CHAPTER TWELVE

In Vitro Reconstitution and Affinity Purification of Catalytically Active Archaeal Box C/D sRNP Complexes

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Contents

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Abstract

Archaeal box C/D RNAs guide the site-specific 2′-*O*-methylation of target nucleotides in ribosomal RNAs and tRNAs. *In vitro* reconstitution of catalytically active box C/D RNPs by use of *in vitro* transcribed box C/D RNAs and recombinant core proteins provides model complexes for the study of box C/D RNP assembly, structure, and function. Described here are protocols for assembly of the archaeal box C/D RNP and assessment of its nucleotide modification activity. Also presented is a novel affinity purification scheme that uses differentially tagged core proteins and a sequential three-step affinity selection protocol that yields fully assembled and catalytically active box C/D RNPs. This affinity selection protocol can provide highly purified complex in sufficient quantities not only for biochemical analyses but also for biophysical approaches such as cryoelectron microscopy and X-ray crystallography.

1. Introduction

The box C/D RNAs constitute large populations of small noncoding RNAs found in both eukaryotic and archaeal organisms, where their primary function is to guide the site-specific 2′-*O*-methylation of nucleotides located in various target RNAs. Guide sequences within each box C/D RNA base pair to complementary sequences in the target RNA, thereby designating specific nucleotides for posttranscriptional modification (Bachellerie *et al.*, 2002; Maxwell and Fournier, 1995; Terns and Terns, 2002; Tollervey, 1996). Archaeal box C/Ds RNAs are defined by highly conserved boxes C and D located near their 5′ and 3′ termini and internally located C′ and D′ boxes (Dennis *et al.*, 2001; Omer *et al.*, 2000). Both the external boxes C and D and the internal C′ and D′ boxes fold to establish kink-turn or K-turn motifs. It is these highly structured K-turn (box C/D) and K-loop (C′/D′) motifs that serve as binding platforms for the box C/D RNP core proteins (Hama and Ferre-D'Amare, 2004; Moore *et al.*, 2004; Omer *et al.*, 2002; Suryadi *et al.*, 2005; Tran *et al.*, 2003). Archaeal core proteins ribosomal protein L7, Nop56/58, and fibrillarin bind both box C/D and C′/D′ motifs to establish individual RNP complexes. It is the core proteins, working in concert with the guide regions located immediately upstream of the D and D′ boxes, that direct the 2′-*O*-methylation of targeted nucleotides (Omer *et al.*, 2002; Tran *et al.*, 2003).

Investigation of box C/D RNP structure and function has been greatly facilitated in recent years with the establishment of *in vitro* systems that assemble catalytically active archaeal sRNP complexes by use of *in vitro* transcribed sRNAs and recombinant sRNP core proteins purified from bacterial expression systems. Several laboratories, including our own, have used these *in vitro* assembled complexes to investigate the assembly,

structure, and methylation function of this RNA-protein enzyme (for a review see Dennis and Omer, 2006). Presented here are detailed protocols for the assembly of a *Methanocaldococcus jannaschii* box C/D sRNP and the assessment of the complex's methylation capabilities. Also described here is a novel sRNP isolation protocol involving three sequential affinity selection steps that use differentially tagged fibrillarin core proteins and an oligonucleotide complementary to the sRNA. This purification scheme yields highly purified and fully assembled archaeal box C/D sRNPs in sufficient quantities for not only biochemical analyses but also for biophysical approaches such as cryoelectron microscopy and X-ray crystallography. Although specifically designed for the *M. jannaschii* sR8 box C/D sRNP, this approach can be easily modified for the isolation of other sRNP complexes assembled either *in vitro* or possibly *in vivo* in the cell.

2. Cloning, Expression, and Preparation of *M. jannaschii* Box C/D sRNP Core Proteins

Genes encoding the *M. jannaschii* core proteins L7, Nop56/58, and fibrillarin were PCR-amplified from isolated genomic DNA, inserted into bacterial expression vectors, and recombinant proteins expressed and then purified by use of affinity and cation-exchange chromatography as previously outlined (Tran *et al.*, 2003). The cloning, expression, and purification of each core protein are presented here in greater detail.

2.1. Cloning of *M. jannaschii* L7, Nop56/58, and fibrillarin genes

Ribosomal protein L7, fibrillarin, and Nop56/58 gene coding sequences are PCR amplified from *M. jannaschii* genomic DNA. DNA oligonucleotide primers are synthesized for each core protein gene and used for PCR amplification. The L7 and fibrillarin upstream and downstream primers contain 5′ terminal Nde*I* and Bam*HI* restriction sites, respectively, whereas the Nop56/58 primers contain Nco*I* and Bam*HI* restriction sites. After PCR amplification, resulting DNA fragments are digested with the appropriate restriction endonucleases and ligated into similarly digested pET28a (Novagen) protein expression vectors by standard methods. Plasmid constructs are transformed into *E. coli* cells and colonies screened for plasmids containing core protein sequences by PCR amplification by use of L7-, Nop56/58-, or fibrillarin-specific primers. Selected colonies are cultured in liquid LB broth with antibiotics and plasmid DNA harvested with Wizard Midiprep Kits (Promega). Expression of recombinant L7 and fibrillarin

proteins produces N-terminal and thrombin-cleavable 6×-histidine tags. The expressed Nop56/58 recombinant protein is untagged. To generate FLAG-tagged fibrillarin, the fibrillarin coding sequence is PCR amplified from genomic *M. jannaschii* DNA and ligated into the pET28a vector engineered to encode a FLAG peptide (DYKDDDDK) in place of the His tag. The sequences of all core protein plasmid constructs are verified by DNA sequencing.

