RNA-Guided RNA modification: functional organization of the archaeal H/ACA RNP

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In eukaryotes and archaea, uridines in various RNAs are converted to pseudouridines by RNA-guided RNA modification complexes termed H/ACA RNPs. Guide RNAs within the complexes base-pair with target RNAs to direct modification of specific ribonucleotides. Cbf5, a protein component of the complex, likely catalyzes the modification. However, little is known about the organization of H/ACA RNPs and the roles of the multiple proteins thought to comprise the complexes. We have reconstituted functional archaeal H/ACA RNPs from recombinant components, defined the components necessary and sufficient for function, and determined the direct RNA–protein and protein–protein interactions that occur between the components. The results provide substantial insight into the functional organization of this RNP. The functional complex requires a guide RNA and each of four proteins: Cbf5, Gar1, L7Ae, and Nop10. Two proteins interact directly with the guide RNA: L7Ae and Cbf5. L7Ae does not interact with other H/ACA RNP proteins in the absence of the RNA. We have defined two novel functions for Cbf5. Cbf5 is the protein that specifically recognizes and binds H/ACA guide RNAs. In addition, Cbf5 recruits the two other essential proteins, Gar1 and Nop10, to the pseudouridylation guide complex.

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guide RNA with the target RNA positions the substrate nucleotide for 2′-O-methylation by bifilarin (Cavaille et al. 1996; Kiss-Laszlo et al. 1996).

Much less is known about the structure and function of the pseudouridylate guide RNPs or H/ACA RNPs. Four proteins have been identified as components of H/ACA RNPs: Chf5, Gar1, L7Ae (Nhp2 in eukaryotes), and Nop10 (Bousquet-Antonelli et al. 1997; Henras et al. 1998; Lafontaine et al. 1998; Watkins et al. 1998; Dragon et al. 2000; Watanabe and Gray 2000; Rozhdestvensky et al. 2003). In yeast, these proteins are found associated with H/ACA guide RNAs, and disruption of the corresponding genes affects pseudouridylation (Bousquet-Antonelli et al. 1997; Henras et al. 1998; Lafontaine et al. 1998; Watkins et al. 1998). The sequence and structure of Chf5 suggest that it is a pseudouridine synthase (Koonin 1996, Lafontaine et al. 1998; Zebarjadian et al. 1999; Charette and Gray 2000; Hoang and Ferre-D’Amare 2001). The precise roles of the other proteins are not known. It is not known whether these four proteins comprise the full set of proteins required for RNA-guided pseudouridylation. It is also not known whether the essential roles of the proteins in vivo reflect direct involvement in modification or critical upstream functions (e.g., stabilization or trafficking of guide RNAs). The pseudouridylation guide RNAs are comprised of one to three hairpins, each of which contains a bipartite guide sequence within an internal loop (pseudouridylation pocket) and is followed by a conserved sequence element, either box H or box ACA [see Fig. 1A; Balakin et al. 1996; Ganot et al. 1997b; Tang et al. 2002]. Due largely to the technical difficulties that have been encountered with the protein components of H/ACA RNPs from eukaryotes, there is very little information about the organization and composition of functional complexes.

