

# Sequential 2'-O-Methylation of Archaeal Pre-tRNA<sup>Trp</sup> Nucleotides Is Guided by the Intron-encoded but *trans*-Acting Box C/D Ribonucleoprotein of Pre-tRNA\*

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***Haloferax volcanii* pre-tRNA<sup>Trp</sup> processing requires box C/D ribonucleoprotein (RNP)-guided 2'-O-methylation of nucleotides C34 and U39 followed by intron excision. Positioning of the box C/D guide RNA within the intron of this pre-tRNA led to the assumption that nucleotide methylation is guided by the *cis*-positioned box C/D RNPs. We have now investigated the mechanism of 2'-O-methylation for the *H. volcanii* pre-tRNA<sup>Trp</sup> *in vitro* by assembling methylation-competent box C/D RNPs on both the pre-tRNA and the excised intron (both linear and circular forms) using *Methanocaldococcus jannaschii* box C/D RNP core proteins. With both kinetic studies and single nucleotide substitutions of target and guide nucleotides, we now demonstrate that pre-tRNA methylation is guided in *trans* by the intron-encoded box C/D RNPs positioned in either another pre-tRNA<sup>Trp</sup> or in the excised intron. Methylation by *in vitro* assembled RNPs prefers but does not absolutely require Watson-Crick pairing between the guide and target nucleotides. We also demonstrate for the first time that methylation of two nucleotides guided by a single box C/D RNA is sequential, that is, box C'/D' RNP-guided U39 methylation first requires box C/D RNP-guided methylation of C34. Methylation of the two nucleotides of exogenous pre-tRNA<sup>Trp</sup> added to an *H. volcanii* cell extract also occurs sequentially and is also accomplished in *trans* using RNPs that pre-exist in the extract. Thus, this *trans* mechanism is analogous to eukaryal pre-rRNA 2'-O-methylation guided by intron-encoded but *trans*-acting box C/D small nucleolar RNPs. This *trans* mechanism could explain the observed accumulation of the excised *H. volcanii* pre-tRNA<sup>Trp</sup> intron *in vivo*. A *trans* mechanism would also eliminate the obligatory refolding of the pre-tRNA that would be required to carry out two *cis*-methylation reactions before pre-tRNA splicing.**

Eukaryotic cells possess numerous small nucleolar RNAs (snoRNAs),<sup>1</sup> the primary function of which is to guide the

2'-O-methylation and pseudouridylation of specific nucleotides within pre-rRNA and other target RNAs (1–7). The box C/D snoRNAs direct 2'-O-methylation of specific nucleotides within target RNAs. Members of this snoRNA family are defined by conserved box C (RUGAUGA) and D (CUGA) consensus sequences located in the 5' and 3' termini, respectively, of the snoRNA. Often, imperfect copies called C' and D' boxes are found internally. Regions of 10–21 nucleotides located upstream of boxes D and D' function as guide sequences, pairing with those regions in rRNA containing the nucleotide to be modified. The nucleotide sugar to be methylated resides within the snoRNA-rRNA duplex and is located 5 nucleotides upstream of box D or D'.

Box C/D RNAs are also found in Archaea, in which they are designated snoRNA-like RNAs or sRNAs (8–11). Archaeal sRNAs are generally smaller than the eukaryal snoRNAs and typically possess C' and D' boxes that vary little from the terminal box C and D sequences. The primary function of the archaeal box C/D sRNAs also is to guide the 2'-O-methylation of targeted nucleotides, and their mechanism of nucleotide modification is analogous to the eukaryal snoRNAs.

Both the eukaryal and archaeal box C/D RNAs are bound to core proteins to establish snoRNP and sRNP complexes, respectively. Four snoRNP core proteins are bound to the box C/D snoRNAs: fibrillarin (Nop1p), Nop56p, Nop58p (Nop5p), and the 15.5-kDa (Snu13p) protein (4–7). The methylation activity resides in the proteins of the snoRNP, with fibrillarin functioning as the methyltransferase. The differential distribution of the 15.5-kDa Nop56 and Nop58 proteins on the box C/D and C'/D' motifs establishes an asymmetric snoRNP complex (12, 13). The archaeal box C/D sRNP complex possesses three core proteins. Ribosomal protein L7Ae (the archaeal homolog of the eukaryal 15.5-kDa protein), afibrillarin, and aNop5p (a single homolog of eukaryal Nop56p and Nop58p) bind both the terminal C/D and internal C'/D' RNA motifs to establish a symmetrical RNP complex (4, 5, 14–19). *In vitro* assembly systems using purified archaeal sRNAs and recombinant core proteins have reconstituted enzymatically active box C/D sRNPs (16, 18, 20, 21). Core protein binding follows an order of assembly in which L7Ae binds first followed by aNop5p and then afibrillarin. Efficient catalysis requires that the box C/D and C'/D' RNPs be juxtaposed within the full-length sRNA (18).

The tRNA<sup>Trp</sup> of *Haloferax volcanii* is derived from an intron-containing pre-tRNA (22) and possesses 2'-O-methylated nucleotides at positions 34 (Cm) and 39 (Um) (where “m” is

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<sup>1</sup> The abbreviations used are: sno, small nucleolar; sRNA, sno-like

RNA; sRNP, sno-like RNP; RNP, ribonucleoprotein; nt, nucleotide; AdoMet, S-adenosylmethionine.

2'-O-methylation of the residue) (23). Analysis of the pre-tRNA<sup>Trp</sup> structure (see Fig. 1, upper left) revealed that the intron contains box C/D and C'/D' motifs with guide sequences complementary to the pre-tRNA regions encompassing modified nucleotides Cm34 and Um39, respectively (8, 10, 24). Subsequent investigations that involved deleting regions of the pre-tRNA intron and then examining nucleotide methylation *in vitro* led to the conclusion that these intron-encoded box C/D motifs are indeed responsible for guiding the methylation of pre-tRNA<sup>Trp</sup> nucleotides C34 and U39 (21, 24). These investigations also led to the proposal that the box C/D RNPs of the unspliced intron are responsible for guiding in *cis* the methylation of the pre-tRNA<sup>Trp</sup> nucleotides (21, 24). The presence of a *cis* mechanism for intramolecular methylation would be unique and provide a stark contrast from the box C/D RNP-guided *trans*-methylation in eukaryal and other archaeal systems. Although box C/D snoRNAs are frequently found in the introns of eukaryal pre-messenger RNAs, they are excised from the host pre-mRNA as snoRNPs before they guide the 2'-O-methylation of target nucleotides.

In this investigation, we have assembled *in vitro* a box C/D sRNP using recombinant *Methanocaldococcus jannaschii* core proteins L7Ae, aNop5p, and afibrillarlin and *H. volcanii* pre-tRNA<sup>Trp</sup>. This complex is enzymatically active and methylates tRNA<sup>Trp</sup> nucleotides C34 and U39. However, the experiments demonstrated that modification of the pre-tRNA target nucleotides was accomplished in *trans* using box C/D RNPs assembled in another unspliced pre-tRNA or in the excised intron (both linear and circular). Certain non-Watson-Crick pairings between target and guide nucleotides permitted 2'-O-methylations, although less efficiently. Nucleotide modification was also sequential, that is, modification of U39 guided by the C'/D' RNP first required methylation of C34 guided by the box C/D RNP. Pre-tRNA<sup>Trp</sup> methylation carried out in an *H. volcanii* cell extract confirmed the sequential methylation of these two nucleotides guided by *trans*-acting box C/D RNP complexes already present in the extract. Collectively, these observations indicate that the sequential methylation of the *H. volcanii* pre-tRNA<sup>Trp</sup> nucleotides guided by the intron-encoded box C/D RNP occurs via an intermolecular or *trans* mechanism rather than an intramolecular or *cis* mechanism as previously assumed. This *trans* mechanism could explain the accumulation of the excised introns of this pre-tRNA *in vivo* (25).

#### EXPERIMENTAL PROCEDURES

**DNA Template Construction and Site-directed Mutagenesis**—The following DNA oligonucleotide primers were used in PCR-amplification of plasmid pVT9P11 (25) to produce DNA templates for *in vitro* transcription: 1) TAATACGACTCACTATAGGGGCTGTGGCCAAGC; 2) TGGG-GCCGGAGGGATTGAAAC; 3) TCAGTATATCAGCTGGAGTGT; 4) TAATACGACTCACTATAGGCTTGGCGCCCGGA; and 5) ATCTCCGG-TGGGCACCT. Primer pairs 1 and 2, 1 and 3, and 4 and 5 were used to prepare full-length *H. volcanii* pre-tRNA<sup>Trp</sup> (177 nt), 5'-half pre-tRNA (78 nt), and intron RNA (102 nt), respectively. Specific nucleotide mutations at pre-tRNA target or guide positions C34G (C at position 34 mutated to G), C34U, C34A, G117C, G117A, G117U, U39A, and A70U were introduced into the pVT9P11 templates using the QuikChange site-directed mutagenesis kit (Stratagene) and appropriate DNA oligonucleotides. These residues correspond to target or guide nucleotides of the box C/D and C'/D' motifs contained within the *H. volcanii* pre-tRNA<sup>Trp</sup>.