2.2. Recombinant core protein expression in bacterial cells

Core proteins are expressed in the Rosetta (DE3) strain of *E. coli* (Novagen), a protein expression host carrying a plasmid encoding rare tRNA codons to help enhance levels of recombinant protein synthesis. Competent cells are transformed with individual plasmids and spread onto LB agar plates containing kanamycin (30 μg/ml) and chloramphenicol (34 μg/ml). A single cell colony is selected and grown in 1 liter of LB broth containing antibiotics with shaking at 37° until the cell culture reaches an optical density at 600 nm (OD_{600}) of 0.8. Expression of His-L7, His-fibrillarin, and FLAG-fibrillarin is initiated by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M* and shaking is continued for 4 h at 37°. For expression of Nop56/58, the transformed cell culture is grown at 37° with shaking to an OD_{600} of 0.5 and then moved to 15° and shaken for an additional 30 min. Nop56/58 expression is induced with the addition of IPTG (0.4 m*M*) and shaking continued at 15° for an additional 24 h. IPTG induces the expression of a genomically encoded bacteriophage T7 RNA polymerase gene under control of the *lacUV5* promoter. The expressed T7 RNA polymerase transcribes the plasmid-encoded core protein genes under control of the pET28a vector's T7 promoter.

After recombinant core protein expression, cells are pelleted by centrifugation at 10,000*g* for 10 min at 4° and then resuspended in 5 ml of buffer D (20 m*M* HEPES, pH 7.0, 100 m*M* NaCl, 3 m*M* $MgCl_2$, 20% glycerol [w/v]) per gram of cell paste. A protease inhibitor cocktail (Cocktail Set VII, Calbiochem) at a final concentration of 1% (w/v) and 10 U/ml of Benzonase Nuclease (Novagen) are added to prevent protein degradation and promote nucleic acid degradation, respectively. To lyse cells, the cell suspension is sonicated (Fisher Sonic Dismembrator, Model 150) at maximum power for 30 sec (×3) on ice, with 1-min cooling intervals. The lysate is then mixed by rocking for 1 hr at room temperature to facilitate nucleic acid degradation. Degradation of nucleic acids at this step is crucial for preparation of the Nop56/58 core protein, because contaminating nucleic acids bind this core protein tightly, resulting in protein precipitation and greatly reduced yields. Cell lysates are then separated into soluble and insoluble protein fractions by ultracentrifugation at 38,000*g* for 30 min at 4°. Most recombinant protein remains in the soluble fraction. Nop56/58

core protein is expressed at lower levels than either L7 or fibrillarin, and only 50% of the total Nop56/58 protein is soluble. However, without low temperature expression, nearly all the Nop56/58 protein is found in the insoluble fraction. These soluble fractions can be stored at −80° for several weeks before continuing with chromatographic enrichment of the individual core proteins.

2.3. Affinity chromatographic isolation of L7 and fibrillarin core proteins

His-tagged L7 (14.7 kDa, 13 kDa without the His tag) and His-tagged fibrillarin (28 kDa) are isolated from soluble protein fractions to >90% purity by use of a single nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography step (Fig. 12.1A, lanes 1 and 2). The soluble lysate fraction containing recombinant, His-tagged L7 or fibrillarin protein is applied at room temperature to 4 ml of buffer D–equilibrated Ni^{2+}-charged Ni-NTA His-bind resin (Novagen) suspended in a 20 ml (1.5 × 10 cm) chromatography column. This resin has an estimated binding capacity of 8 mg of protein/ml of resin. The column is then washed with 25 bed volumes of buffer D_{300} (buffer D with 300 m*M* NaCl) containing 40 m*M* imidazole. Glycerol may be omitted from the wash buffer to increase column flow rates. Bound core protein is eluted with 2 bed volumes of buffer D containing 250 m*M* imidazole and collected in 1–2 ml fractions. Elution fractions containing the core protein are pooled and dialyzed against 100 volumes of buffer D overnight at 4° to remove imidazole. The His-tag may be removed at this point by digesting the isolated core protein with thrombin (~5 U/ml) either before or during dialysis. Protein concentrations are estimated by UV absorbance at 280 nm by use of Beer's Law and extinction coefficients of 5,240 cm^{-1} M^{-1} (experimentally determined) and 29,900 cm^{-1} M^{-1} (calculated) for L7 and fibrillarin, respectively. Use of dye-based assays, such as the Bradford assay to determine protein concentrations, is suitable for fibrillarin. However, L7 is not proportionately stained with respect to protein concentration.

FLAG-tagged fibrillarin (28 kDa) is affinity selected from the soluble cell sonicate with ANTI-FLAG M2 affinity agarose (Sigma). Fibrillarin lysate is applied twice at room temperature to 5 ml of ANTI-FLAG M2 agarose resin previously equilibrated in buffer D and packed in a 20-ml chromatography column (estimated resin binding capacity of 0.5–1.0 mg of fibrillarin per ml of resin). The affinity column with bound fibrillarin is then washed with 25 bed volumes of buffer D_{300} (300 m*M* NaCl). FLAG-fibrillarin can be eluted with 3 bed volumes of buffer D containing 100 μg/ml of FLAG peptide (Sigma). However, an alternative and more economical approach is to elute bound FLAG-fibrillarin with 0.1 *M* glycine, pH 3.5, containing 100 m*M* NaCl. The eluted fractions are collected into 1.5-ml Microfuge tubes containing 1/10 the elution volume of 1 *M* Tris, pH 8.0 (the Tris pH

