wide range of substrate RNA shapes. **B.** Illustration of how two helical junctions facilitating transitions between the two pseudocontinuous helices in the U65 3' guide-substrate RNA complex (PDB ID: 2P89, Ref. 40). Rotations of the junction nucleotides are indicated by arrows. Small gray plates indicate the stacking sites of the pseudocontinuous helices of the junctions. **C.** In the L7Ae-minus structure (PDB ID: 2RFX, Ref. 43), protein residues are observed to line the minor groove of the pseudocontinuous helix comprised of P1 and SH2. Note that junction J2 is an important site for protein interactions.

Figure 3. Structural comparison of the L7Ae-minus (substrate-bound) and L7Ae-plus (substrate-free) RNP structures reveals a critical role of L7Ae in positioning the guide-substrate complex. **A.** Superimposed guide RNA structures of the L7Ae-plus (Ref. 36, +L7Ae) and L7Ae-minus (Ref. 43, -L7Ae) complexes. To facilitate visualization, the upper stem of the guide RNA of the L7Ae-minus structure was extended to include the kink-turn motif and the bound Gar1 and L7Ae proteins are removed. **B.** A schematic depicting protein-facilitated structural transitions of the H/ACA guide RNA (yellow cylinders) that place the substrate RNA (red) in the active site. Directions of motions are indicated by dashed arrows.