**In Vitro RNA Synthesis**—Generally, *in vitro* transcription was carried out in 20- $\mu$ l reactions at 37 °C for 2–3 h in buffer containing 40 mM Tris-Cl, pH 7.9, 6 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 2 mM spermidine, 10  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]ATP (specific activity, 3000 Ci/mmol) (MP Biomedicals), 0.6 mM unlabeled ATP, and unlabeled GTP, CTP, and UTP each at 1.0 mM, PCR-amplified DNA, and 50 units of T7 RNA polymerase (New England Biolabs). Radiolabeled RNA transcripts were purified by denaturing PAGE, and amounts were approximated by Cerenkov counting. High specific activity transcripts were prepared similarly except that unlabeled ATP was omitted.

**RNP Assembly and Electrophoretic Mobility Shift Assay**—Recombinant *M. jannaschii* L7Ae, aNop5p, and afibrillarlin proteins were prepared, and RNP complexes were assembled as described previously (18). Briefly, ~0.2 pmol of radiolabeled RNA was incubated at 70 °C with 10 pmol of L7Ae in 20- $\mu$ l reactions (20 mM HEPES, pH 7.0, 150 mM NaCl, 0.75 mM dithiothreitol, 1.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 10% glycerol) for 10 min. Assembly of higher order RNP complexes was accomplished by incubating 10 pmol of L7Ae, 32 pmol of aNop5p, and 33 pmol of afibrillarlin with radiolabeled RNA transcripts in the presence of 10  $\mu$ g of *Escherichia coli* tRNA. Assembled RNP complexes were resolved by electrophoretic mobility shift assay using 4% polyacrylamide gels containing 25 mM potassium phosphate, pH 7.0, and 2% glycerol as described previously (18). Resolved RNPs were visualized by phosphorimaging using a Packard Cyclone system.

**In Vitro RNP-directed Nucleotide 2'-O-Methylation and Thin Layer Chromatography Analysis of Modified Nucleotides**—Generally, RNP complexes were assembled in the presence of 0.05 mM S-adenosylmethionine (AdoMet) using recombinant core proteins and 0.2 pmol of [ $\alpha$ -<sup>32</sup>P]ATP-labeled RNAs as described above. After incubation at 70 °C for 2 h, radiolabeled RNA was purified by phenol/chloroform extraction and ethanol precipitation. In cases in which two different transcripts were included in the reaction, the amount of each transcript was 0.1 pmol. RNA samples were digested with RNase T2, and the digestion products were resolved on cellulose plates (EM Science) using two-dimensional TLC. The solvents for TLC were isobutyric acid, 0.5 N NH<sub>4</sub>OH (5:3, v/v) for the first dimension and isopropanol/HCl/H<sub>2</sub>O (70:15:15, v/v/v) for the second dimension (23). Radiolabeled nucleotides resolved by TLC analysis were visualized and quantified by phosphorimaging. The identity of dinucleotides was established based on our previous study (23). A small aliquot of RNA was obtained before the RNase T2 digestion and saved. These RNAs were checked by denaturing PAGE for RNA stability and integrity, especially for those cases in which methylation was not observed. Target nucleotide 2'-O-methylation was calculated by dividing the amount of radioactivity in the corresponding dinucleotide spot on the TLC plate by 33 (number of A residues in the pre-tRNA) of the sum of the total radioactivity in all spots and expressed as the percentage of modification.

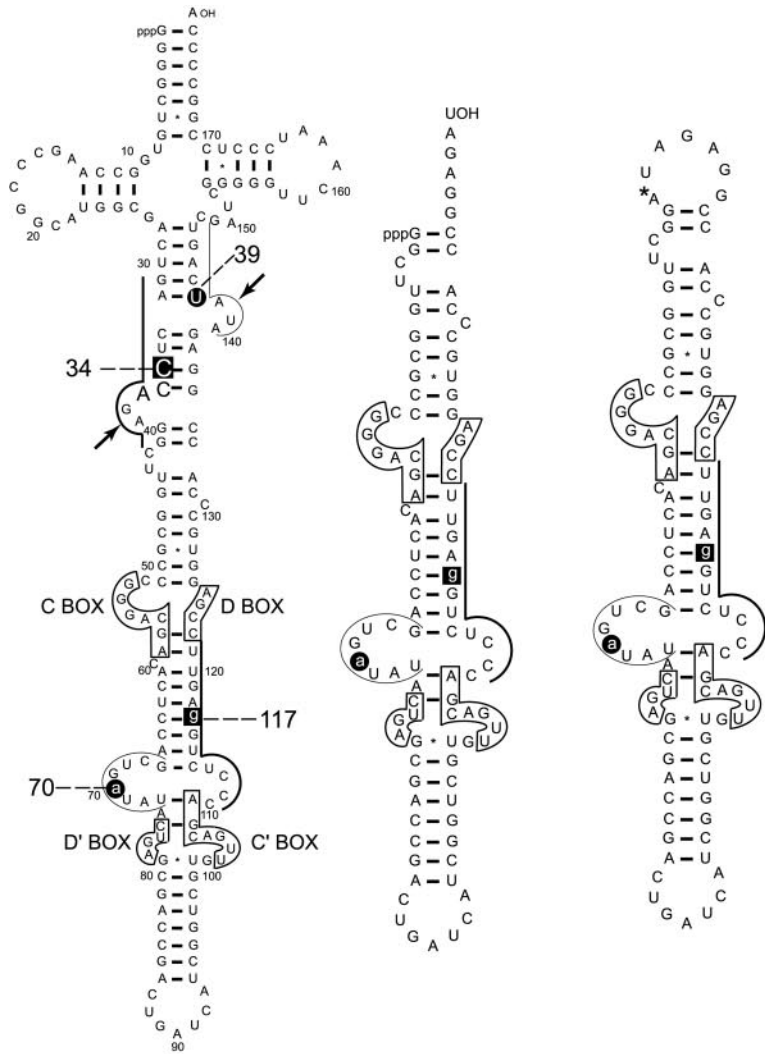
The amount of radiolabeled wild type pre-tRNA transcript used in the reactions and their incubation times varied (up to 2 h) for the kinetic study experiments (see Fig. 2D). Incubation at 70 °C beyond this time leads to RNA degradation. In some reactions, small amounts (<0.08 fmol) of high specific activity transcript were included as tracers to quantify the minimal levels of detectable modification. Two specific and more sensitive experiments using the G117C mutant pre-tRNA were carried out. In one experiment, instead of the typical 0.2 pmol, 2 pmol of pre-tRNA was used for the standard 2-h reaction. For wild type pre-tRNA, more than 80 and 40% of C34 and U39, respectively, were methylated under these conditions (see Fig. 2D). In another experiment, 0.2 pmol of normally radiolabeled and 2.25 fmol of high specific activity tracer transcript (in place of the typical <0.08 fmol transcript) were used in the reaction. We are able to detect methylation of a given residue as low as 2.5% under these conditions.

**Pre-tRNA Methylation in Cell Extracts**—Some *in vitro* methylation reactions were carried out in cell extracts. Extracts were prepared by growing *H. volcanii* cells to an A<sub>550</sub> density of 0.5–0.6 as described previously (23). Pelleted cells were resuspended in three volumes (w/v) of solution D (3.4 M KCl, 0.1 M MgOAc, 10 mM Tris-Cl, pH 7.6) and lysed by three passages through a French pressure cell at 20,000 psi. The lysate was cleared by centrifugation at 10,000  $\times$  g for 10 min followed by two additional centrifugations each at 32,000  $\times$  g for 30 min. Glycerol was added to a final concentration of 20%, and the cell extract was stored at -70 °C. Approximately 0.4 pmol of radiolabeled RNA was incubated at 37 °C for 1 h in a 35- $\mu$ l reaction containing 30  $\mu$ l of cell extract and 0.05 mM AdoMet. Purification of the RNA, RNase T2 digestion, and TLC analysis of digested nucleotides was performed as described above.

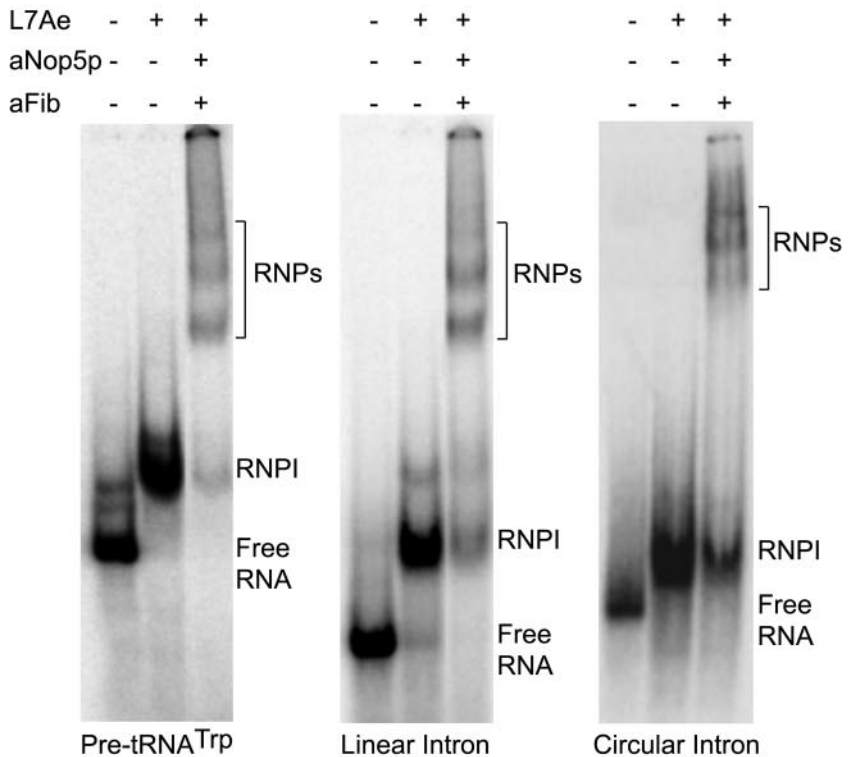
**Pre-tRNA Splicing Reactions**—Splicing endonuclease and ligase reactions to produce linear and circular forms of *H. volcanii* pre-tRNA<sup>Trp</sup> introns were carried out as described previously (25, 26). RNA products were separated by denaturing PAGE, and specific introns were eluted from the gels.