In this work we report the first reconstitution of RNA-guided RNA pseudouridylation from recombinant components. We have reconstituted functional H/ACA RNPs using components from the hyperthermophilic archaeon Pyrococcus furiosus. Our results demonstrate that each of four proteins, Chf5, Gar1, L7Ae, and Nop10, and the guide RNA are essential, and that set of five components is sufficient for function in vitro. The reaction depends upon the pseudouridylation pocket, K-turn, and box ACA sequence within the guide RNA. We have also mapped the direct RNA–protein and protein–protein interactions between the components of the archaeal pseudouridylation guide complex. Surprisingly, we have found that Chf5, the presumptive pseudouridine synthase, interacts directly and specifically with the H/ACA guide RNA. The interaction of Chf5 with the guide RNA depends on the signature motif, box ACA, and the pseudouridylation pocket (and also to some extent on sequences in the terminal loop of the hairpin), but does not depend on the K-turn. In addition, the archaeal Chf5 protein can specifically recognize eukaryotic H/ACA RNAs. Our results suggest that the number of molecules of Chf5 bound to an H/ACA RNA correlates with the number of RNA hairpin units. As has been reported previ-
ously (Rozhdestvensky et al. 2003), L7Ae also interacts directly with the H/ACA RNA via the K-turn. Our work indicates that L7Ae does not interact independently with the other protein components of the RNP and also is not required for the interaction of the other proteins with the guide RNA. The other two essential proteins, Gar1 and Nop10, do not interact with the guide RNA in the absence of other proteins. We have found that Gar1 and Nop10 each interact independently with Cbf5, which mediates the association of these two proteins with the H/ACA guide RNA.

Results

Requirements for RNA-guided RNA pseudouridylation

Proteins with sequence homology to the four proteins associated with eukaryotic pseudouridylation guide RNPs are encoded in archaean genomes, but with the exception of L7Ae, these proteins have not been characterized (Watanabe and Gray 2000; Rozhdestvensky et al. 2003). In order to assess the potential role of the four archaean proteins in RNA-guided pseudouridylation, we investigated whether a functional RNP complex could be reconstituted in vitro using proteins and RNAs from P. furiosus.

We used Pf9, a single hairpin H/ACA RNA from P. furiosus, as the guide RNA for the majority of our work. Pf9 was identified as a potential noncoding RNA by Klein et al. (2002) in a computational screen for GC-rich regions in the AT-rich genomes of hyperthermophilic archaea. We have determined that this RNA is an H/ACA RNA (see Fig. 1A) and verified the corresponding modification at the predicted target site (U910) in H/ACA RNAs in archaea (Rozhdestvensky et al. 2003). In order to assess the potential role of the four archaean proteins in RNA-guided pseudouridylation, we investigated whether a functional RNP complex could be reconstituted in vitro using proteins and RNAs from P. furiosus.

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We incubated the radiolabeled substrate RNA with unlabeled Pf9 guide RNA and various combinations of the four purified proteins (Fig. 1C). To test for pseudouridylation of the substrate, we extracted and nuclease-digested the RNA, separated uridines and pseudouridines by thin layer chromatography, and examined the products by autoradiography. No pseudouridylation was observed in the absence of proteins (Fig. 1C, lane 1) or in the absence of Pf9 RNA (Fig. 1D, lane 4). In addition, no single protein, including the pseudouridine synthase Cbf5, was found to catalyze pseudouridylation of the rRNA substrate (data not shown). However, pseudouridylation was observed upon addition of all four proteins and the guide RNA (Fig. 1C, lane 2). Importantly, the absence of any one protein from the reaction resulted in substantial loss or elimination of pseudouridylation activity (Fig. 1C, lanes 3–6). The results indicate that these four proteins, which were implicated in RNA-guided pseudouridylation on the basis of homology to eukaryotic H/ACA RNP proteins, function in this process in P. furiosus. Moreover, our results demonstrate for the first time that the activity of an H/ACA guide RNP depends on all four proteins, Cbf5, Gar1, Nop10, and L7Ae, as well as the guide RNA in vitro.

We then tested the importance of conserved elements of the guide RNA in function (Fig. 1D). We incubated the substrate RNA with the four proteins and various Pf9 mutant RNAs. Disruption of box ACA, the pseudouridylation pocket, or the K-turn eliminated or severely reduced function (Fig. 1D, cf. lanes 1–3 and 5). Thus, function of the complex in vitro also depends on at least three important elements of the guide RNA: the signature motif [box ACA], the pseudouridylation pocket, and the L7Ae-binding site [K-turn] (see Rozhdestvensky et al. 2003).

