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Archaeal Guide RNAs Function in rRNA Modification in the Eukaryotic Nucleus

Wayne A. Speckmann,[1] Zhu-Hong Li,[1] Todd M. Lowe,[2] Sean R. Eddy,[3] Rebecca M. Terns,[1,4] and Michael P. Terns[1,4]

[1]Department of Biochemistry and Molecular Biology
University of Georgia
Athens, Georgia 30602
[2]Department of Genetics
Stanford University School of Medicine
Stanford, California 94305
[3]Howard Hughes Medical Institute
Department of Genetics
Washington University School of Medicine
St. Louis, Missouri 63110

Summary

In eukaryotes, many Box C/D small nucleolar RNAs base pair with ribosomal RNA through short complementary guide sequences, thereby marking up to 100 individual nucleotides of ribosomal RNA for 2′-*O*-methylation [1–3]. Function of the eukaryotic Box C/D RNAs depends upon interaction with at least six known proteins [4–10]. Box C/D RNAs are not known to exist in *Bacteria* but were recently identified in *Archaea* by biochemical analysis and computational genomic screens [11, 12] and have likely evolved independently in *Archaea* and *Eukarya* for more than 2000 million years [13–15]. We have microinjected Box C/D RNAs from *Pyrococcus furiosus*, a hyperthermophilic archaeon, into the nuclei of oocytes from the aquatic frog *Xenopus laevis*. Our results show that Box C/D RNAs derived from this prokaryote are retained in the nucleus, localize to nucleoli, and interact with the *X. laevis* Box C/D RNA binding proteins fibrillarin, Nop56, and Nop58. Furthermore, we have demonstrated the ability of archaeal Box C/D RNAs to direct site-specific 2′-*O*-methylation of ribosomal RNA. Our studies have revealed the remarkable ability of archaeal Box C/D RNAs to assemble into functional RNA-protein complexes in the eukaryotic nucleus.

Results and Discussion

The numerous Box C/D RNAs that guide modification of rRNA in eukaryotic cells are characterized by two very short conserved sequence elements, Box C (UGAUGA) and Box D (CUGA), found near the 5′ and 3′ termini of the RNAs, respectively. Two additional elements, Box C′ (UGAUGA) and Box D′ (CUGA), are also present within Box C/D guide RNAs but are usually less highly conserved [16]. Box C/D RNAs base pair with rRNA via complementary guide sequences (10 to 21 nucleotides in length) found immediately upstream of Box D (or Box D′). Interaction of a Box C/D RNA with rRNA results in 2′-*O*-methylation of the rRNA at the base-paired position five nucleotides upstream of Box D (or D′) [2, 17]. The eukaryotic Box C/D RNAs function as ribonucleoprotein (RNP) complexes. At least four proteins are core components of the RNPs: fibrillarin (the likely methyltransferase [18, 19]), Nop56, Nop58, and p15.5 kDa [4–6, 8, 9]. Two additional proteins, p50 and p55, also interact with the RNAs and are required for the stability and/or function of the RNAs [7, 10]. Box C and Box D are required for interaction of the RNAs with proteins and for the biogenesis, transport, and function of the eukaryotic Box C/D RNAs [1, 17, 20, 21].

The discovery of homologous RNAs containing Box C and Box D elements in at least six archaeal species [11, 12] suggested the remarkable possibility that the essential elements of the Box C/D RNAs had not diverged in *Archaea* and *Eukarya* over a vast period of independent evolution. However, the minimal features required for assembly of a functional Box C/D RNP in eukaryotes have not yet been defined. The archaeal Box C/D RNAs do not possess significant sequence homology with eukaryotic Box C/D RNAs outside of the Box C, C′, D, and D′ sequence elements [11, 12]. Moreover, the identified archaeal Box C/D RNAs are more compact than eukaryotic Box C/D RNAs (e.g., on average 56 nucleotides in *P. furiosus* versus 104 in *Saccharomyces cerevisiae*) [11, 22].