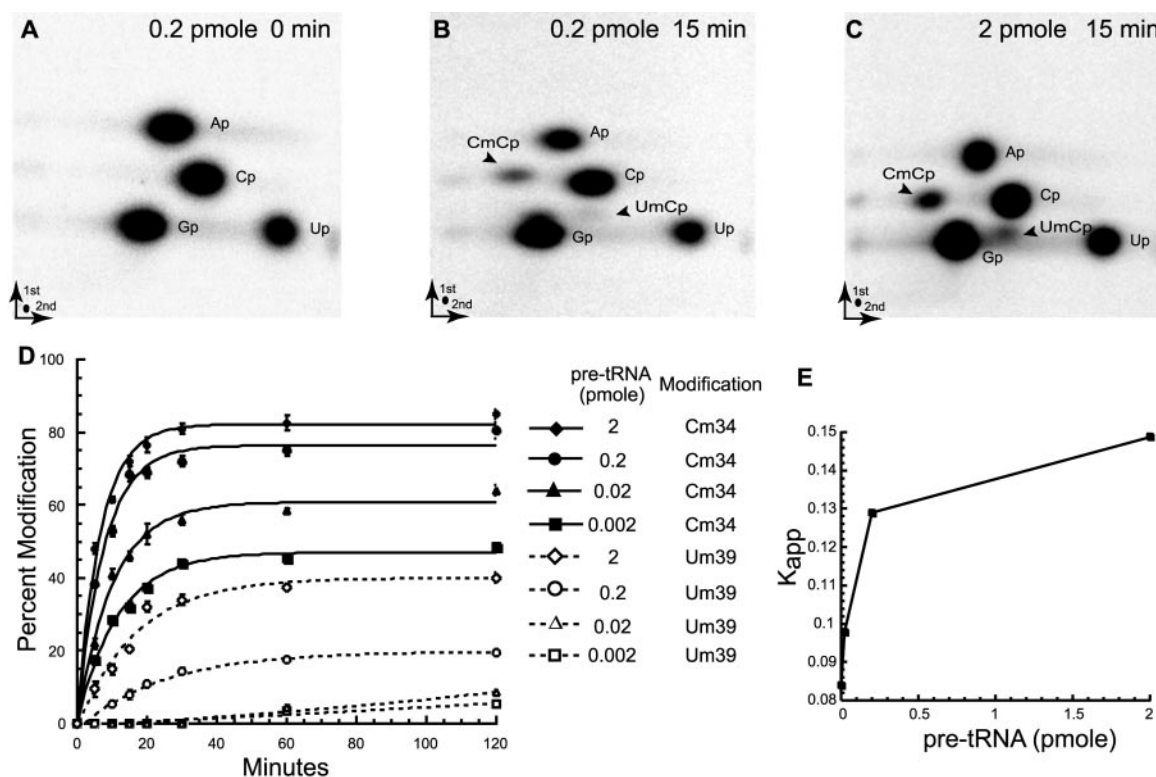
#### RESULTS

***M. jannaschii* sRNP Core Proteins Bind the *H. volcanii* Pre-tRNA<sup>Trp</sup> and Its Derivative Introns in Vitro to Assemble Box C/D RNP**—The folded *H. volcanii* pre-tRNA<sup>Trp</sup> transcript shown in Fig. 1 (upper left) contains the bulge-helix-bulge motif



**FIG. 1. *In vitro* assembly of the intron-encoded box C/D RNPs of pre-tRNA<sup>Trp</sup>.** Upper panels, primary sequences and predicted secondary structures of the *H. volcanii* pre-tRNA<sup>Trp</sup> (left) and its linear (middle) and circular (right) intron. The tRNA anticodon sequence (CCA) is indicated in large letters in the pre-tRNA. The exon-intron junctions, designated by arrows, are located within the bulge-helix-bulge structure required for pre-tRNA splicing. Boxes C, D, C', and D' are enclosed and designated. Complementary guide and target sequences are designated by the thick lines (box C/D) and thin lines (C'/D'). The target nucleotides in the pre-tRNA are numbered C34 and U39 according to the standard tRNA numbering system. Complementary guide (lowercase) and target (uppercase) nucleotide pairs (g117:C34 and a70:U39) are indicated in black squares (C/D motif) and black circles (C'/D' motif), respectively. The sequence of the linear intron is that of the *in vitro* transcribed RNA. (For transcriptional efficiency, this RNA begins with a G residue. It is identical to that produced by endonucleolytic cleavage during splicing except that it is missing a 5'-A residue and also lacks the 5'-hydroxyl and 3'-cyclic phosphate termini.) The position of the junction phosphate within the circular intron formed after pre-tRNA splicing (25) is marked by an asterisk. Lower panels, radiolabeled, full-length pre-tRNA<sup>Trp</sup> linear and circular intron RNAs were incubated with recombinant *M. jannaschii* sRNP core proteins to assemble box C/D RNPs. Assembled sRNP were resolved by native PAGE, and their migration position was revealed by phosphorimaging. Individual radiolabeled RNAs are designated at the bottom of each panel, and the combinations of sRNP core proteins incubated with each RNA are shown at the top. Migration positions of free RNA, L7Ae-bound RNA (RNPI), and higher order RNA-protein complexes (RNPs) are indicated.





**FIG. 2. The kinetics of *H. volcanii* pre-tRNA<sup>Trp</sup> methylation indicate that nucleotides C34 and U39 are modified sequentially by *trans*-acting box C/D RNPs.** Radiolabeled pre-tRNA<sup>Trp</sup> transcripts (0.2 pmol) were incubated with recombinant *M. jannaschii* sRNP core proteins in the presence of AdoMet for 0 min (A) and 15 min (B). TLC analysis of RNase T2-digested pre-tRNA revealed C34 and U39 methylation with the appearance of dinucleotides CmCp and UmCp, respectively. C, methylation reaction was identical to B except that the pre-tRNA concentration was 2.0 pmol. D, the time course of nucleotide C34 and U39 methylation at different pre-tRNA concentrations is shown. Dinucleotides CmCp and UmCp produced at different time points were quantified by phosphorimaging analysis and plotted with respect to reaction time. Each curve, except for the U39 methylation curves at the two lowest pre-tRNA concentrations, was fitted as a single exponent. E, reaction rates ( $k_{app}$ ) were calculated from each fitted C34 methylation curve and plotted versus pre-tRNA substrate concentration (pmol).

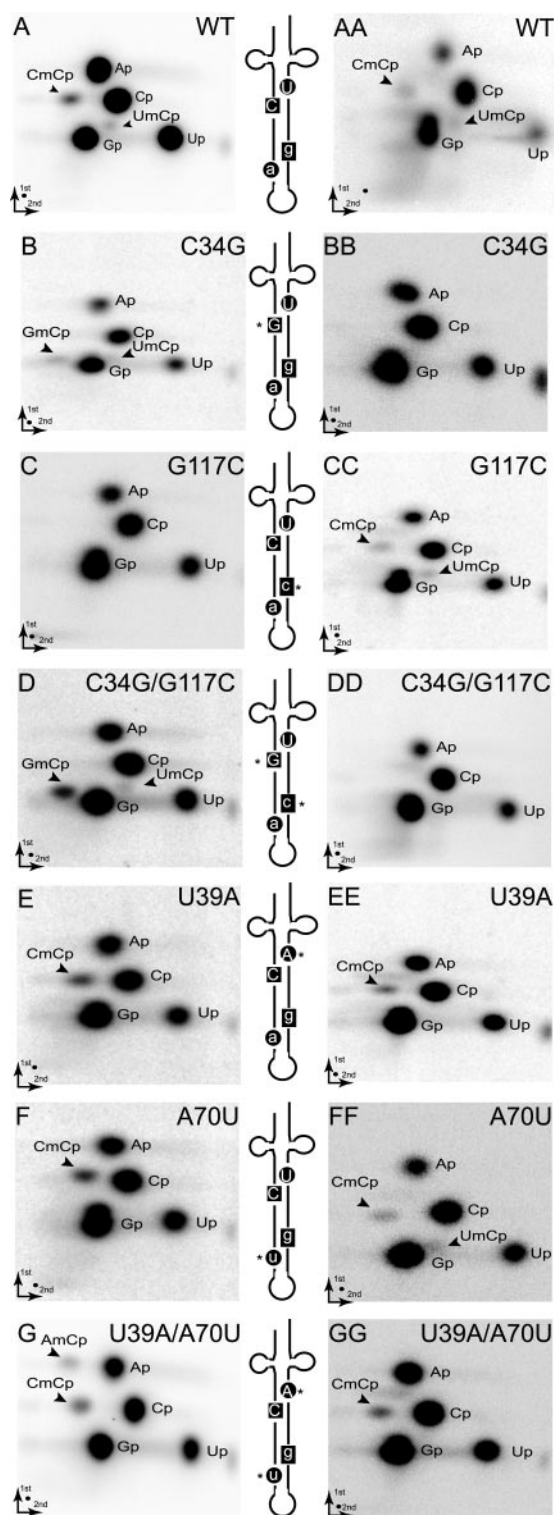
recognized by the archaeal splicing endonuclease and possesses intron-encoded boxes C/D and C'/D' in their characteristic conformation. Pre-tRNA is spliced after nucleotide methylation because the two exon-intron junctions are located within the target sequences of the box C/D RNPs. Both the linear intron formed after endonuclease cleavage of the pre-tRNA and the circular intron formed after ligation of the excised intron (25) retain the essential features of the box C/D sRNA motifs (Fig. 1, upper middle and right); thus, both forms of the intron have the potential to guide the intermolecular, *trans*-2'-O-methylation of other pre-tRNA<sup>Trp</sup> molecules.