Mechanism of association of Cbf5 with H/ACA guide RNAs

One key issue is the mechanism by which the enzyme (Cbf5) associates with the guide RNAs. In the case of C/D modification guide RNPs, it is clear that the association of the enzyme [fibulillarin] depends on prior binding of the other protein components of the RNP (Omer et al. 2002; Rashid et al. 2003; Tran et al. 2003). Interestingly, the protein that recognizes C/D RNAs and initiates assembly of the C/D complex is a common component of C/D and H/ACA RNPs in archaea: L7Ae (Kuhn et al. 2002, Rozhdestvensky et al. 2003). Furthermore, L7Ae has also been shown to bind directly to archaeal H/ACA RNAs via K-turns (Rozhdestvensky et al. 2003). Therefore it seemed likely that L7Ae might also be involved in the assembly of the H/ACA proteins on H/ACA RNAs in archaea.

We tested the ability of each of the four H/ACA RNP proteins to interact with the H/ACA guide RNA [Pf9] in the absence of the other proteins by gel mobility shift assay (Fig. 2A). Consistent with a previous study (Rozhdestvensky et al. 2003), we found that L7Ae interacts with Pf9 and that the interaction depends on the K-turn motif of the RNA (Fig. 2A,B). Surprisingly however, we found that Cbf5 also interacts with Pf9 in the absence of the other H/ACA RNP proteins. The apparent Kd of the interaction between Cbf5 and Pf9 (estimated as the concentration of protein resulting in half-maximal binding of the input RNA) was ~450 nM [Fig. 2C; data not shown]. Cbf5 failed to interact with P. furiosus C/D RNAs sR2 and sR29 and human tRNA methyltransferases (Fig. 2D), indicating that the direct interaction of Cbf5 with the guide RNA is specific. Finally, we found that Nop10
Figure 2. Cbf5 interacts directly and specifically with Pf9 H/ACA guide RNA. Direct interactions of proteins with radiolabeled RNAs were investigated by native gel mobility shift analysis and autoradiography. (A) Pf9 RNA was incubated with L7Ae. (B) The K-turn of Pf9 was disrupted and the mutant protein (−) was incubated with increasing concentrations of Cbf5 (0–2000 nM) to assess the apparent Kd of the observed interaction. (C) Pf9 RNA was incubated with L7Ae. (D) Cbf5 was incubated with non-H/ACA RNAs including P. furiosus H/ACA sRNAs sR2 and sR29 and a human tRNA to assess the specificity of the observed interaction.

and Gar1 do not interact with the guide RNA independently (tested over a range of protein concentrations up to 600 nM and 10 µM, respectively, data not shown). These results indicate that both the pseudouridylase synthase Cbf5 and L7Ae interact directly with the guide RNA, but that the interactions of Gar1 and Nop10 with the guide RNA are likely mediated by the other proteins.

To identify the elements of the guide RNA that are important for its recognition by the modifying enzyme, we tested a series of Pf9 mutants and fragments in gel mobility shift assays (Fig. 3). We found that box ACA is essential, but not sufficient, for recognition by Cbf5. Mutation of box ACA eliminated the interaction observed with wild-type Pf9 (Fig. 3B). However, an RNA comprised of box ACA and the lower stem of the Pf9 hairpin was not sufficient for Cbf5 binding (Fig. 3C). The pseudouridylation pocket also plays an important role in the interaction of Cbf5 with Pf9. Addition of the pseudouridylation pocket to the lower stem and box ACA resulted in significant binding by Cbf5 (Fig. 3D). In addition, the elimination of the pseudouridylation pocket in the context of the full-length Pf9 RNA substantially reduced the ability of Cbf5 to interact with the guide RNA (Fig. 3E). However, while box ACA and the pseudouridylation pocket are necessary for recognition by Cbf5, it appears that these two are not the only elements that contribute to Cbf5 binding (Fig. 3D), and that another important element present in the upper region of the hairpin of the RNA is required for full binding activity.