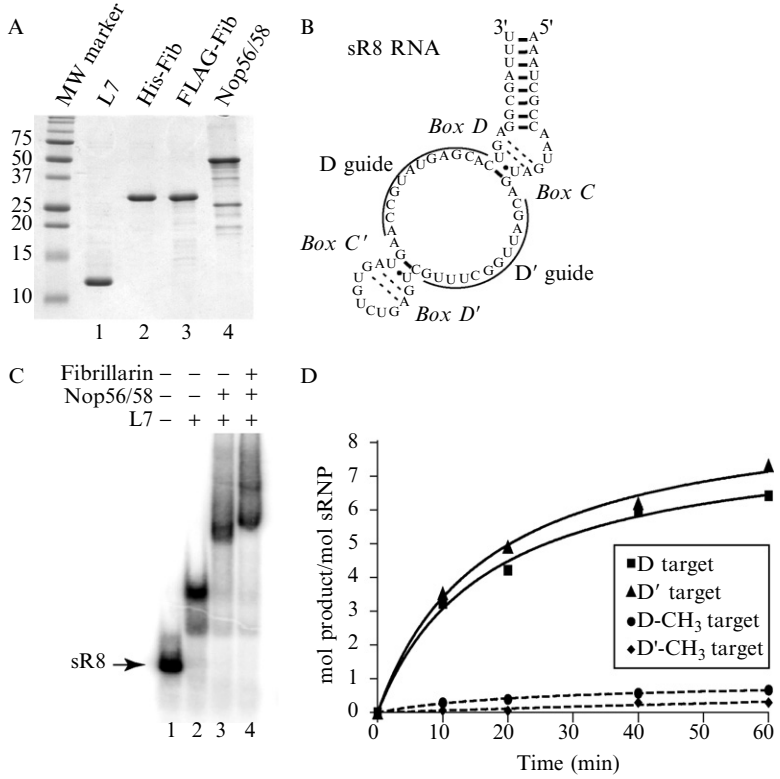

Figure 12.1 Archaeal box C/D recombinant core protein isolation, *in vitro* sRNP assembly, and *in vitro* sRNP-guided nucleotide 2′-*O*-methylation. (A) Coomassie Brilliant Blue–stained SDS-polyacrylamide gel of isolated *M. jannaschii* box C/D sRNP core proteins used for *in vitro* sRNP assembly. (B) Sequence and folded secondary structure of the *M. jannaschii* sR8 box C/D sRNA used for *in vitro* sRNP assembly. (C) Electrophoretic mobility-shift analysis revealing the hierarchal binding of the sRNP core proteins to 5′-radiolabeled sR8 sRNA in assembly of the box C/D sRNP. (D) *In vitro* methylation of D and D′ target RNAs by the *in vitro* assembled sR8 box C/D sRNP. The lack of methylation at the target nucleotide when this nucleotide is already methylated at the ribose 2′ position (D-CH_3 and D′-CH_3 targets) demonstrates nucleotide-specific modification of each target RNA by the *in vitro* assembled sRNP.

8.0 buffer reestablishes the eluate pH to approximately 7.0). Both elution protocols yield FLAG-tagged fibrillarin that efficiently assembles box C/D sRNPs that are active for sRNP-guided nucleotide methylation. ANTI-FLAG M2 resin has a lower binding capacity than the Ni-NTA resin. Therefore, this chromatography step is typically repeated several times with the same fibrillarin lysate, and the eluted fibrillarin fractions are pooled. Pooled

fibrillarin fractions are then concentrated 10–15 fold using sequentially a 50-ml Amicon Centricon Concentrator (10,000 MWCO, Millipore) and a 15-ml spin concentrator (Vivaspin 15R, Vivascience) before dialysis against 100 volumes of buffer D for 16 h at 4°. This single-affinity selection step yields FLAG-tagged fibrillarin at >85% homogeneity (Fig. 12.1A, lane 3). FLAG-tagged fibrillarin concentrations are determined either by a Bradford assay or UV absorbance at 280 nm with a calculated extinction coefficient of 31,400 cm^{-1} M^{-1}. Isolated L7 and fibrillarin protein preparations can be stored at −80° for up to 1 y.

2.4. Isolation of Nop56/58 core protein by cation-exchange chromatography

Purification of recombinant Nop56/58 (48 kDa) by cation-exchange chromatography takes advantage of this core protein's strong positively charged character. Initial chromatographic isolation of Nop56/58 used SP Sepharose Fast Flow cation-exchange resin (Amersham Biosciences) (Tran *et al.*, 2003). We now routinely use heparin agarose, because this resin more efficiently binds Nop56/58, has a higher binding capacity, and yields a more homogeneous protein preparation. Affinity-tagged versions of Nop56/58 previously tested in our laboratory did not bind their affinity resins. Notably, however, an N-terminally His-tagged version of Nop56/58 has been successfully purified with Ni-NTA metal affinity chromatography (developed by the Brown laboratory, Wake Forest University), and this affinity-purified Nop56/58 is comparable to protein isolated by use of cation exchange chromatography (Zhang *et al.*, 2006).

The Nop56/58 lysate is applied twice at room temperature to 10 ml of buffer D–equilibrated heparin agarose (MP Biomedicals) packed in a 40-ml chromatography column (1.5 × 20 cm). The resin with bound Nop56/58 is then washed with 30 bed volumes of buffer D_{800} (800 m*M* NaCl). Bound Nop56/58 is eluted with 3 bed volumes of high-salt buffer D_{1300} (1.3 *M* NaCl). The Nop56/58 eluate is diluted with buffer D to a final NaCl concentration of 500 m*M* and then concentrated to approximately 2 ml by use of the concentration techniques outlined earlier for FLAG-tagged fibrillarin. Concentrated Nop56/58 is then centrifuged at 14,000*g* at room temperature for 15 min to pellet insoluble protein. Nop56/58 is stored in buffer D_{500} to avoid aggregation and precipitation, although buffers with lower salt concentrations are suitable for lower Nop56/58 concentrations. This single purification step typically yields Nop56/58 protein to approximately 60–70% homogeneity (Fig. 12.1A, lane 4). A Bradford assay estimates the concentration of isolated Nop56/58, and this core protein can be stored at −80° for up to 1 y.