*H. volcanii* pre-tRNA<sup>Trp</sup> as well as the linear and circular forms of its spliced intron can bind recombinant *M. jannaschii* box C/D sRNP core proteins *in vitro* to assemble sRNP complexes (Fig. 1, lower panels). Consistent with previous investigations (16, 18, 20, 21), electrophoretic mobility shift assay also demonstrated that core protein binding is ordered, with L7Ae binding first followed by aNop5p and then afibrillarlin (data not shown). The mouse U14 box C/D core motif (19) is an effective competitor for protein binding (data not shown), indicating that core protein binding is specific for the box C/D motif. When the RNA products of a pre-tRNA<sup>Trp</sup> splicing reaction were incubated with L7Ae, only the introns (both linear and circular forms) bound this core protein (data not shown). Thus, box C/D RNP assembly requires the box C/D and C'/D' RNA motifs contained within the intron of this pre-tRNA.

**In Vitro Assembled Box C/D RNPs Guide the 2'-O-methylation of Target Nucleotides in Pre-tRNA<sup>Trp</sup>**—Box C/D RNPs assembled on the *H. volcanii* pre-tRNA<sup>Trp</sup> with the three recombinant *M. jannaschii* sRNP core proteins guided the AdoMet-dependent, 2'-O-methylation of C34 and U39 target

nucleotides in the tRNA<sup>Trp</sup> precursor. Following RNP assembly and incubation in the presence of AdoMet, TLC of the nucleotides generated by RNase T2 digestion of the [ $\alpha$ -<sup>32</sup>P]ATP-labeled pre-tRNA<sup>Trp</sup> transcript revealed radiolabeled residues corresponding to the dinucleotides CmCp and UmCp (where "p" is 3'-phosphate of the residue) (Figs. 2, B and C, and 3A). (RNase T2 cleaves after every ribonucleotide to produce ribonucleoside 3'-monophosphate (Np), except when nucleotides are methylated at the 2' position of the sugar.) Dinucleotides CmCp and UmCp are derived from 2'-O-methylation of residues C34 and U39, respectively. Specificity of methylation for these two nucleotides has been confirmed by dNTP concentration-dependent, primer extension (27) analysis (data not shown) as well as by mutation studies described later. Nucleotide methylation required box C/D RNP assembly because neither dinucleotide was observed when any one of the three sRNP core proteins was omitted from the reaction (data not shown). These studies therefore demonstrate that the *in vitro* assembled box C/D RNPs function to guide the 2'-O-methylation of pre-tRNA<sup>Trp</sup> nucleotides C34 and U39. The amount of the CmCp dinucleotide was always greater than the UmCp dinucleotide (Fig. 2, B and C, and Fig. 3A), suggesting that modification of U39 follows that of C34 and/or occurs at a significantly slower rate.

**The Kinetics of C34 and U39 Methylation Indicate That These Modifications Occur Sequentially and Are Guided via a *trans* Mechanism**—A time course study of C34 and U39 nucleotide methylation was carried out in which the concentration of the pre-tRNA substrate was increased while the amount of core proteins was held constant. The initial rates and the extent (total percentage) of methylation for both nucleotides increased



**FIG. 3. 2'-O-Methylation of pre-tRNA<sup>Trp</sup> nucleotides C34 and U39 is sequential; pre-tRNA<sup>Trp</sup> nucleotide methylation in an *H. volcanii* cell extract is also sequential but utilizes pre-existing box C/D RNPs.** Radiolabeled wild type and mutant pre-tRNA<sup>Trp</sup> transcripts possessing altered guide and/or target nucleotides were incubated for 2 h with recombinant *M. jannaschii* sRNP core proteins in the methylation reactions. A–G, TLC analyses of RNase T2-digested pre-tRNAs are shown. The pre-tRNAs used in each reaction are indicated in the individual panels. Pre-tRNAs with wild type and mutant guide and/or target nucleotides are illustrated at the side (mutated nucleotides are designated by asterisks). Target (uppercase) and guide (lowercase) nucleotides are shown with target:guide nucleotide pairs for the box C/D motif and C'/D' motif indicated in black squares and black circles, respectively. AA–GG, radiolabeled wild type and mutant pre-tRNA<sup>Trp</sup> transcripts possessing altered guide and/or target nucleotides

as pre-tRNA concentrations increased (Fig. 2D). Reaction rates for C34 methylation ( $k_{app}$ ) were calculated and then plotted versus pre-tRNA substrate concentration (Fig. 2E). Consistent with a *trans* reaction,  $k_{app}$  increased as pre-tRNA substrate concentration increased. Lower reaction rates for U39 methylation, particularly at low substrate concentrations, caused a similar plot of the methylation rate of this nucleotide to be more problematic. However, visual inspection of the initial reaction rate for U39 at substrate concentrations of 0.2 and 2 pmol (Fig. 2D) clearly revealed increasing rates with respect to increasing pre-tRNA concentration. Indeed, the  $k_{app}$  rates at these two concentrations revealed values of 0.041 (0.2 pmol) and 0.059 (2 pmol). The fact that these rates are significantly less than C34 methylation rates at these same pre-tRNA substrate concentrations is consistent with the sequential methylation of these two target nucleotides (see below). Collectively, these kinetics are consistent with an intermolecular or *trans* reaction mechanism, thus suggesting that C34 and U39 nucleotide methylation do not occur via an intramolecular or *cis* mechanism as assumed previously (see below). Interestingly, inspection of the C34 methylation rate at higher substrate concentrations revealed the deviation of this curve from linearity (Fig. 2E). This suggests the presence of more than one step in the methylation reaction, with a second step becoming rate-limiting when saturating levels of pre-tRNA substrate are used. Such a second step could include a conformational change in the pre-tRNA substrate required for methylation (see under “Discussion”).

At the highest pre-tRNA concentration (2 pmol), the sum of C34 and U39 modifications exceeded 100% at time points of 30 min or more (Fig. 2D). This indicated that at least some transcripts are modified at both positions and precluded the possibility that, in this assay, only one of the nucleotides could be modified in any given pre-tRNA molecule. In these studies it was also noted that for all pre-tRNA concentrations tested, C34 methylation was more rapid than that of U39 (Fig. 2D). Additionally, there was a lag in U39 methylation, and its rate of modification as well as its amount was always lower than that of C34. These kinetics suggest that the methylation of these two nucleotides in a pre-tRNA molecule is sequential, with U39 methylation occurring after C34 is modified (see below).

**2'-O-Methylation of Pre-tRNA<sup>Trp</sup> Nucleotides C34 and U39 Is Sequential and Prefers but Does Not Require Watson-Crick Pairing between the Target and Guide Nucleotides—Analysis of 2'-O-methylated pre-tRNAs having single mutations at the target and/or corresponding guide nucleotide revealed that Watson-Crick pairing between guide and target residues was not absolutely required for methylation.** The importance of target and guide nucleotide pairing was first assessed for the box C/D RNA motif by mutating target nucleotide C34 and/or corresponding guide nucleotide G117. These experiments revealed that the non-canonical base pairs G:G, U:G, A:G, C:A, and C:U yielded methylated target residues, albeit at lower efficiencies (pre-tRNA mutants C34G, C34U, C34A, G117A, and G117U in Table I and Fig. 3B). For all of these non-canonical pairs, the amount of modification at position 34 exceeded that of U39, again consistent with sequential modification of these two nucleotides as observed in our kinetic studies of the wild type pre-tRNA. Surprisingly, mutation of G117 to C117 to establish C:C pairing of target and guide nucleotides resulted in the loss of methylation of both C34 and U39 (pre-

were incubated in an *H. volcanii* cell extract, and nucleotide methylation was assessed with the TLC analysis of RNase T2-digested pre-tRNAs. Pre-tRNAs for each reaction are indicated in the individual panels and illustrated at the side (mutated nucleotides are designated by asterisks).