The K-turn is important for the interaction of L7Ae with Pf9 (Fig. 2B); however it is not essential for the interaction of Cbf5 (Fig. 3F). On the other hand, we found that replacement of the terminal loop of the hairpin with a stable tetra-loop significantly reduced binding (Fig. 3G).

Although neither the sequence nor length of the terminal loops of H/ACA RNAs are thought to be conserved, we noticed a “GAG” sequence present within the terminal loop of several archaeal H/ACA sRNAs. [This bears some similarity to the CAB box that has been found to be important in the localization of certain guide RNAs to Cajal bodies in eukaryotes [Richard et al. 2003]]. Mutation of the GAG sequence significantly reduced Cbf5 binding (Fig. 3H), suggesting that this sequence within the terminal loop also plays a role in the interaction of Cbf5 with H/ACA guide RNAs.

We also examined the interaction of Cbf5 with other H/ACA RNAs in gel mobility shift assays. Pf3 is a double hairpin H/ACA RNA from P. furiosus [Klein et al. 2002; Rozhdestvensky et al. 2003]. Interestingly, we found that Pf3 formed two distinct complexes with Cbf5 (Fig. 4A). The second complex [Fig. 4A, marked with * * *], which comigrates with a band observed with RNA alone, appears with increasing concentrations of Cbf5. We did not observe the formation of more than one specific complex with the single hairpin RNA Pf9, even at protein concentrations up to 6 µM [Fig. 2C; data not shown]. The results suggest that Cbf5 interacts with each of the two hairpins of a double guide RNA. Mutation of the two ACA elements found in Pf3 disrupted the interaction of Cbf5 with Pf3 (Fig. 4B). In addition, we tested two types of eukaryotic H/ACA RNAs—a small nucleolar or snRNA and a small Cajal body or scaRNA. U65 is a typical, human H/ACA snoRNA with two hairpins that guides rRNA modification in the nucleolus (Ganot et al. 1997b). U92 is a double hairpin H/ACA scaRNA that guides RNA-guided RNA pseudouridylation.
interaction of proteins with the two hairpins in eukaryotic double guide H/ACA RNAs.

In summary, these results indicate that Cbf5, the pseudouridine synthase, interacts with H/ACA guide RNAs specifically and independently of the other proteins of the pseudouridylation guide complex. Our mutational analysis indicates that Cbf5 depends upon box ACA, the pseudouridylation pocket, and sequences within the terminal loop of the hairpin for interaction with the H/ACA RNA. Moreover, it appears that the number of molecules of Cbf5 that binds a guide RNA correlates with the number of hairpins.

Protein–protein interactions within the archael H/ACA RNP

Our results indicate that both Cbf5 and L7Ae interact directly and independently with H/ACA guide RNAs, however the means of association of Gar1 and Nop10 with the RNP was still unclear. In addition, we were very interested in identifying protein–protein interactions between components of the complex. We investigated the protein–protein interactions by incubating various combinations of the recombinant proteins [shown in input [I] lanes in Fig. 5], one of which was His-tagged [indicated with an asterisk in Fig. 5], and identifying the proteins associated with the tagged protein by affinity chromatography [shown in bound [B] lanes in Fig. 5]. Bovine serum albumin [BSA] was included in all incubations to assess the extent of nonspecific interactions, but was not detected in the affinity-purified samples [Fig. 5]. The results indicate that Cbf5 interacts directly with each Gar1 and Nop10 [Fig. 5, lanes 1–4]. Gar1 and Nop10 do not interact with one another [Fig. 5, lanes 5,6], but Gar1 does copurify with tagged Nop10 in the presence of Cbf5 [Fig. 5, lanes 13,14], indicating that these three proteins form a heterotrimeric complex in which each Gar1 and Nop10 are bound to Cbf5. At the same time, no interaction was observed between L7Ae and either Gar1, Nop10, or Cbf5 [Fig. 5, lanes 7–12]. Moreover, when all four proteins were coincubated, Cbf5, Gar1, and Nop10 copurified, but L7Ae did not, suggesting that L7Ae does not interact with the other protein components of the H/ACA RNP in the absence of the guide RNA [Fig. 5, lanes 15,16].