Analysis of the sequences of archaeal homologs of the eukaryotic Box C/D RNA-associated proteins suggests that the archaeal Box C/D RNAs interact with a simpler set of proteins. For example, archaeal fibrillarin homologs lack the glycine arginine-rich (GAR) domain that is characteristic of fibrillarin in eukaryotes [18, 23, 24]. Moreover, five of the eukaryotic proteins, Nop56, Nop58, p50, p55, and p15.5 kDa, appear to be products of gene duplications that have occurred since the divergence of *Archaea* and *Eukarya* [7, 20, 23]. For example, Nop56, Nop58, and the spliceosomal protein Prp31 are related eukaryotic proteins all represented by a single archaeal homolog [23]. Thus, the six eukaryotic Box C/D RNA-associated proteins are represented by just four homologous proteins in archaea [20]. All six of the eukaryotic Box C/D RNA-associated proteins are essential [6, 10, 25, 26], indicating that the duplicated genes (paralogs) have evolved to encode proteins with distinct and critical functions in eukaryotes. We were interested to know whether the eukaryotic proteins would recognize archaeal Box C/D RNAs.

In this work, we have tested the consequences of the evolutionary distance and extant differences between the Box C/D RNAs and associated proteins in *Archaea* and *Eukarya*. We cloned the coding sequence of four (of 52) Box C/D RNAs predicted to exist in *P. furiosus*: sR2, sR3, sR29, and sR42 ([11] and equivalent to sR10, 25, 35, and 30, respectively, in [12]). We also confirmed by RT-PCR analysis that the computationally predicted *P. furiosus* sR2, sR3, sR29, and sR42 are expressed RNAs (data not shown). In eukaryotes, Box C/D RNAs are actively retained in the nucleus and function within

[4]Correspondence: mterns@bmb.uga.edu [M.P.T.], rterns@bmb.uga.edu [R.M.T.]

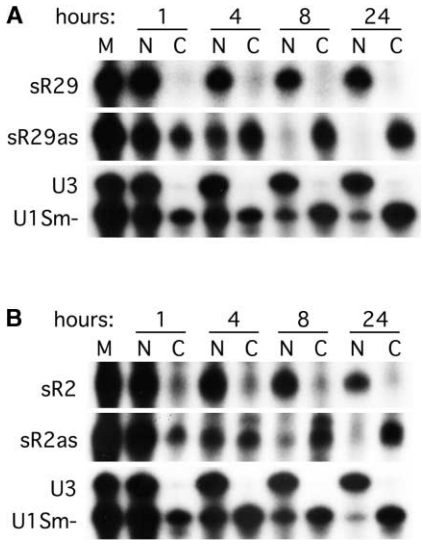

Figure 1. Archaeal Box C/D RNAs Are Stable and Retained in the Nucleus of *Xenopus laevis* Oocytes

In vitro transcribed, ^{32}P-labeled *P. furiosus* sR29 (A) and sR2 (B) and coinjected *X. laevis* U3 Box C/D snoRNA and U1sm- snRNA present in the nucleus (N) and cytoplasm (C) 1, 4, 8, and 24 hr after microinjection into *X. laevis* oocyte nuclei. Antisense (as) sR29 (A) and sR2 (B) RNA panels are from separate experiments. Marker lanes (M) show RNAs prior to microinjection.