3. Cloning and *In Vitro* Transcription of Archaeal Box C/D sRNAs

The *M. jannaschi* sR8 box C/D sRNA (Fig. 12.1B) gene was originally PCR amplified from genomic DNA and then cloned into a pUC19 plasmid (Tran *et al.*, 2003). *In vitro* transcription of sR8 sRNA is accomplished by first generating DNA templates from this plasmid by PCR amplification. The upstream PCR primer possesses a T7 promoter sequence (22 nucleotides) at the 5′ terminus followed by the first 22 nucleotides of sR8 coding sequence, whereas the downstream primer is complementary to the last 23 nucleotides of sR8 coding sequence. PCR amplification of the sR8 pUC19 plasmid by use of these primers generates a DNA template for *in vitro* T7 RNA polymerase transcription. A standard 100 μl PCR amplification reaction of 35 cycles produces approximately 8–10 μg of DNA template from 50 ng of plasmid DNA. Ampliscribe Flash T7 Transcription Kits (Epicentre) are used to synthesize sRNA transcripts following the manufacturer's protocol, except that the 37° incubation is extended to 5 h. Transcribed RNA is phenol-chloroform extracted, ethanol precipitated, and then resuspended in Tris-Borate-EDTA (TBE) buffer containing 80% formamide. The RNA is resolved on denaturing 6% polyacrylamide-TBE gels containing 7 *M* urea. RNA bands are visualized by UV shadowing for excision from the gel. sRNA is eluted from the gel slice (×3) at room temperature for 45–60 min with 2 ml of elution buffer (10 m*M* Tris, pH 7.4, 0.3 *M* sodium acetate, 5 m*M* EDTA, 0.1% SDS) per gram of gel by use of the crush-and-soak method. Eluted RNA is ethanol precipitated, resuspended in water, and the RNA concentration determined by absorbance at 260 nm. RNA is then aliquoted, dried, and stored at −80°. This *in vitro* transcription protocol produces approximately 50–80 μg of gel-purified sR8 sRNA per μg of DNA template for a standard 20 μl transcription reaction.

4. *In Vitro* Assembly of the *M. jannaschii* sR8 Box C/D sRNP Complex

Core protein binding capabilities and sRNP assembly are assessed by use of electrophoretic mobility-shift analysis (EMSA) (Tran *et al.*, 2003). *In vitro* assembly of the *M. jannaschii* sR8 box C/D sRNP is accomplished by incubating 5′-radiolabeled sR8 sRNA (0.2 pmol and 1×10^4 cpm) with 20 pmol of L7, 32 pmol Nop56/58, and 32 pmol fibrillarin in assembly buffer (20 m*M* HEPES, pH 7.0, 150 m*M* NaCl, 1.5 m*M* $MgCl_2$, 10% glycerol) containing tRNA (1.5 mg/ml). Binding capabilities of individual core

proteins are determined by sequentially adding L7, Nop56/58, and then fibrillarin as assembly of the complex requires ordered binding of the core proteins. Assembly of the sRNP complex is accomplished by incubating sRNA and core proteins at 75° for 10 min. *In vitro* assembly of the sRNP requires elevated temperatures to facilitate sRNA remodeling required for core protein binding (Gagnon *et al.*, 2006). Partial or completely assembled complexes are resolved on native 4% polyacrylamide gels containing 25 m*M* potassium phosphate buffer, pH 7.0, and 2% glycerol. After electrophoresis, gels are dried, and assembled RNPs visualized by autoradiography or PhosphorImager analysis. Figure 12.1C shows a representative EMSA analysis of the sequential binding of sRNP core proteins to radiolabeled sR8 sRNA.

5. Assessment of *In Vitro* Assembled *M. jannaschii* Box C/D sRNP Methylation Activity

Methyltransferase activity of the *in vitro* assembled *M. jannaschii* sR8 box C/D sRNP is assessed with an *in vitro* methylation assay. Assembled sRNP complexes are incubated in the presence of the methyl donor *S*-adenosyl-L-[*methyl*-^{3}H] methionine (SAM) (Amersham Pharmacia) and synthetic target RNA oligonucleotide substrates (Dharmacon) that are complementary to the D or D′ guide regions. Methylation activity is assessed by measuring the incorporation of [^{3}H]–CH_3 into these target RNAs. Assembly reactions of 80 μl and approximately 0.5 μM assembled sRNP are incubated on ice and mixed with 30 μl of assembly buffer containing 12 μM target RNA substrate(s) and 15 μM SAM (5 μCi of [^{3}H]-SAM at a 1:50 ratio with nonradioactive SAM). This SAM concentration is sufficient for this methylation assay, although recent work has indicated that higher concentrations of SAM can drive the reaction to yield higher levels of target RNA methylation (Hardin and Batey, 2006). We have established that the length of target RNA oligonucleotide substrates affects the level of RNA methylation. Extending the target RNA at both 5′ and 3′ termini by 4–5 nucleotides beyond that region that base pairs with the sRNA guide sequence significantly increases [^{3}H]–CH_3 incorporation (Appel and Maxwell, 2007). Negative controls are target RNAs already possessing a 2′-*O*-CH_3 at the target nucleotide or target RNAs with a deoxynucleotide replacing the target ribonucleotide.

Target RNA methylation is initiated by incubating the assembled reactions at 68°. Aliquots of 20 μl are removed at the desired time points and spotted onto 2-cm filter discs (3 *M* Whatman paper). After drying, the filters are washed in 10% trichloroacetic acid (TCA) and then three times in 5% TCA. Washed and dried filters are suspended in scintillation fluid and

counted in a liquid scintillation counter. Results of a typical *in vitro* methylation activity assay are shown in Fig. 12.1D. Methylation activity of the sRNP is reported as moles of methylated target RNA per mole of sRNP. Conversion of cpms to moles of incorporated [^{3}H]–CH_3 is accomplished by spotting 1 μCi of [^{3}H]–SAM onto control filters, determining the cpms/uCi, and then calculating the moles of incorporated CH_3 using the specific activity of the [^{3}H]–SAM (Ci/mole) provided by the manufacturer and the molar ratio of radioactive and nonradioactive SAM in the reaction. This value is then reported with respect to the moles of assembled sRNP in the reaction.