In vitro assembly of an H/ACA RNP

We next examined the assembly of the H/ACA RNP in gel mobility shift assays [Fig. 6]. As we have shown, Cbf5 and L7Ae [but not Gar1 and Nop10] interact directly with the single hairpin guide RNA Pf9, and the interaction of Cbf5 with Pf9 depends on box ACA [Figs. 2, 3, 6 [lanes 1–5,10–14]]. In protein–protein interaction assays we found that Gar1 and Nop10 interact with Cbf5 in the absence of the guide RNA [Fig. 5] and thus hypothesized that Cbf5 mediates the interaction of these two proteins with the RNP. Here we show that addition of each Nop10 and Gar1, and both Nop10 and Gar1 to Cbf5, in gel mobility shift assays results in stepwise supershifts of the RNA relative to Cbf5 alone [Fig. 6, lanes 4,6–8]. Like the interaction of Cbf5 alone, these interactions are dependent on box ACA [Fig. 6, lanes 13,15–17]. We did not observe a shift in the mobility of Pf9 with the combination of Gar1 and Nop10 in the absence of Cbf5 [data not shown]. In addition, Gar1 and Nop10 did not supershift the L7Ae–Pf9 RNA complex [data not shown]. These results indicate that Cbf5 mediates the interaction of both Gar1 and Nop10 with the H/ACA RNP. Addition of L7Ae resulted in a further supershift of the complex formed by Cbf5, Gar1, and Nop10 with Pf9 [Fig. 6, lane 9, asterisk], indicating that L7Ae can interact with Pf9 in the context of the complex formed with the other three proteins. Together, our results indicate that a functional H/ACA RNP is formed by the independent
binding of each Cbf5 and L7Ae to distinct sites on the guide RNA and by independent binding of Gar1 and Nop10 to Cbf5.

Discussion

RNA-guided RNA pseudouridylation

Pseudouridylation is the most common RNA modification and occurs in tRNA, rRNA, snRNA, snoRNA, and likely other noncoding RNAs (Ofengand and Fournier 1998; Charette and Gray 2000; Yu et al. 2005). There is mounting evidence that pseudouridines occur in functionally important RNA domains and play a vital role in RNA-mediated cellular processes including pre-mRNA splicing and ribosome function (Yu et al. 1998; King et al. 2003; Donmez et al. 2004; Zhao and Yu 2004). Pseudouridylation of RNA is an evolutionarily ancient process catalyzed by a large family of enzymes known as pseudouridine synthases [Koonin 1996; Ofengand et al. 2001].

There are two distinct mechanisms by which pseudouridine synthases select target uridine residues for isomerization. In all known instances in eubacteria, pseudouridylations are carried out by dedicated pseudouridine synthases that each recognize one or a small set of similar RNA substrates (Koonin 1996; Charette and Gray 2000). Most known pseudouridine synthases are of this type. However, in archaia and eukaryotes, many pseudouridylations are introduced by RNA-guided pseudouridine synthases [Yu et al. 2005]. The RNA-guided system is versatile and employs armies of H/ACA guide RNAs to direct a common pseudouridine synthase to many different sites. The RNA-guided pseudouridine synthases are members of the TruB subfamily and are called Cbf5[p] in yeast (Jiang et al. 1993) and archaia (Watanabe and Gray 2000), dyskerin in humans (Heiss et al. 1998), and NAP57 in rat (Meier and Blobel 1994). Three additional proteins are associated with the RNA-guided pseudouridine synthases: Gar1, Nop10, and L7Ae (Nhp2 in eukaryotes). However, the roles of these additional proteins in pseudouridylation are not known. The results presented here provide a substantial amount of new information on the structure and function of the RNP that catalyzes RNA-guided RNA pseudouridylation in archaea.