TABLE I  
 Methylation of pre-tRNAs

Pre-tRNA	Target <sup>a</sup>	Guide <sup>a</sup>	Cm34 <sup>b</sup>	Um39	Gm34	Am	Fig.
			%	%	%	%	
Single pre-tRNA							
WT <sup>c</sup>	C34	G117	81 ± 2	20 ± 1			3A
C34G	G34	G117		13 ± 1	29 ± 3		3B
C34U	U34	G117		98 ± 4 <sup>d</sup>			NS <sup>g</sup>
C34A	A34	G117		8 ± 1		10 ± 1 <sup>e</sup>	NS
G117C	C34	C117					3C
G117A	C34	A117	40 ± 4	15 ± 2			NS
G117U	C34	U117	43 ± 3	14 ± 2			NS
C34G/G117C	G34	C117		16 ± 1	65 ± 2		3D
WT	U39	A70	81 ± 2	20 ± 1			3A
U39A	A39	A70	70 ± 2				3E
A70U	U39	U70	71 ± 2				3F
U39A/A70U	A39	U70	59 ± 4			13 ± 1 <sup>f</sup>	3G
Two distinct pre-tRNAs							
C34G and G117C	G34	G117	41 ± 4	12 ± 2	34 ± 3		4A
C34U and G117C	U34	C117					NS
C34U and G117C	C34	G117	32 ± 1	49 ± 1 <sup>d</sup>			NS
C34A and G117C	A34	C117					NS
C34A and G117C	C34	G117	34 ± 2	20 ± 3		14 ± 1 <sup>e</sup>	NS
U39A and A70U	A39	A70	73 ± 4	9 ± 2		10 ± 1 <sup>f</sup>	4B
	U39	U70					

<sup>a</sup> Only relevant target and guide residues are shown.

<sup>b</sup> Percentage of methylation at a position is calculated from the amount of corresponding dinucleotide, as described under "Experimental Procedures."

<sup>c</sup> WT, wild type.

<sup>d</sup> Um is derived by methylation of both U34 and U39 of C34U pre-tRNA. Maximum value is 200% U available among these two positions.

<sup>e</sup> Am is derived by methylation of A34 of C34A pre-tRNA.

<sup>f</sup> Am is derived by methylation of A39 of U39A/A70U pre-tRNA.

<sup>g</sup> NS, not shown.

tRNA mutant G117C in Table I and Fig. 3C). This observation was verified by more sensitive analyses of nucleotide methylation. Specifically, a high concentration (2 pmol) of G117C pre-tRNA was used in one experiment, whereas a very high amount of high specific activity G117C tracer was included in another. Both experiments yielded negligible and non-quantifiable amounts of modified nucleotides, confirming that C:C pairing between the C34 target and C117 guide nucleotides of G117C pre-tRNA did not result in C34 methylation during our standard analysis. However, methylation of both mutant G34 and wild type U39 was restored in the double mutant pre-tRNAs (C34G/G117C) in which Watson-Crick pairing between the guide and target nucleotides is re-established by creating a second, compensatory mutation, *i.e.* C34G, in the target nucleotide of the G117C pre-tRNA (mutant C34G/G117C in Table I and Fig. 3D). The absence of U39 methylation in the single mutant G117C and its restoration in double mutant C34G/G117C support the conclusions of our kinetic studies indicating that modification of U39 requires prior modification of nucleotide 34. The G117C mutation that disrupts box C/D-guided methylation at position 34 also disrupts U39 methylation guided by the non-mutated, wild type box C'/D' motif; the pre-tRNA here contains normal guide A70 nucleotide, which is complementary to the U39 target (Fig. 3C). On the other hand, alternative Watson-Crick as well as non-canonical base pairs between the target and guide nucleotides of the box C/D motif that promote methylation at position 34 do allow subsequent C'/D' RNA-guided U39 methylation (Fig. 3, B and D, and mutants C34G, C34U, C34A, G117A, G117U, and C34G/G117C in Table I). The fact that U39 methylation levels were below those at position 34 in these cases (Table I) is again consistent with sequential nucleotide methylation.

Similar mutational analyses were carried out for target nucleotide U39 methylation guided by the C'/D' RNP. Interestingly, both purine (A:A) and pyrimidine (U:U) non-Watson-Crick pairs did not allow methylation at position 39 (Fig. 3, E

and F, and mutants U39A and A70U in Table I), although compensatory mutations that restored Watson-Crick pairing also restored methylation guided by this RNP (Fig. 3G and mutant U39A/A70U in Table I). The RNA duplex of eight base pairs formed between the box C'/D' guide sequence and its complementary target sequence containing U39 is inherently less stable than that of the 11 base pairs formed between the box C/D guide sequence and its target sequence containing C34 (see Fig. 1). This may suggest that more stable RNA:RNA duplex formation is required to allow non-Watson-Crick pairing between guide and target nucleotides to permit nucleotide modification. Further analysis of additional mutants will be required to conclusively demonstrate this possibility. Strikingly, the loss of methylation at position 39 resulting from mutation of the C'/D' target or guide nucleotide had no effect on the C34 methylation guided by the box C/D motif RNP (Fig. 3, E and F, and mutants U39A and A70U in Table I). These results demonstrate that C34 modification is not dependent on U39 modification and are again consistent with the sequential methylation of these two pre-tRNA nucleotides.

*Unspliced Pre-tRNA<sup>Trp</sup> Can Guide the Intermolecular or trans-2'-O-Methylation of Pre-tRNA<sup>Trp</sup> Nucleotides at Positions 34 and 39*—Our kinetic studies suggested an intermolecular or *trans* mechanism for the methylation of pre-tRNA nucleotides C34 and U39. To assess this possibility, an assay was developed in which two different tRNA<sup>Trp</sup> precursors were co-incubated with recombinant core proteins in the same methylation reaction. The first pre-tRNA of this pair was the box C/D mutant G117C. This pre-tRNA presents a C34:C117 target guide nucleotide pair and is incapable of box C/D-guided methylation (Fig. 3C and Table I). The second pre-tRNA mutant of the pair possessed various mutations at target site 34 (mutants C34G, C34U, or C34A; three different mutant target nucleotides but not the wild type C). The box C/D guide nucleotide of this second pre-tRNA was always G117 (wild type) and would therefore be capable of guiding C34 (wild type) methylation of

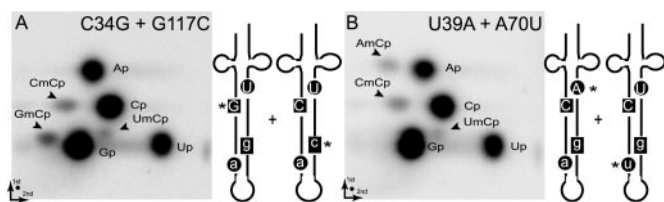


FIG. 4. The intron-encoded box C/D RNPs of *H. volcanii* pre-tRNA<sup>Trp</sup> act in *trans* to guide nucleotide methylation of the corresponding target nucleotides in another pre-tRNA transcript. Pairs of pre-tRNA<sup>Trp</sup> transcripts possessing singly mutated target or guide nucleotides were co-incubated with recombinant *M. jannaschii* sRNP core proteins in methylation reactions. A and B, TLC analyses of the RNase T2-digested pre-tRNA pairs are shown. The pre-tRNA pairs are indicated in the individual panels with the wild type and mutant guide or target nucleotides illustrated at the side (mutated nucleotides are designated by asterisks). Target (uppercase) and guide (lowercase) nucleotides are shown with target:guide nucleotide pairs for the box C/D motif and C'D' motif indicated in black squares and black circles, respectively.

the first pre-tRNA using a *trans* mechanism. Thus, the appearance of the dinucleotide CmCp in TLC analysis for any of these three co-incubated pre-tRNA pairs could only result from intermolecular or *trans*-methylation of the non-mutated target nucleotide C34 of the first pre-tRNA by the non-mutated guide G117 nucleotide of the second pre-tRNA and not by *cis*-methylation by either of the two pre-tRNAs in the assay. For all three pre-tRNA pairs tested (Table I and Fig. 4A), a CmCp dinucleotide was detected, indicating that *trans*-methylation does occur. The appearance of the dinucleotides GmCp, UmCp, and AmCp for the respective pairs (Table I) indicated 2'-O-methylation of target nucleotide 34 in the second pre-tRNA molecule. These second pre-tRNA methylations could result from *trans*-methylation but are not inconsistent with a *cis* mechanism. The appearance of UmCp for all three pairs was the product of sequential box C/D'-guided U39 modification for both pre-tRNAs, except for mutant C34U in which methylation of residue U34 in the second pre-tRNA molecule would also contribute to UmCp levels.