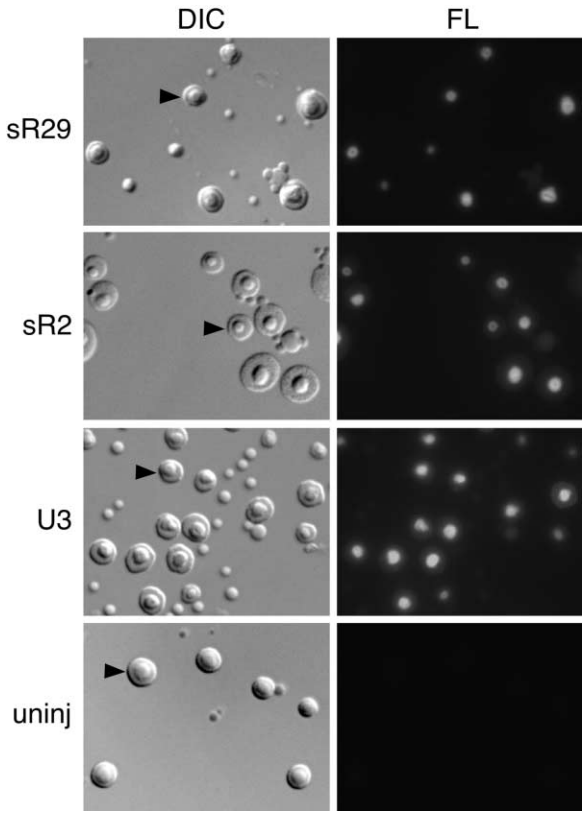

Figure 2. Targeting of *Pyrococcus furiosus* Box C/D RNAs to *Xenopus laevis* Oocyte Nucleoli

Nuclear spreads prepared 1 hr after microinjection of in vitro transcribed, fluorescein-labeled RNAs (*P. furiosus* sR29 and sR2 and *X. laevis* U3 Box C/D RNA) into oocyte nuclei were analyzed by differential interference contrast (DIC) and fluorescence (FL) microscopy. An uninjected control (uninj) is also shown. A representative nucleolus is indicated in each DIC panel (arrowhead). Other intranuclear structures visible in the DIC panels include snurposomes and Cajal (coiled) bodies. Localization of eukaryotic Box C/D RNAs but not other cellular RNAs to the dense fibrillar region of nucleoli was described previously [28].

the nucleolus [21, 27]—structures that are not known to exist in archaea. To test the fate of archaeal Box C/D RNAs in a eukaryotic cell, radiolabeled RNAs were produced by in vitro transcription and microinjected into nuclei of *Xenopus laevis* oocytes. The nuclei were dissected from the oocytes at various times after microinjection, and the nucleocytoplasmic distribution of the RNAs was determined (Figure 1). Remarkably, the four archaeal RNAs were stable and retained in the nucleus for up to 24 hr after microinjection (Figure 1, sR3 and sR42 not shown). In contrast, antisense transcripts of the archaeal RNAs were exported to the cytoplasm similar to spliceosomal U1Sm-snRNA (Figure 1). To determine whether the *P. furiosus* Box C/D RNAs localized to nucleoli, we analyzed nuclear spreads prepared 1 hr after microinjection of fluorescently labeled RNAs. The archaeal RNAs were targeted specifically to the central, dense fibrillar region of nucleoli in a pattern indistinguishable from eukaryotic snoRNAs (Figure 2, sR3 and sR42 not shown, and [28]). Thus, the archaeal RNAs were retained in the nucleus and targeted to nucleoli in *X. laevis* oocytes.

In eukaryotes, Box C/D RNAs interact with fibrillarin, Nop56, and Nop58 [4, 8, 9]. Fibrillarin shares structural features with methyltransferases and is thought to catalyze the 2′-*O*-ribose methylation of rRNA, guided by the individual associated snoRNAs [5, 19]. Nop56 and Nop58 are both required for rRNA modification, but the precise roles of the proteins are not known [8, 9, 26]. We tested whether the archaeal RNAs interacted with eukaryotic Nop56, Nop58, and fibrillarin by immunoprecipitation of the endogenous *X. laevis* proteins 24 hr after microinjection of archaeal and control RNAs. Coinjected eukaryotic Box C/D RNAs (U8 and/or U14) but not spliceosomal snRNAs (U1 and U6) are associated with each of the proteins (Figure 3). Interestingly, U3 RNA, a Box C/D snoRNA involved in rRNA processing rather than modification, was associated with fibrillarin and Nop56 but not extensively with Nop58 (Figure 3). All four of the *P. furiosus* Box C/D RNAs were coimmunoprecipitated with endogenous *X. laevis* fibrillarin and both Nop56 and Nop58 (Figure 3, sR3 and sR42 not shown). Our results demonstrate that the endogenous eukaryotic proteins, including both the Nop56 and Nop58 paralogs, interact with the archaeal Box C/D RNAs.