6. Sequential Affinity Chromatographic Purification of *In Vitro* Assembled Box C/D sRNPs

6.1. An overview

Affinity purification with tagged proteins or RNAs has proved to be a powerful approach for isolating multicomponent protein and RNA–protein complexes from isolated cellular extracts (Rigaut *et al.*, 1999; Schimanski *et al.*, 2005; Srisawat and Engelke, 2002; Waugh, 2005). Compared with more traditional fractionation techniques such as gradient sedimentation centrifugation, gel filtration, and ion exchange chromatography, affinity chromatography typically yields highly purified complexes in only one or two isolation steps. Therefore, we have developed a tandem affinity purification protocol for the rapid isolation of *in vitro* assembled archaeal box C/D sRNPs. This protocol uses three affinity selection steps that are designed to isolate fully assembled and catalytically active sRNP.

Archaeal box C/D sRNPs are assembled by use of large preparations of *in vitro* transcribed box C/D sRNA and the three recombinant sRNP core proteins. Assembled sRNPs are first selected by use of an oligonucleotide complementary to the sRNA. Two approaches can be used in this step. An sR8 sRNA engineered with a poly-A tail (14 adenines) at the 3′ end is used in sRNP assembly, and selection is carried out with oligo-dT cellulose resin. Alternately, a biotinylated DNA oligonucleotide complementary to the sR8 D guide region is hybridized to the assembled sRNP and then selected with streptavidin resin. For both approaches, the sRNA and bound core proteins are efficiently eluted from the respective resins at elevated temperature. We have noted, however, that sRNP affinity selected with the biotinylated oligonucleotide exhibits a 20–30% reduction in methylation activity guided by the terminal box C/D RNP. Subsequently, assembled complexes are sequentially selected by fibrillarin's FLAG tag and His tag by

use of ANTI-FLAG M2 and Ni-NTA affinity resins, respectively. Fibrillarin is the third and final core protein to bind the box C/D and C′/D′ motifs. Sequential affinity selection of the *in vitro* assembled sRNP possessing the two tags assures that each isolated complex contains two fibrillarin proteins and is thus a fully assembled sRNP with catalytically active box C/D and C′/D′ RNPs. This is particularly important for biophysical analyses such as cryoelectron microscopy or X-ray crystallography, where a homogeneous population of complexes is crucial for analysis.

Selection of the sRNP by use of complementary oligonucleotides as the first selection step effectively eliminates free core proteins not bound to the sRNA. Removal of free proteins is particularly advantageous at this point in the isolation protocol. The highly charged character of free Nop56/58 can cause aggregation problems. Protein aggregation may be minimized by use of higher salt buffers and working in the presence of very low concentrations of SDS (~0.007%), nonionic detergents (0.1% Triton X-100), or nonspecific RNA (1–2 mg/ml tRNA). These added components could increase sRNP yields, although small amounts of detergent may be carried through the purification process and be present in the final purified sRNP fraction. Nop56/58 and fibrillarin also efficiently dimerize in the absence of sRNA and sequential isolation of the sRNP complexes solely by means of the tagged fibrillarin core proteins results in isolation not only of the sRNP but also of free Nop56/58-fibrillarin dimers. ANTI-FLAG M2 affinity chromatography is the second step in sRNP purification, because this resin exhibits low binding capacity and low elution efficiency. By placing this affinity step second in the purification protocol, we are able to use larger amounts of resin and elute the sRNP in larger elution volumes for more efficient recovery. As the final selection step, the Ni-NTA resin binds the His-tagged fibrillarin with high affinity. The large capacity of this resin facilitates elution of more concentrated sRNP in smaller final volumes. We have found that this order of affinity selection steps is most efficient for purification of fully assembled and methylation-competent sRNP.

A flowchart for the tandem affinity selection and purification of *in vitro* assembled archaeal box C/D sRNP complexes is presented in Fig. 12.2. The starting amounts of assembled sRNP may be reduced or scaled up, depending on the quantity of purified sRNP desired. For the particular sRNP purification experiment shown here, additional components such as detergents were omitted. sRNP obtained from the final Ni-NTA affinity column can be exchanged with any buffer of choice during concentration, depending on the requirements of the planned experiments, although buffers of higher ionic strength help to reduce sRNP aggregation. sRNP fractions applied to each resin, collected flow through fractions, and subsequently eluted sRNP fractions are analyzed on an SDS-polyacrylamide gel and shown in Fig. 12.3A.

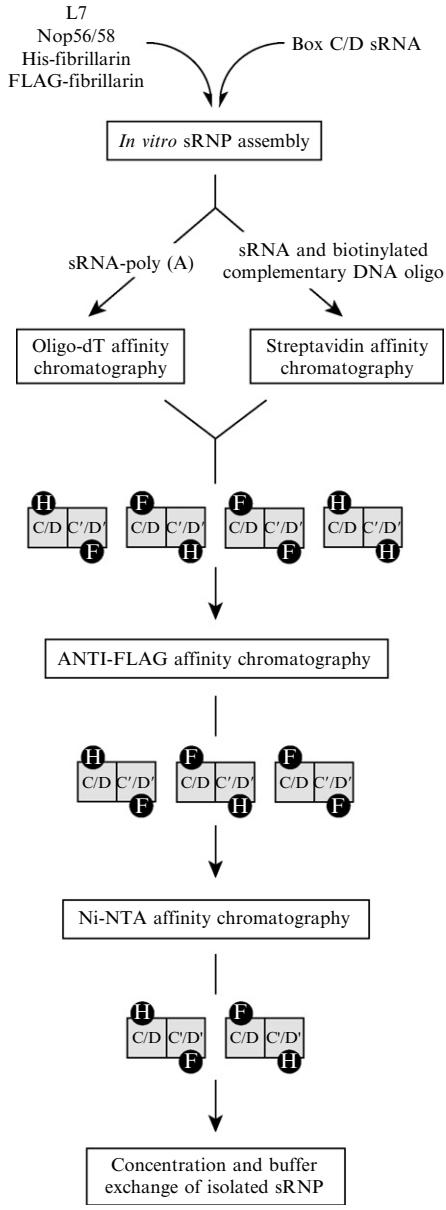

Figure 12.2 Sequential affinity purification of *in vitro* assembled archaeal box C/D sRNP.