Similar results were obtained when C/D'-guided modification of pre-tRNA residue at position 39 was assessed (Fig. 4B and Table I). For this experiment, pre-tRNA mutants U39A and A70U were co-incubated in a methylation reaction. Previous analysis (Fig. 3, E and F, and Table I) clearly demonstrated the inability of target nucleotide 39 to be modified in either of these mutants (A39:A70 and U39:U70 target:guide pairs). However, co-incubation of these two mutants would restore Watson-Crick pairing of target and guide nucleotides (A39:U70 or U39:A70) between the two pre-tRNAs. The appearance of AmCp and UmCp dinucleotides (Fig. 4B and Table I) clearly demonstrated that each pre-tRNA was indeed guiding modification of residue 39 in the other tRNA precursor via a *trans* mechanism. Of course, the appearance of dinucleotide CmCp was a product of box C/D-guided modification in both pre-tRNAs, a prerequisite for nucleotide 39 modification. Collectively, these experiments demonstrate that *trans*-methylation can occur between two identical pre-tRNAs as well as between two distinct pre-tRNAs sharing compatible target and guide nucleotides.

*H. volcanii* Pre-tRNA<sup>Trp</sup> C34 and U39 Methylation in an *H. volcanii* Cell Extract Is Also Sequential and Guided Exclusively via a *trans* Mechanism Utilizing Pre-existing Box C/D RNPs Contained in the Extract—Previous investigators (21, 24) have examined archaeal pre-tRNA<sup>Trp</sup> 2'-O-methylation *in vitro* using both recombinant proteins and cell extracts and have concluded that nucleotide modification occurs via a *cis* mechanism. This conclusion contrasts with our observations of *trans*-

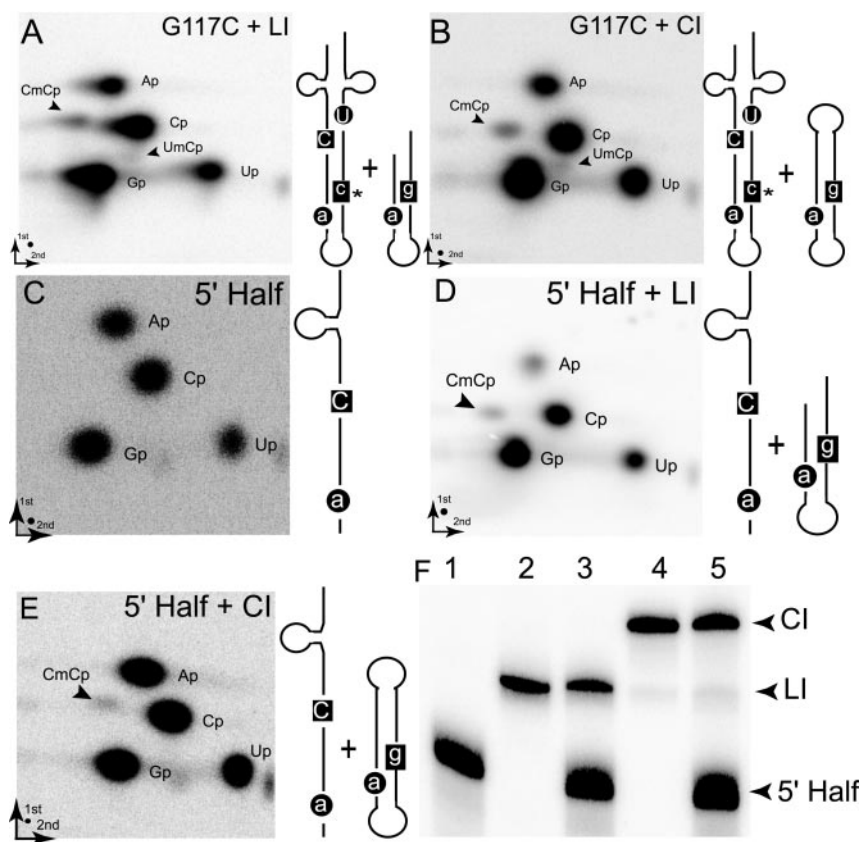
methylation when the pre-tRNA<sup>Trp</sup> box C/D RNPs are reconstituted with recombinant proteins. We therefore decided to also carry out pre-tRNA<sup>Trp</sup> methylation in the cell extracts (Fig. 3, AA–GG) using the same pre-tRNA mutants used to study methylation of the box C/D RNP complexes assembled with recombinant proteins (Fig. 3, A–G). TLC analyses of the reaction products produced for the various pre-tRNA mutants incubated in cell extracts is presented in parallel in Fig. 3 to allow direct comparison of the two *in vitro* methylation systems. Incubation of wild type *H. volcanii* pre-tRNA<sup>Trp</sup> transcripts in *H. volcanii* cell extract clearly resulted in the 2'-O-methylation of C34 and U39 in the pre-tRNA as anticipated (Fig. 3AA). A time course analysis of nucleotide methylations in cell extract (data not shown) for the wild type pre-tRNA<sup>Trp</sup> revealed similar reaction kinetics as that with the complexes assembled with recombinant proteins, consistent with the sequential methylation of C34 and U39.

Methylation of neither G34 (mutant) nor U39 (wild type) nucleotide of the C34G pre-tRNA was observed when this pre-tRNA was incubated in the extract (Fig. 3BB), even when the pre-tRNA substrate concentration was doubled and the cell extract was increased 10-fold (data not shown). The lack of G34 methylation was unexpected because the same mutant pre-tRNA assembled with recombinant proteins was able to utilize this purine:purine/target:guide pair (G34:G117) to guide methylation (Fig. 3, compare B with BB). This suggested that the cell extract system does not permit non-Watson-Crick pairing for box C/D-guided methylation. The coincident lack of U39 methylation, however, would be anticipated because the methylation of this target nucleotide sequentially follows and requires nucleotide 34 modification. Unexpectedly, when pre-tRNA G117C, a mutant pre-tRNA that does not show any modification when incubated with recombinant proteins (Fig. 3C), was incubated in the cell extract, both target nucleotides C34 and U39 were methylated (Fig. 3CC). This suggested that mutant G117C is functioning as the methylation substrate in the cell extract. The results of these C34G and G117C pre-tRNA mutants prompted us to consider the possibility that pre-tRNA modifications in the cell extract were occurring exclusively via a *trans* mechanism and were utilizing endogenous box C/D RNPs.

To examine these possibilities, additional pre-tRNA<sup>Trp</sup> mutants were incubated in the cell extract, and methylation of the two target nucleotides was assessed. Surprisingly, incubation of the pre-tRNA double mutant C34G/G117C, which contains the compensatory G34:C117 pair, resulted in no methylation of either G34 or U39 target nucleotides (Fig. 3DD). This pre-tRNA double mutant did show both G34 and U39 methylation when it was incubated with recombinant core proteins (Fig. 3D). The lack of G34 methylation in the cell extract suggests the inability of this double mutant pre-tRNA to guide its own methylation in *cis* as well as to guide methylation of another identical pre-tRNA in *trans*. Again, a lack of U39 modification in the absence of nucleotide 34 modification in the double mutant is consistent with the normal sequential methylation of the two target nucleotides. In sum, the results of C34G and G117C single mutants and their combinatorial double mutant are consistent with the proposition that the pre-tRNA fails to assemble a competent box C/D RNP in the cell extract but serves as a pre-tRNA substrate for *trans*-methylation guided by endogenous RNP complexes.

Incubation of pre-tRNA mutant U39A in the extract (Fig. 3EE) resulted in C34 methylation but not mutant target A39 methylation, again suggesting that modification of target nucleotide 34 occurs independently of nucleotide 39. Target nucleotide C34 would be methylated by endogenous RNP using

**FIG. 5. Linear and circular forms of the *H. volcanii* pre-tRNA<sup>Trp</sup> intron act in trans as guide RNAs for 2'-O-methylation.** Linear (LI) and circular (CI) forms of the *H. volcanii* pre-tRNA<sup>Trp</sup> intron were isolated and used with recombinant *M. jannaschii* sRNP core proteins in methylation reactions, which also contained radiolabeled pre-tRNA mutant G117C that is itself incapable of guiding methylation. Nucleotide methylation was assessed by TLC analysis of RNase T2-digested pre-tRNAs (A and B). Schematic presentations of the RNAs used in the reactions are indicated at the side, with mutated nucleotides indicated with asterisks. Similar analyses are shown using the 5'-half of pre-tRNA<sup>Trp</sup> as substrate (nucleotides 1–78) incubated alone (C, negative control) and with linear and circular introns (D and E). F, the circular intron remains circular during intron-guided 2'-O-methylation. Aliquots of input RNAs both before and after the methylation reaction (but before RNase T2 digestion) were resolved by denaturing PAGE and visualized by phosphorimaging. *lane 1*, input 5'-half pre-tRNA transcript; *lane 2*, input linear intron; *lane 3*, 5'-half pre-tRNA and linear intron after methylation; *lane 4*, input circular intron; *lane 5*, 5'-half pre-tRNA and circular intron after methylation. Circular intron derivatives of pre-tRNA<sup>Trp</sup> run slower than the corresponding linear intron in denaturing PAGE (25).



wild type guide nucleotide G117, but target nucleotide A39 cannot be methylated by A70 guide nucleotide of either the U39A mutant pre-tRNA (*cis* or *trans*) or the wild type endogenous RNP. Pre-tRNA mutant A70U, which is not methylated at U39 with recombinant proteins (Fig. 3F), does reveal U39 methylation in the cell extract (Fig. 3FF). This is again consistent with the utilization of endogenous RNP containing wild type A70 guide nucleotides. Finally, the pre-tRNA double mutant U39A/A70U, in which Watson-Crick pairing (A39:U70) between target and guide is restored, thus producing A39 methylation with the recombinant proteins (Fig. 3G), failed to be methylated at A39 in the cell extracts (Fig. 3GG). Although this double mutant could, in principle, be guided by its own pre-tRNA either in *cis* or *trans*, the absence of wild type target nucleotide U39 prevented its methylation via endogenous box C/D RNPs. This is similar to the absence of nucleotide 34 methylation of the double mutant C34G/G117C (Fig. 3DD) when incubated in the cell extract.