Evidence suggests that the Box C/D RNAs also direct 2′-*O*-methylation of rRNA in archaea. Each archaeal RNA is predicted to guide modification of 1 or 2 rRNA sites that can be deduced from the internal complementary sequences using the "Box D + 5 nucleotide" rule derived from studies in eukaryotes [2, 3, 11, 12]. The total number of modifications, as well as the positions of some specific sites that have been mapped in archaeal species, are consistent with predictions based on the identified archaeal Box C/D RNAs [11, 12]. In addition,

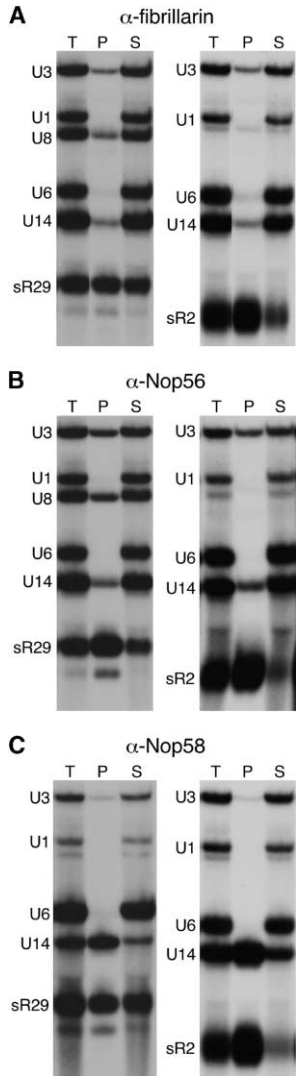

Figure 3. Archaeal Box C/D RNAs Interact with Eukaryotic Proteins Fibrillarin, Nop56, and Nop58

P. furiosus sR29 and sR2 were coinjected with *Xenopus laevis* Box C/D RNAs U3, U8, and/or U14 and with spliceosomal snRNAs U1 and U6. The RNAs present in the nuclear extract prior to immunoprecipitation (20% of total, T), immunoprecipitated (P) with antibodies against fibrillarin (A), Nop56 (B), or Nop58 (C) and remaining in the supernatant (20% of supernatant, S) are shown.

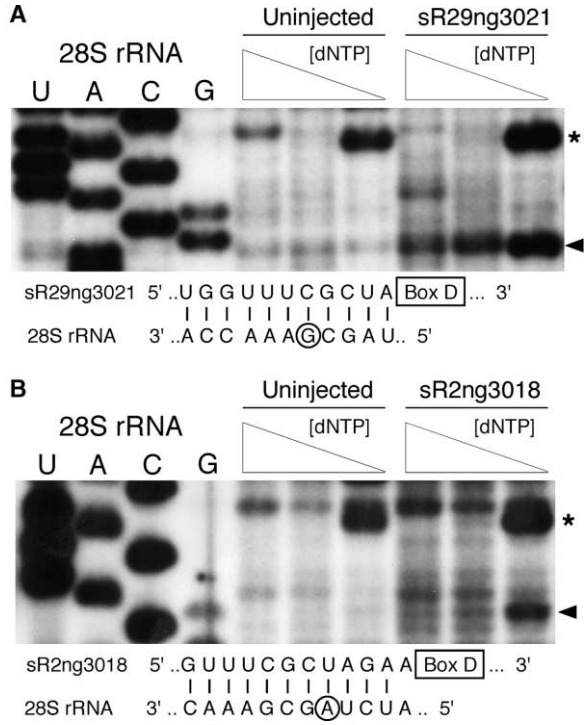

Figure 4. Archaeal Box C/D RNAs Guide 2′-*O*-Ribose Methylation of *Xenopus laevis* rRNA