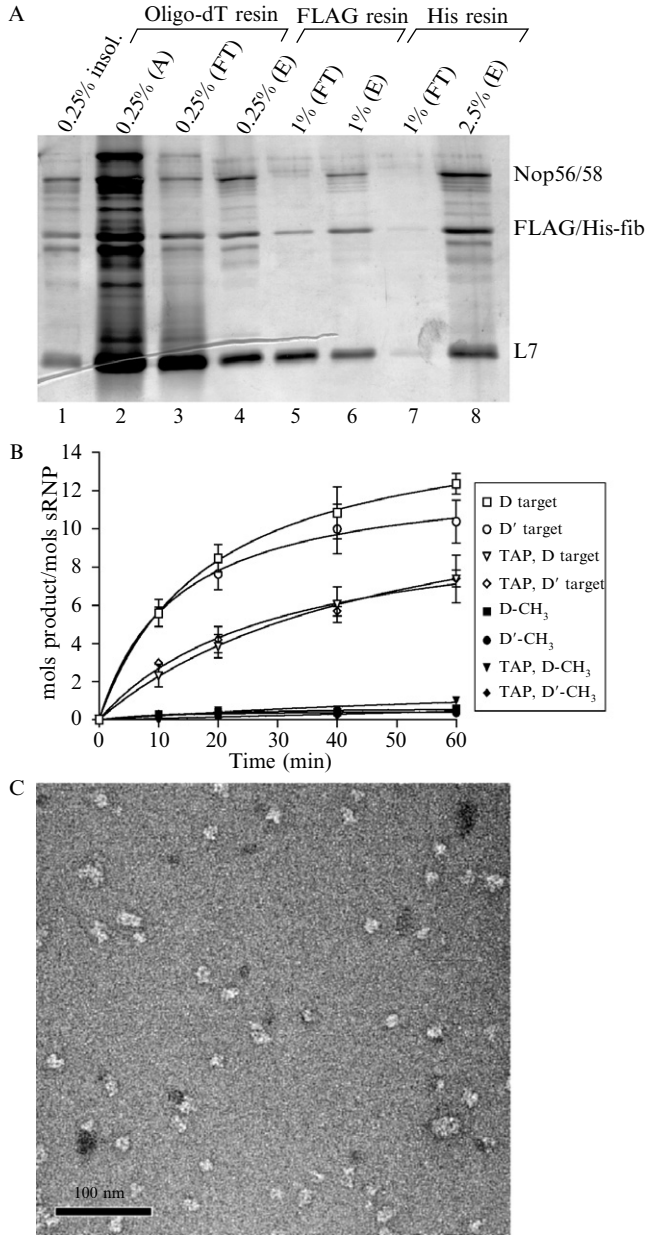

Figure 12.3 SDS-polyacrylamide gel electrophoretic analysis, methyltransferase activity, and electron microscopy of *in vitro* assembled *M. jannaschii* sR8 box C/D sRNPs. (A) SDS-polyacrylamide gel electrophoretic analysis of sRNP fractions. Silver-stained sRNP fractions analyzed from the individual affinity columns include the sRNP sample applied (A); the flow-through fraction of unbound material (FT);

6.2. Affinity chromatography buffers

10× Binding buffer (BB): 100 m*M* HEPES, pH 7.0, 1.0 *M* NaCl
Buffer R: 20 m*M* HEPES, pH 7.0, 0.1 *M* NaCl, 1 m*M* EDTA
Buffer D: 20 m*M* HEPES, pH 7.0, 3 m*M* $MgCl_2$, 100 m*M* NaCl, 20% glycerol
Buffer E: 20 m*M* HEPES, pH 7.0, 1.5 m*M* $MgCl_2$, 500 m*M* NaCl
Buffer G: 0.1 *M* glycine, pH 3.5, 100 m*M* NaCl

6.3. Preparation of the affinity chromatography resins

1. Oligo-dT cellulose (Ambion): Equilibrate 4 ml of resin packed in a 20 ml (1.5 × 10 cm) chromatography column with buffer E.

1A. Streptavidin agarose (Novagen): Equilibrate 3 ml of resin packed in a 20 ml (1.5 × 10 cm) chromatography column with buffer D. Bind 3 mg of biotinylated DNA oligonucleotide (Integrated DNA Technologies) complementary to the D guide sequence (underlined) by incubating the oligonucleotide (5′-(biotin)-ACAGTCAT CGCTTGCTCATACGGTCTC-3′) and resin for 10 min at room temperature. Then equilibrate the resin with buffer E.

2. ANTI-FLAG M2 agarose (Sigma): Equilibrate 3.0 ml of resin packed in a 20 ml (1.5 × 10 cm) chromatography column with buffer E.
3. Ni-NTA His-Bind Resin (Novagen): Charge 1.5 ml of resin packed in a 20-ml chromatography column (1.5 × 10 cm) with 5 ml of 50 m*M* $NiSO_4$. Then equilibrate the resin with buffer E containing 25 m*M* imidazole.