Collectively, pre-tRNA methylation carried out in the cell extract led to the following conclusions: (a) pre-tRNA<sup>Trp</sup> precursors added to the cell extract function only as substrate RNAs and not as guide RNAs; (b) the failure of exogenously added pre-tRNAs to guide methylation suggests that these pre-tRNAs are not assembling functional C/D RNPs; (c) pre-existing wild type C/D box guide sRNPs are acting in *trans* to methylate the exogenously added pre-tRNAs at both target positions; (d) 2'-O-methylation of residues at positions 34 and 39 occurs sequentially in the cell extract as it does when the C/D RNPs are assembled with recombinant core proteins; and (e) methylation of target nucleotide 39 requires prior and independently occurring modification of target nucleotide 34.

**Both Linear and Circular Forms of the Excised *H. volcanii* Pre-tRNA<sup>Trp</sup> Intron Can Act as Guide RNAs for the *trans*-Methylation of Pre-tRNA<sup>Trp</sup>**—Excised linear and circular forms

of the pre-tRNA<sup>Trp</sup> intron possess both box C/D and C'/D' motifs and assemble box C/D RNPs (Fig. 1). We therefore assessed the ability of these excised introns to guide the methylation of their pre-tRNA target nucleotides via a *trans* mechanism. Both linear and circular introns were isolated and used in separate methylation reactions with recombinant core proteins that also contained G117C pre-tRNA mutant (C34:C117 pair), a pre-tRNA unable to methylate its C34 target nucleotide (see Fig. 3C). In reactions with either a circular or linear intron, pre-tRNA nucleotide C34 was methylated (Fig. 5, A and B). The methylation of C34, in turn, permitted the sequential methylation of U39 and hence the appearance of the dinucleotide UmCp. A time course study using linear introns as guide RNAs also confirmed the sequential modification of these two nucleotides with respect to relative amounts of CmCp versus UmCp produced and a coincident lag in U39 methylation (data not shown). Similarly, both linear and circular forms of the excised intron are able to guide methylation of C34 within the 78-nt 5' halfmer pre-tRNA molecule (Fig. 5, D and E), thus demonstrating that *trans*-methylation of C34 does not require a folded, full-length pre-tRNA. Electrophoretic analysis of these RNAs after completion of the pre-tRNA methylation confirmed that the circular intron had not been linearized during the reaction (Fig. 5F), thus demonstrating that circular RNA can also assemble a functional guide RNP. Finally, we have also attempted to methylate target nucleotide U39 of a pre-tRNA 3' halfmer using both linear and circular introns. Strikingly, neither form of the intron-encoded RNP was able to modify U39, either when the 3' halfmer was alone or co-incubated with the 5' halfmer (data not shown). Absence of U39 methylation in these cases may suggest that this nucleotide modification requires a correctly folded, full-length pre-tRNA precursor or that misfolding of the 3' halfmer does not allow it to properly pair with the intron-encoded guide RNP.

## DISCUSSION

Eukaryal and archaeal box C/D RNP-guided 2'-O-methylation characteristically requires the intermolecular or *trans* base pairing of an RNA sequence containing the target nucleotide with the box C/D RNA guide sequence. The discovery in some Euryarchaeota of an intron-encoded box C/D RNA that can guide the 2'-O-methylation of target nucleotides in the same unspliced pre-tRNA led to the postulation of a unique *cis* mechanism for nucleotide modification (8, 24). Indeed, previous reports concerning 2'-O-methylation of the pre-tRNA<sup>Trp</sup> have assumed such a mechanism (21, 24). However, we now demonstrate an intermolecular or *trans* mechanism for pre-tRNA<sup>Trp</sup> nucleotide methylation guided by both *in vitro* assembled RNPs and endogenous RNPs of the cell extract. The increases in reaction rate of nucleotide methylation with increasing pre-tRNA substrate concentrations clearly suggested that methylation was occurring via the intermolecular pairing of target substrate and guide RNAs. Site-directed mutagenesis of both pre-tRNA<sup>Trp</sup> target and guide nucleotides confirmed this *trans* mechanism, and the demonstration that pre-tRNA<sup>Trp</sup> methylation in the cell extract is guided by pre-existing box C/D RNPs in the extract has reaffirmed this mechanism. All of the results detailed in this investigation are consistent with a *trans* mechanism. Some of our observations from the *in vitro* system using recombinant proteins and most of our observations from the cell extract system cannot be explained by a *cis* mechanism.

*H. volcanii* pre-tRNA<sup>Trp</sup> can be assembled *in vitro* into a functional box C/D RNP with recombinant core proteins, but RNP assembly apparently does not occur in the *H. volcanii* cell extract. Thus, pre-existing box C/D RNPs in the extract are responsible for guiding the methylation of exogenously added pre-tRNA substrate. Clearly, the difference in protein composition (isolated recombinant core proteins *versus* total cell protein extract) between the two *in vitro* systems and their ability or inability to assemble functional box C/D RNPs are responsible for the observed differences in methylation results for the same pre-tRNA substrates (Fig. 3, compare A–G with AA–GG). Differences in methylation capabilities among various pre-tRNA<sup>Trp</sup> substrates assembled with recombinant core proteins and total cell extracts have been observed previously (compare results of Ref. 21 with 24). This suggests that caution must be exercised when using these different *in vitro* box C/D RNP assembly systems because the results obtained in one system may not be directly comparable with those observations and conclusions made in the other. Observed differences may be attributed to additional (accessory?) proteins available in the extract that affect the efficiency of the methylation reaction that are not present in the minimal system using recombinant core proteins.

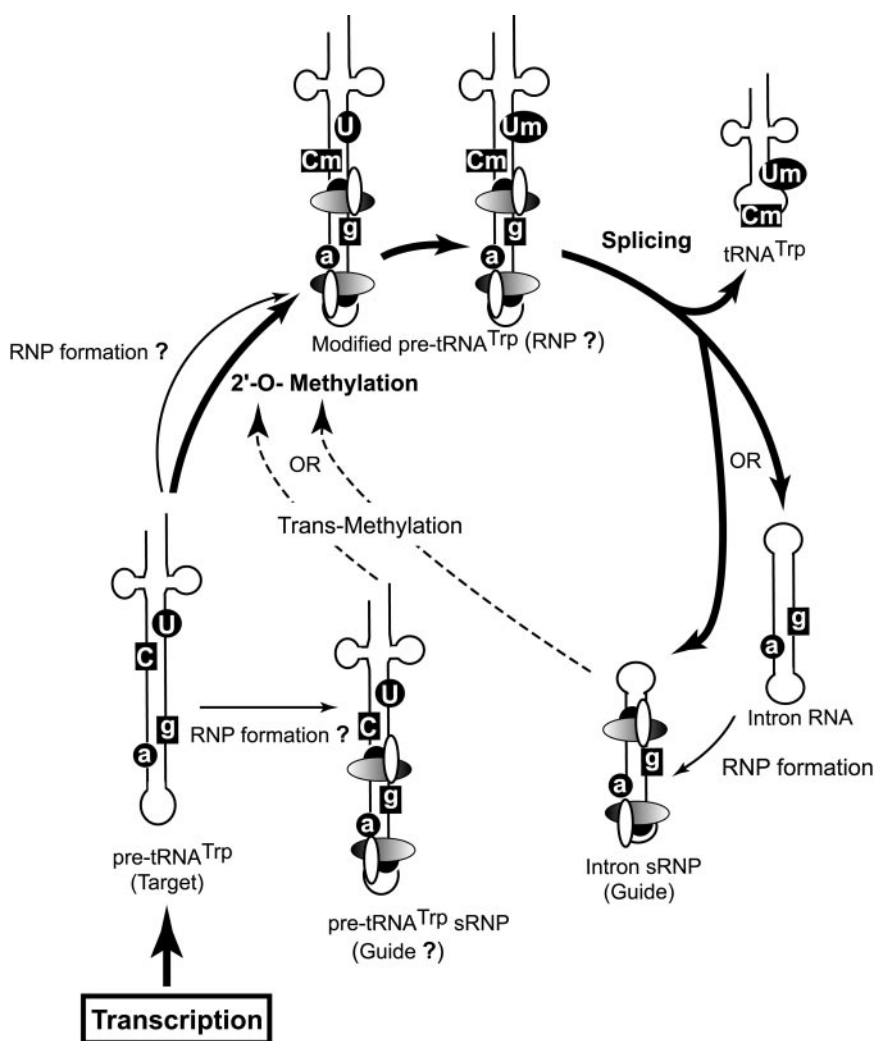
Mutagenesis experiments revealed that Watson-Crick base pairing between target and guide nucleotides was preferred but not absolutely required for the target nucleotide 34 methylation by recombinant proteins. Utilization of non-canonical pairing between target and guide nucleotides has also been noted previously for eukaryotic box C/D snoRNAs *in vivo* (28). Notably, disruption of Watson-Crick pairing between the C'/D' target nucleotide 39 and its guide nucleotide for the tested pairs resulted in a complete loss of methylation. Interestingly, a comparison of the RNA duplexes formed between the target and guide sequences for the box C'/D' and C/D motifs revealed significant differences in helix stability. The box C/D target:guide duplex has 11 base pairs, six of which are G-C, whereas the C'/D' duplex has eight base pairs, only two of which are G-C. Thus, a reduction in target-guide duplex stability may require more stringent pairing of the target and guide nucleotides as noted previously (28).