The pseudouridine synthase Cbf5 interacts directly with H/ACA guide RNAs via the conserved box ACA element

Box ACA is the signature sequence element of H/ACA RNAs. In eukaryotes, mutational analysis has demon-
strated that box ACA is essential for multiple aspects of H/ACA RNA biogenesis and function, including RNP assembly [Filipowicz and Pogacic 2002; Kiss 2002; Terns and Terns 2002]. It was therefore thought that box ACA served as an important protein-binding site, but the identity of the box ACA-binding factor remained elusive. The work presented here demonstrates that the pseudouridine synthase itself, Cbf5, is the RNA-binding protein that specifically recognizes box ACA in archaea. We show that mutation of box ACA abolishes Cbf5 binding [Figs. 3, 6]. Like other TruB-family pseudouridine synthases, Cbf5 contains a domain that is involved in interaction with substrate RNA [Aravind and Koonin 2001; Hoang and Ferre-D’Amare 2001], but an additional RNA-binding motif that might have predicted the ability of Cbf5 to interact selectively with H/ACA guide RNAs was not recognized and should now be a focus of further investigation.

Analysis of hundreds of eukaryotic and archaeal pseudouridylation guide RNAs has revealed a conserved (~14 nt) distance between box ACA of the guide RNA and the unpaired target uridine of the substrate RNA positioned within the pseudouridylation pocket [Ganot et al. 1997a]. Our finding that Cbf5 interacts with box ACA may provide an explanation: The fixed distance may simply reflect the physical spacing between the domains of Cbf5 that interact with box ACA and catalyze pseudouridylation of the target uridine.

**Organization of functional pseudouridylation guide RNP**

Our findings provide a clear model for the basic organization of the archaeal pseudouridylation guide RNP, in which Cbf5 and L7Ae bind independently to distinct sites on the guide RNA, and Gar1 and Nop10 interact with Cbf5 [Fig. 7].

L7Ae interacts directly with the K-turn of the guide RNA [Fig. 2; Rozhdestvensky et al. 2003], but we did not find evidence of interaction of L7Ae with the other proteins in the absence of the guide RNA [Fig. 5]. Moreover, the interaction of the other proteins with the RNA did not depend on the presence of L7Ae [Fig. 6], indicating that L7Ae does not nucleate the assembly of the H/ACA RNP as it does the C/D RNP [Omer et al. 2002].

Cbf5 also interacts directly with the guide RNA and we found that box ACA, the pseudouridylation pocket, and the terminal loop of the hairpin appear to be important for this interaction [Figs. 2, 3], suggesting extensive contact between Cbf5 and the guide RNA. Our data indicate that Gar1 and Nop10 each interact directly with Cbf5, but not with the other proteins or with the guide RNA in the absence of Cbf5 [Figs. 2, 5]. The interaction of Gar1 and Nop10 with Cbf5 mediates the interaction of these proteins with the complex [Fig. 6]. Further, our results indicate that these three proteins can form a heterotrimeric Cbf5/Gar1/Nop10 complex that can interact...
with the guide RNA (Figs. 5, 6; O.A. Youssef, R.M. Terns, and M.P. Terns, unpubl.). Based on our results it seems equally possible that these three proteins assemble on the guide RNA sequentially or as a preformed complex.

Eukaryotic pseudouridylation guide RNPs

The components of eukaryotic and archaeal pseudouridylation guide RNPs are generally well conserved, suggesting that the organization and function of the components will be fundamentally similar in the two systems. Unfortunately, detailed analysis of functional eukaryotic H/ACA RNPs has not been reported to date. However, two recent studies describe interactions between various components of eukaryotic H/ACA RNPs—one in a mammalian system and one in yeast [Henras et al. 2004; Wang and Meier 2004]. The interactions observed in the yeast study [Henras et al. 2004] are in agreement with those reported here, while there are significant differences in the interactions observed in the mammalian system [Wang et al. 2000].