6.4. Assembly of the sR8 sRNP complex

sRNP are assembled *in vitro* by use of *in vitro* transcribed sRNA and recombinant core proteins, with the amount of assembled sRNP dependent on the desired amount of purified complex. Table 12.1 lists the components required for assembling 12 nmol (∼2.5 mg) of sRNP complex. Approximately

affinity column–bound and eluted sRNP fraction (E). Percentage of the total sample volume used for each electrophoretic analysis is indicated above the lane. (B) Assessment of the methyltransferase activity of tandem affinity-purified box C/D sRNP (TAP) compared with *in vitro* assembled but not affinity-selected complexes. Both affinity-selected and nonpurified sR8 box C/D sRNP methylate D and D′ targets, indicating fully assembled and catalytically active box C/D and C′/D′ RNPs. D and D′ targets possessing a 2′-O-CH_3 at the target nucleotide (negative controls) are not methylated, demonstrating nucleotide-specific modification for both affinity-purified and nonpurified complexes. (C) Electron micrograph of *in vitro* assembled and tandem affinity-purified *M. jannaschii* sR8 box C/D sRNP. Electron micrograph courtesy of Franziska Bleichert, Vinzenz Unger, and Susan Baserga (Yale University).

Table 12.1 *In vitro* sRNP assembly

Reaction component	Concentration	Volume (μl)	Approx. mass (μg)
L7	100 μM	250	325
Nop56/58	100 μM	250	1200
His-fibrillarin	100 μM	125	350
FLAG-fibrillarin	100 μM	125	350
Buffer D[a]	1×	500	—
poly (A) sRNA	38 μM	320	320
NaCl	5 M	155	—
Binding buffer[b]	10×	250	—
H_2O	—	525	—
Final		2500	2545

[a] 20 mM HEPES, pH 7.0, 0.1 M NaCl, 3 mM $MgCl_2$, 20%(w/v) glycerol.
[b] 100 mM HEPES, pH 7.0, 1.0 M NaCl.

12 nmol (320 μg) of sR8 sRNA, 24 nmol of L7 (325 μg), 24 nmol of Nop56/58 (1200 μg), and 12 nmol (350 μg) each of FLAG-tagged and His-tagged fibrillarin are incubated in a final assembly volume of 2.5 ml. Core proteins are stored in buffer D except for Nop56/58, which is stored in buffer D containing 0.5 M NaCl to maintain protein solubility. The final assembly buffer (20 mM HEPES, pH 7.0, 0.5 M NaCl, 1.5 mM $MgCl_2$, and 10% glycerol [w/v]) contains 0.5 M NaCl to minimize protein and sRNP aggregation. After mixing components, the assembly reaction is heated to 75° for 5 min and then cooled to room temperature. Centrifugation of the assembly reaction at 14,000g for 5 min at room temperature removes insoluble materials from the supernatant fraction containing assembled sRNP. Shown in Fig. 12.3A are the sRNP fractions obtained from the sequential affinity selection steps.

6.5. Tandem affinity purification of *in vitro* assembled box C/D sRNP

6.5.1. Affinity selection step 1: oligo dT cellulose chromatography

1. Mix the assembled sRNP (~12 nmol in 2.5 ml; Fig. 12.3A, lane 2) with 4 ml of oligo dT cellulose resin equilibrated with buffer E in a 20-ml chromatography column.
2. Bind the sRNP to the oligo dT resin by rocking the column for 5 min at room temperature and then continue rocking for 30 min at 4°.
3. Begin chromatography by collecting the flow through fraction (Fig. 12.3A, lane 3) and then washing the oligo dT resin with 20 bed volumes of cold (4°) buffer E. Maintain the column at 4° during washing to stabilize hydrogen bonding of the poly (A) tail to the oligo dT cellulose.

4. Transfer the column with bound sRNP to a 50° incubator and heat the column and resin for 20 min.
5. Elute the sRNP with 4 bed volumes of buffer E heated to 50° (Fig. 12.3A, lane 4).
6. Cool the sRNP eluate to room temperature for subsequent affinity selection on ANTI-FLAG M2 agarose.
7. Regenerate the oligo dT cellulose resin by washing sequentially with 5 bed volumes each of buffer R, 0.1 *M* NaOH, and H_2O.
8. Equilibrate the resin with buffer R containing 0.05% sodium azide and store at 4°. (For long-term storage, after the water wash, rinse the resin with ethanol, dry the resin, and store at −20°.)

6.5.2. Alternative affinity selection step 1: streptavidin affinity chromatography

1. Mix the assembled sRNP with 3 ml of buffer E–equilibrated streptavidin resin bound with the biotinylated DNA oligonucleotide and suspend in a 20-ml chromatography column.
2. Bind the sRNP to the streptavidin resin by rocking the column for 5 min at room temperature and then continue rocking for 30 min at 4°.
3. Begin chromatography by collecting the flow through fraction and then washing the resin with 20 bed volumes of cold buffer E. Maintain the column with resin at 4° during washing to stabilize hybridization of the biotinylated DNA oligonucleotide to the sRNA.
4. Transfer the affinity column with resin-bound sRNP to a 60° incubator and equilibrate the column for 20 min at this elevated temperature.
5. Elute bound sRNP with 4 bed volumes of buffer E heated to 60°.
6. Cool the eluted sRNP fraction to room temperature for subsequent affinity selection on ANTI-FLAG M2 agarose.
7. Regenerate the DNA oligonucleotide-streptavidin resin by washing with 6 bed volumes of buffer R heated to 60°.
8. Equilibrate the resin in buffer R containing 0.05% sodium azide and store at 4°.

6.5.3. Affinity selection step 2: ANTI-FLAG M2 affinity chromatography

1. Mix the cooled sRNP eluate (12–16 ml) with 3 ml of buffer E–equilibrated ANTI-FLAG M2 agarose suspended in a 20-ml chromatography column.
2. Bind the sRNP to the agarose resin by incubating for 15 min at room temperature.