The guide sequences upstream of Box D in sR29 and sR2 were replaced with sequences complementary to *X. laevis* 28S rRNA to generate sR29ng3021 (A) and sR2ng3018 (B), respectively. The predicted sites of *Xenopus* rRNA modification (paired with the base five nucleotides upstream of Box D) are indicated (G3021 and A3018, circled). Reverse transcriptase primer extension pause assays [22, 35] performed on nuclear RNA from oocytes following injection of sR29ng3021, sR2ng3018, or no RNA (uninjected) are shown. Arrowheads indicate dNTP concentration-dependent pauses observed (typically one nucleotide 3′ of the modified base [35]) specifically in the presence of the archaeal RNAs. An endogenous rRNA modification (asterisk) is detected in the absence and presence of the archaeal RNAs. Adjacent lanes show reference sequence ladders produced with the primer utilized in the pause assays.

the RNAs interact with the presumptive methyltransferase, fibrillarin, in archaea [11]. However, the lack of practical gene disruption and transformation systems in the organisms in which the RNAs have been identified has impeded demonstration of the function of the Box C/D RNAs in archaea. We have tested the ability of the archaeal RNAs to direct modification of rRNA in the *X. laevis* oocyte. The guide sequences in the archaeal RNAs are not complementary to *X. laevis* rRNA due to rRNA sequence divergence and the lack of conservation of sites of rRNA 2′-*O*-methylation between *Archaea* and *Eukarya*. Therefore, we replaced the endogenous 10 and 11 nucleotide guide sequences of sR29 and sR2 with sequences complementary to *Xenopus laevis* 28S rRNA designed to direct 2′-*O*-methylation of C3021 and A3018, respectively. Injection of each new guide RNA resulted in the appearance of a novel modification at the targeted site of *Xenopus* 28S rRNA (Figure 4). The results support the hypothesis that the Box C/D RNAs guide 2′-*O*-methylation of RNA in archaea. Moreover, our findings demonstrate that the archaeal Box C/D RNAs can guide rRNA modification in the *Xenopus* oocyte.

The results described here are a unique demonstration of the ability of a class of RNAs to be recognized, be transported, and function in an organism from another domain of life. The accurate nuclear retention and subnuclear localization of RNAs from a prokaryote in a eukaryotic cell suggest that these processes are mediated by elements that existed in common ancestral RNAs prior to the divergence of *Archaea* and *Eukarya* and development of the eukaryotic nucleus. Our findings demonstrate that the RNA-protein interactions essential for the transport and function of Box C/D RNAs have not changed significantly since the divergence of *Arch-*

aea and *Eukarya*. We propose that the remarkable recognition of archaeal RNAs by eukaryotic proteins reflects a limitation on divergence imposed by the requirement for coevolution of the Box C/D proteins with each of multiple individual RNAs.