Using mammalian proteins expressed in rabbit reticulocyte lysates, Wang and Meier (2004) found a heterotrimeric protein complex with a different composition—comprised of the mammalian homologs of Chf5, Nop10, and L7Ae, rather than Chf5, Nop10, and Gar1 [Fig. 5]. In addition, they found that Nop10 is essential for interaction between the mammalian Chf5 and L7Ae homologs, and thus appears to play the central role in this complex [Wang and Meier 2004], while Chf5 is at the core of the archaeal complex, interacting independently with each Gar1 and Nop10 [Fig. 5]. In the mammalian system, specific recognition of H/ACA RNAs required all three components of the trimeric complex [Wang and Meier 2004]. On the other hand, we have found that archaeal Chf5 interacts specifically with guide RNAs in the absence of the other proteins [Figs. 2–4], and that Gar1 and Nop10 do not observably increase the affinity of the interaction [data not shown].

On the other hand, the data from yeast suggest that the organization of the yeast H/ACA RNP resembles the archaeal complex. In studies with complexes expressed and assembled in vivo and purified from Saccharomyces cerevisiae, Henras et al. (2004) also found that Chf5(p), Gar1(p), and Nop10(p) can form a complex independent of both L7Ae (Nhp2p) and guide RNA.

At present it is not clear whether the observed discrepancies between the mammalian system and the archaeal and yeast systems reflect fundamental differences in the RNPs or the limitations of experimental approaches. The authors of the mammalian study note that no significant pseudouridylase activity could be detected with the complexes assembled in the mammalian system [Wang and Meier 2004]. The functionality of the purified yeast complexes was not reported. The eukaryotic H/ACA RNP proteins, and especially Chf5, are challenging to express and purify [Wang et al. 2000; Henras et al. 2004]. It is possible that both the lack of functionality of the mammalian proteins and the observed differences result from production of defective [perhaps misfolded] mammalian proteins in vitro. A better understanding of the extent of differences between the eukaryotic and archaeal RNPs awaits more detailed structural studies of functional eukaryotic complexes.

Roles of the H/ACA RNP proteins in RNA-guided pseudouridylation

All evidence indicates that Chf5 is the pseudouridine synthase [i.e., catalyzes breakage of the N1–C1’ glycosidic bond and reattachment of the free uridine base to the ribose via a C5–C1’ glycosidic bond]. The sequence and structure of the protein is very similar to other known pseudouridine synthases [Koonin 1996; Charrette and Gray 2000; Hoang and Ferre-D’Amare 2001], and in yeast, mutation of the predicted catalytic aspartate [universally conserved in all pseudouridine synthases] prevents RNA-guided pseudouridylation in vivo [Lafontaine et al. 1998; Zebarjadian et al. 1999]. Our work establishes two additional key roles for Chf5: direct recognition of the guide RNA and recruitment of both Gar1 and Nop10 [Figs. 2, 5].

What are the roles of the other proteins? It is clear that L7Ae interacts directly with the guide RNA [Fig. 2; Rozhdestvensky et al. 2003]. In addition, our results indicate that L7Ae does not interact independently with the other proteins and is not responsible for the recruitment of the other proteins to the complex [Figs. 5, 6; data not shown]. One conceivable role of L7Ae is alteration of the structure of the guide RNA [e.g., introduction of a kink in the upper stem] to induce a conformation in the RNA or RNP that is important for pseudouridylation.