3. Collect the flow through (Fig. 12.3A, lane 5) and reapply this eluate to the affinity column twice more, each time incubating the sRNP eluate with the resin for 15 min at room temperature.
4. Wash the agarose resin with 20 bed volumes of buffer E at room temperature.
5. Release the bound sRNP by resuspending the agarose resin in 1 bed volume of buffer E containing 110 μg/ml of FLAG peptide (Sigma) and incubate for 10 min at room temperature.
6. Collect the column eluate containing the sRNP.
7. Resuspend the agarose resin twice more in 1 bed volume of buffer E with FLAG peptide, each time incubating the resin for 10 min at room temperature before collecting the eluate.
8. Pool all three sRNP eluate fractions (Fig. 12.3A, lane 6) for subsequent Ni-NTA affinity chromatography.
9. Regenerate the agarose resin by washing twice with 4 bed volumes of buffer G. Do not allow this resin to be suspended in buffer G for more than 20 min. Wash the resin with buffer R.
10. Equilibrate the agarose resin in buffer R containing 0.05% sodium azide and store at 4°.

6.5.4. Affinity selection step 3: Ni-NTA His-Bind resin affinity chromatography

1. Mix the pooled eluates from ANTI-FLAG M2 affinity chromatography with 1.5 ml of buffer E–equilibrated Ni-NTA His-Bind resin suspended in a 20-ml chromatography column and incubate for 10 min at room temperature.
2. Collect the column flow through fraction (Fig. 12.3A, lane 7) and reapply the eluate to the resin, incubating another 10 min at room temperature.
3. Wash the affinity resin with 20 bed volumes of buffer E containing 25 m*M* imidazole.
4. Release the bound sRNP by resuspending the resin in 2 bed volumes of buffer E containing 200 m*M* imidazole and incubating for 5 min at room temperature.
5. Collect the eluate fraction.
6. Resuspend the resin in an additional 2 bed volumes of buffer E containing 200 m*M* imidazole and incubate for an additional 5 min at room temperature.
7. Collect the second eluate fraction.
8. Pool the two eluates (this pooled eluate may be stored overnight at 4°).
9. Concentrate the pooled eluates to between 200 and 500 μl by use of a 15-ml spin concentrator (Vivaspin 15R, Vivascience) and a microspin concentrator (Microcon YM-3, Millipore). During concentration, an

exchange of buffers to the desired final buffer may be accomplished. Dialysis is not recommended at this step.

10. Regenerate the Ni-NTA His-Bind resin by washing with 5 bed volumes of 100 m*M* EDTA, pH 7.0, followed by washing with water.
11. Equilibrate the Ni-NTA His Bind resin in 20% ethanol and store at 4°.

The yield of purified sRNP is estimated by assessing the amount of RNA and/or protein contained in a small aliquot of complex resolved on polyacrylamide gels. Alternately, the amount of RNA in a phenol-extracted aliquot of sRNP is determined by absorbance at 260 nm. Typically 200–300 pmol (40–60 μg) of purified sRNP is obtained from approximately 2.5 mg of assembled complex. Typical losses are observed for each affinity step, with major losses being at the second and third selection step for the FLAG-tagged and His-tagged fibrillarin proteins. At these steps, significant amounts of sRNP are lost as unselected complexes, because these sRNP are assembled with similarly tagged but unselected fibrillarin proteins at both box C/D and C′/D′ RNPs. Assessment of box C/D RNP-guided nucleotide methylation activity of the purified complexes reveals methylation of target RNAs for both box C/D and C′/D′ RNPs comparable to that of unpurified sRNPs (Fig. 12.3B). Determination of sRNP methylation capabilities for complexes suspended in buffer E with elevated NaCl concentration has no discernible effect on the complex's enzymatic activities. Electron microscopy of isolated sRNP reveals sRNP of homogeneous size with some larger complexes (Fig. 12.3C). The larger complexes are aggregated sRNP, and their presence can be diminished with the addition of ionic (SDS, heparin) compounds, although elevated concentrations can destabilize the sRNP complex.

7. Concluding Remarks

In vitro assembled archaeal sRNPs provide a model complex for the investigation of box C/D RNP assembly, structure, and function. Study of these minimal, yet catalytically active, RNA–protein enzymes will help define the fundamental principles behind RNA-guided nucleotide modification. The archaeal sRNP also serves as a prototype box C/D RNA-guided nucleotide modification enzyme for understanding the more structurally and functionally complex eukaryotic snoRNPs. Affinity purification of completely assembled and catalytically active sRNPs can now provide not only complexes for more detailed biochemical and functional studies but also for various biophysical approaches requiring larger amounts of material. Of particular advantage is the fact that the isolated complexes are homogeneous in composition, a prerequisite for approaches such as cryoelectron microscopy and X-ray crystallography.

The sequential affinity approach described here may well have broader applications in the study of RNA-guided nucleotide modification complexes. This same isolation protocol should be easily adapted to isolate other box C/D complexes and, in principle, *in vitro* assembled H/ACA sRNPs. Perhaps more exciting is the possibility to use this affinity protocol for the isolation of *in vivo* assembled RNP complexes. Expression of tagged core proteins in various cell lines should make affinity selection of the corresponding RNP complexes from cell lysates or various cellular fractions possible. This could ultimately lead to the identification of additional or ''accessory'' proteins associated with a family of complexes, thus defining novel proteins important for RNP biogenesis and/or function. Also possible may be the selection of specific RNP complexes from a homogenous RNP population by use of oligonucleotides complementary to a given sRNA or snoRNA species. Again, such an approach could lead to the identification of accessory proteins unique to a specific RNP complex. Although these suggested approaches have yet to be tested, they have the potential to greatly facilitate more detailed examinations of the diverse and highly conserved populations of RNA-guided nucleotide modification complexes.

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