The pre-tRNA<sup>Trp</sup> mutants of this study possessed only site-specific, single nucleotide substitution in guide and/or target nucleotides. These minimal changes in primary sequence and RNA folded structure would be expected to present essentially native, folded pre-tRNA substrates for investigation of these RNA-guided nucleotide modification reactions. Previous investigations (21, 24) in other laboratories have also examined the roles of the intron-encoded box C/D and C'/D' motifs in arriving at their conclusion that pre-tRNA<sup>Trp</sup> methylation is accomplished via a *cis* mechanism. However, these studies utilized pre-tRNA mutant substrates containing small deletions (*i.e.* removal of boxes D or D') as well as the removal of significant portions (~45 and 75 nt) of the intron. We suspect that such changes are likely to alter the folding of the pre-tRNA transcript, thus complicating the interpretation of results regarding the effects these mutations have on box C/D- and C'/D'-guided methylation.

For many of our pre-tRNA mutants incubated as single precursors bound with recombinant sRNP core proteins, methylation could occur via either a *cis* mechanism involving a single molecule or a *trans* mechanism involving two identical molecules. Fortunately, however, one of our pre-tRNA mutants (G117C), presenting as a non-canonical target:guide pair (C34:C117), did not support *in vitro* assembled box C/D RNP-guided methylation as a single pre-tRNA transcript. This mutant thus provided a pre-tRNA substrate that could be exploited to demonstrate the *trans* mechanism for guided methylation. These experiments specifically used two distinct pre-tRNAs with various combinations of altered target and guide nucleotides. Results of these experiments conclusively demonstrated box C/D RNP-guided *trans*-methylation. Further analysis using linear and circular forms of the excised intron confirmed the *trans* mechanism. A similar methylation system guided by a linear, derivatized form of the *Archaeoglobus fulgidus* pre-tRNA<sup>Trp</sup> intron has been described previously (20). However, those experiments utilized RNA oligonucleotides as target RNA substrates. Therefore, box C/D- and C'/D'-guided methylation was not carried out with the corresponding target nucleotides positioned in a pre-tRNA<sup>Trp</sup> context as occurs *in vivo*. Obviously, these RNA substrates would also not provide the opportunity to investigate the temporal relationship of the two-nucleotide modification reactions.

Our experiments have demonstrated that methylation of box C'/D'-guided nucleotide 39 occurs after methylation of box C/D-guided nucleotide 34. To our knowledge, this is the first example of sequential methylation guided by box C/D RNP complexes in either Archaea or Eukaryota. Sequential nucleotide methylation was first suggested in our kinetic studies in which U39 methylation lagged behind C34 methylation in all cases. Lower levels of methylation at position 39 compared with position 34 were observed in all cases as well. Subsequent mutagenesis experiments confirmed this order of nucleotide methylation in which all mutants disrupting position 34 methylation resulted in the loss of methylation at position 39. In direct contrast, methylation of C34 was independent of U39 modification. The sequential methylation of nucleotides guided by the box C/D and C'/D' motifs of tRNA<sup>Trp</sup> intron raises interesting questions about the temporal relationship of the methylation reactions of other box C/D RNAs that guide more than one nucleotide modification reaction. Is sequential modification a common characteristic of guide RNAs that direct two modification reactions? Is the sequence of modification always C/D-guided and then C'/D'-guided? Is the order of methylation in the target RNA always 5'-nucleotide first and then 3'-nucleotide? Does the second nucleotide modification reaction require conformational changes in target and/or guide RNA? Does

FIG. 6. **Proposed model for the trans-2'-O-methylation of *H. volcanii* pre-tRNA<sup>Trp</sup> in vivo.** Thick arrows indicate the pre-tRNA<sup>Trp</sup> processing pathway proceeding from transcription of the pre-tRNA through nucleotide methylation and splicing to the production of tRNA<sup>Trp</sup> and excised intron. RNP assembly is denoted by thin arrows, and nucleotide modification guided intermolecularly by the intron-encoded box C/D RNP is denoted by dashed arrows.



methylation by the box C/D RNP convert a juxtaposed but inactive C'/D' complex into an active form, possibly involving inter-RNP interactions? Kinetic analysis of the C34 methylation reaction indicating more than one step is consistent with the notion that conformational changes in the sRNA or sRNP could play an important role in catalysis. Our establishment of an *in vitro* pre-tRNA<sup>Trp</sup> nucleotide methylation system using recombinant core proteins now provides the opportunity to address some of these questions directly.

It has been assumed that *H. volcanii* pre-tRNA<sup>Trp</sup> methylation of nucleotides C34 and U39 in *H. volcanii* cell extracts were carried out via a *cis* mechanism (21, 24). However, our results clearly demonstrate that pre-tRNAs added to the cell extract do not serve as guide RNAs. We suspect that free core proteins, even if present in sufficient concentrations in the extract, fail to assemble methylation-competent box C/D RNPs on the full size pre-tRNA. Methylation of pre-tRNA nucleotides C34 and U39 in these extracts indicated that endogenous tRNA<sup>Trp</sup> precursors or excised introns possessing assembled box C/D RNPs are responsible for the observed nucleotide methylations; these reactions clearly involve a *trans* mechanism. As mentioned above, the previous conclusion regarding a *cis* mechanism for pre-tRNA<sup>Trp</sup> methylation was based on the results of experiments incubating deletion mutant pre-tRNAs in the extract (24). Some of these pre-tRNAs could not be methylated in the extract. Again, we suspect that the structural changes in those pre-tRNA substrates resulting from deletion of the box elements or large regions of intron likely

resulted in structural rearrangements of the pre-tRNA molecule. These alterations could have significantly affected the capabilities of these mutant pre-tRNAs to function as target RNA substrates. Structural rearrangements within the pre-tRNA interrupting its pairing with the endogenous box C/D RNP of the cell extract would not allow methylation.

Our results suggest a novel model for intron-encoded, box C/D RNP-guided 2'-O-methylation and splicing of the *H. volcanii* pre-tRNA<sup>Trp</sup> in vivo (Fig. 6). Newly transcribed tRNA<sup>Trp</sup> precursors serve as the substrate for box C/D RNP-guided 2'-O-methylation of target nucleotides C34 and U39. These methylation reactions are guided sequentially and in *trans* by pre-existing, intron-encoded, box C/D and C'/D' RNPs. The 2'-O-methylated pre-tRNAs would then be spliced to produce tRNA<sup>Trp</sup> and circular intron. This splicing reaction would produce additional introns that could guide, in *trans*, pre-tRNA<sup>Trp</sup> methylation. The linear introns observed *in vivo* (25) could be either intermediates of the splicing reaction or linearized forms of the circular intron. The point at which the intron-encoded box C/D RNPs are assembled is not clear and could occur at different steps in this pathway or even in stages. The full complement of core proteins could bind the pre-tRNA molecule before methylation, thus providing the possibility that this pre-tRNA could also guide methylation in *trans*. It is also possible that RNP assembly of the pre-tRNA is only partial, perhaps binding only L7Ae. Partial core protein binding could influence the folded structure of the intron and/or contribute to the ability of the substrate to be methylated. Alternatively,

L7Ae could act as a chaperone to help fold the pre-tRNA into a splicing competent structure. In this scenario, L7Ae would remain bound to the spliced introns and subsequently recruit aNop5p and afibrillarin to assemble the complete box C/D sRNPs. Alternatively, box C/D RNP assembly could occur entirely on the excised intron.

Our proposed model could explain two previous observations concerning pre-tRNA methylation and splicing. First, we have noted the accumulation of circular and linear tRNA<sup>Trp</sup> introns *in vivo* (25). Accumulation of these introns is consistent with a *trans*-methylation mechanism as proposed. Second, it has been noted that the two *cis*-methylations and the splicing reaction require mutually exclusive structures of the pre-tRNA. This observation therefore suggests the need for alternatively folded intron structures or formation of a pseudoknot for the two *cis*-methylations before producing a splicing competent pre-tRNA (21, 24). Guiding pre-tRNA methylation in *trans* using the excised intron would eliminate this requirement. Finally, the demonstration that a circular RNA can guide 2'-*O*-methylation of archaeal target RNAs raises the possibility of additional circular RNAs in Archaea guiding nucleotide modification via a *trans* mechanism. The prospect of additional circular RNAs potentially produced through various RNA processing reactions would also raise interesting questions concerning the biogenesis of these guide RNAs and the possible coordinate regulation of nucleotide modification in the biosynthesis of the archaeal target RNAs.

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