Experimental Procedures

Synthesis of RNAs

Sequences of putative Box C/D RNAs were obtained from a computational search of the *P. furiosus* genome [11] and are available at http://rna.wustl.edu/snoRNAdb/. Primers complementary to sR2, sR3, sR29 and sR42 were used for RT-PCR from purified *P. furiosus* total RNA (kindly provided by Gerti Schut, Dr. Amy Grunden, and Dr. Michael Adams, University of Georgia). Primers were designed to include sequences proposed to contribute to terminal stem structures [11] and exogenous T7 promoter sequences for in vitro transcription (underlined): sR2-5′ oligo, TAATACGACTCACTATAGGGG GATGATGAGTTTTTCCC; sR2-3′ oligo, GAGGTCAGTGCATCGGC TCC; sR3-5′ oligo, TAATACGACTCACTATAGGCGATGATGAATAG CAAGCC; sR3-3′ oligo, GGCTCAGGGGGTGTTCACCTC; sR29-5′ oligo, TAATACGACTCACTATAGGGATTAGCGGATGATGTGCCTTGC; sR29-3′ oligo, GAAAAGCGTCAGCTAATACCGAC; sR42-5′ oligo, TAATACGACTCACTATAGGGATAGCTTGATGATGAACTTCACGG; sR42-3′ oligo, GAAAAAGGTAATCAGACGAGAGGG. RT-PCR products were subcloned into pCR2.1-TOPO (Invitrogen) and verified by sequencing. The following primers were used to generate templates for transcription of antisense RNAs (SP6 promoter sequences underlined): as-sR2-5′ oligo, ATTTAGGTGACACTATAGAGGTCAG TGCATCGGCTCC; as-sR2-3′ oligo, GGGGGATGATGAGTTTTTCCC; as-sR29-5′ oligo, ATTTAGGTGACACTATAGAAAAGCGTCAGCTAA TACCGAC; as-sR29-3′ oligo, GGATTAGCGGATGATGTGCCTTGC. In sR2ng3018 and sR29ng3021, the endogenous guide sequence immediately 5′ of Box D in *P. furiosus* sR2 and sR29, respectively, was replaced with sequence complementary to *X. laevis* 28S rRNA by PCR using the 5′ primers listed above with the following 3′ primers: sR2ng3018-3′, GGGGTCAGTTCTAGCGAAACTCATCAC TAATCAGAGTG; sR29ng3021-3′, GAAAAGCGTCAGTAGCGAAAC CAATCATCACCTTTCAGGCTGG. PCR products were utilized as templates for in vitro transcription. Templates for transcription of other RNAs were generated as described previously [28, 29]. SP6 or T7 RNA polymerase was used to produce m^7G-capped (or MepppG-capped, U6 only) RNAs, which were either ^{32}P labeled or fluorescein labeled, as described previously [28, 29].

Analysis of RNA Stability, Nucleocytoplasmic Distribution, Subnuclear Localization, and Protein Binding in *Xenopus laevis* Oocytes

These analyses have been described in detail [28–31]. In brief, 1–3 fmol ^{32}P- or fluorescein-labeled RNA was microinjected into nuclei of *Xenopus* oocytes. At various time points, nuclei were manually dissected from oocytes. Accuracy of microinjection and dissection was monitored via coinjected blue dextran and control RNAs. For stability and nucleocytoplasmic distribution analysis (Figure 1), RNAs were extracted from nuclear and cytoplasmic fractions and analyzed by gel electrophoresis and autoradiography as described previously [29]. Intranuclear localization of RNAs (Figure 2) was assessed in nuclear spreads prepared by manually removing the nuclear membrane from a dissected nucleus and centrifuging the nuclear contents onto a microscope slide [28, 32, 33]. Fluorescein-labeled RNAs were visualized by fluorescence microscopy [28, 34]. Interaction of injected RNAs with endogenous *Xenopus* proteins (Figure 3) was assayed by immunoprecipitation. Polyclonal antibodies against *X. laevis* fibrillarin, Nop56, and Nop58 were generated and verified in Western blots of oocyte cell extracts and recombinant proteins and in immunoprecipitations of in vitro translated proteins (data not shown) as well as by specific coimmunoprecipitation of *X. laevis* Box C/D RNAs as shown in Figure 3. Immunoprecipitations were performed with nuclear extract prepared from five nuclei 24 hr after injection and carried out in Ipp-100T for fibrillarin and in Ipp-200T for Nop56 and Nop58, essentially as described previously [28, 29, 31].

Primer Extension Pause Assays

Mapping of 2′-*O*-methyl sites in *X. laevis* rRNA was based on published protocols [22, 35]. In our experiments, RNA was recovered from nine nuclei isolated 22 hr after microinjection of 3–5 fmol of sR2ng3018 or sR29ng3021. The nuclear RNA was annealed with ^{32}P 5′ end-labeled primer (0.15 pmol/μl) at 60°C for 4 min. Primer extension was carried out at 42°C for 30 min at three dNTP concentrations (1.0, 0.1, and 0.004 mM). The oligo used for primer extension and sequencing reactions was GCAGACTAGAGTCAAGCTCAA CAGGG. All products were analyzed by electrophoresis on 8% polyacrylamide gels and autoradiography.

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