Our finding that Chf5 interacts directly with the guide RNA indicates that the other proteins do not bridge the interaction of the modifying enzyme with the guide RNA. Gar1 and Nop10 both interact with Chf5 [Fig. 5], but this interaction does not apparently increase the affinity of Chf5 for the guide RNA [data not shown]. The association of Gar1 and Nop10 with Chf5 suggests that they may serve auxiliary roles in H/ACA RNP function. For example, these proteins may promote or stabilize the interaction with the substrate rRNA, ensure proper positioning of the target uridine in the active site, or influence substrate rRNA release following catalysis. Site-specific cross-linking studies support an intimate association of Gar1 [as well as Chf5] with the target uridine in the mammalian system [Wang and Meier 2004]. In addition, genetic depletion of Gar1(p) in yeast results in partially assembled RNP complexes that are unable to interact with substrate RNA [Bousquet-Antonelli et al. 1997]. Analysis of the sequence of archaeal Nop10 proteins suggests the potential existence of a zinc-finger motif [D. Baker, J. Omichinski, R. Terns, and M. Terns, unpubl.] and the ability to interact directly with nucleic acids—perhaps substrate RNA.

Importantly, our results establish for the first time that each of the four proteins is essential for RNA-guided pseudouridylation in vitro [Fig. 1]. Previous studies in eukaryotes established that these proteins are associated
with H/ACA guide RNAs and that RNA pseudouridylation is reduced in cells lacking these proteins. Our results indicate that the proteins are not solely required for the stability of the guide RNAs or other upstream functions in vivo, but are also necessary for efficient catalysis of the modification.

Materials and methods

Expression and purification of recombinant proteins

The genes encoding *P. furiosus* Cbf5 (PF1785), Gar1 (PF1791), Nop10 (PF1411), and L7Ae (PF1367) were amplified by PCR from genomic DNA and cloned into modified versions of pET21d and pET24d. The primers used in the PCR reactions are specified in Supplementary Tables 1 and 2. The recombinant proteins were expressed in *Escherichia coli* BL21 codon+ cells (DE3, Invitrogen). The cells were grown to a culture OD_{600} of 0.7, and expression of the proteins was induced with 1 mM IPTG for 4 h at 37 °C. The supernatant was heated at 75–78 °C for 20 min and centrifuged at 45,000 g for 37 °C. The supernatant was filtered (0.8 µm pore size Millex Filter Unit; Millipore) and applied to a Ni-NTA agarose (Qiagen) column equilibrated with Buffer A. Proteins were eluted with Buffer A containing 500 mM imidazole. The protein samples were dialyzed at room temperature against 40 mM HEPES (pH 7.0), 500 mM KCl, 1.5 mM MgCl_{2}. Some samples were concentrated using a PL-10 Microcon filter device (Millipore). The purity of the protein samples was assessed by SDS-PAGE and Coomassie blue protein assay. Input and bound protein samples were analyzed by nondenaturing 6% or 8% polyacrylamide gels containing 20 mM HEPES (pH 7.0), 250 mM KCl, 1.5 mM MgCl_{2}, 0.25 µg/µL *E. coli* tRNA, 0.75 mM DTT, and 10% glycerol. After incubation at 37 °C for 1 h, samples were loaded on nondenaturing 6% or 8% polyacrylamide gels containing 0.5x TBE. Ethidium bromide was performed at 4°C in 0.5x TBE for 12 h at 125 V. The RNA distribution was visualized by autoradiography after gel drying.

In vitro protein/protein interaction assay

Protein samples were dialyzed against Buffer B (20 mM HEPES at pH 7.0, 500 mM KCl, 1.5 mM MgCl_{2}). Approximately equimolar amounts of proteins were incubated for 30 min at 37°C. Bovine serum albumin (Promega) was included as a negative control. Half of the protein mixture was reserved as input sample and concentrated 10-fold using a YM-3 Microcon filter device (Millipore). The other half of the sample was incubated for 10 min at room temperature with 15 µL of Ni-NTA resin (Qiagen) equilibrated in Buffer B. The resin was washed four times with Buffer B plus 20 mM imidazole and 0.1% Triton X-100. Bound proteins were eluted with SDS gel loading buffer and heating. Input and bound protein samples were analyzed by 15% Tris-tricine gel electrophoresis and Coomassie blue protein staining.

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