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LETTER TO THE EDITOR

The 9/4 secondary structure of eukaryotic selenocysteine tRNA: More pieces of evidence

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INTRODUCTION

Selenocysteine biosynthesis and its cotranslational incorporation into selenoproteins are achieved by a complex molecular machinery (reviewed in Hüttenhofer & Böck, 1998). A major wheel in this mechanism is the $tRNA^{Sec}$, which plays a pivotal role. It is first charged with serine by the conventional SerRS, the seryl-residue being further converted in situ to the selenocysteyl-residue by the selenocysteine synthase enzyme. The charged selenocysteyl-$tRNA^{Sec}$ delivers selenocysteine to the nascent polypeptide chain in response to a reprogrammed UGA codon. The classical elongation factors EF-Tu (in bacteria) or EF1-α (in eukaryotes) do not intervene at this stage. Instead, this process requires a selenocysteine-specific translation factor, called SELB in bacteria, but for which no eukaryotic homologue has been cloned yet. Interestingly, antideterminants against EF-Tu binding were found in the *Escherichia coli* $tRNA^{Sec}$ (Rudinger et al., 1996). Based on the several functions that this tRNA has to accomplish, it is reasonable to expect that the $tRNA^{Sec}$ secondary structure should exhibit distinctive structural features deviating from classical elongator tRNAs. In this regard, functional studies, structural probing, and sequence comparisons confirmed the earlier proposal that the bacterial $tRNA^{Sec}$ needs an 8-bp long amino acceptor stem and a 6-bp long D-stem to function, instead of the canonical 7 bp and 3/4 bp, respectively (Leinfelder et al., 1988; Baron et al., 1990, 1993; Tormay et al., 1994).

Regarding the eukaryotic $tRNA^{Sec}$ secondary structure, two hand-folded models were proposed in the literature. In the first one (Diamond et al., 1981), the acceptor stem is 7-bp and the T-stem 5-bp long with a bulged C (7/5 model in Fig. 1A). The second model (Böck et al., 1991) proposed 9 bp for the amino acceptor and 4 bp for the T-stem (9/4 model in Fig. 1B). To test the two models, we had performed a structural analysis with the use of enzymatic and chemical probes (Sturchler et al., 1993). The accumulated data favored the 9/4 model, with the additional finding of an extended 6-bp D-stem (Fig. 1B), resembling the bacterial counterpart in this respect.

In a recent Letter to the Editor of *RNA*, Steinberg et al. (1998) reconsidered our experimentally tested model in the light of data obtained by ourselves and others on the function of $tRNA^{Sec}$ (Wu & Gross, 1993, 1994; Ohama et al., 1994; Sturchler-Pierrat et al., 1995; Amberg et al., 1996) and concluded that the existing data fit the 7/5 better than the 9/4 model. Here, we present new structural data that, taken together with our previously published data (Sturchler et al., 1993), argue that the 9/4 is the only secondary structure model that can rationalize the several lines of evidence provided by the array of various structural probes employed. Lastly, and most convincingly, we show that the secondary structure of the $tRNA^{Sec}$ of the Archae *Methanococcus jannaschii*, owing to sequence constraints, can only fold into the 9/4 model.

EVIDENCE FOR THE 9/4 SECONDARY STRUCTURE MODEL

Chemical probing

In our earlier work (Sturchler et al., 1993), the *Xenopus laevis* $tRNA^{Sec}$ molecule was monitored with a variety of enzymatic and chemical probes that led to a convergence of data in favor of the 9/4 model shown in Figure 1B. The key point was to determine whether

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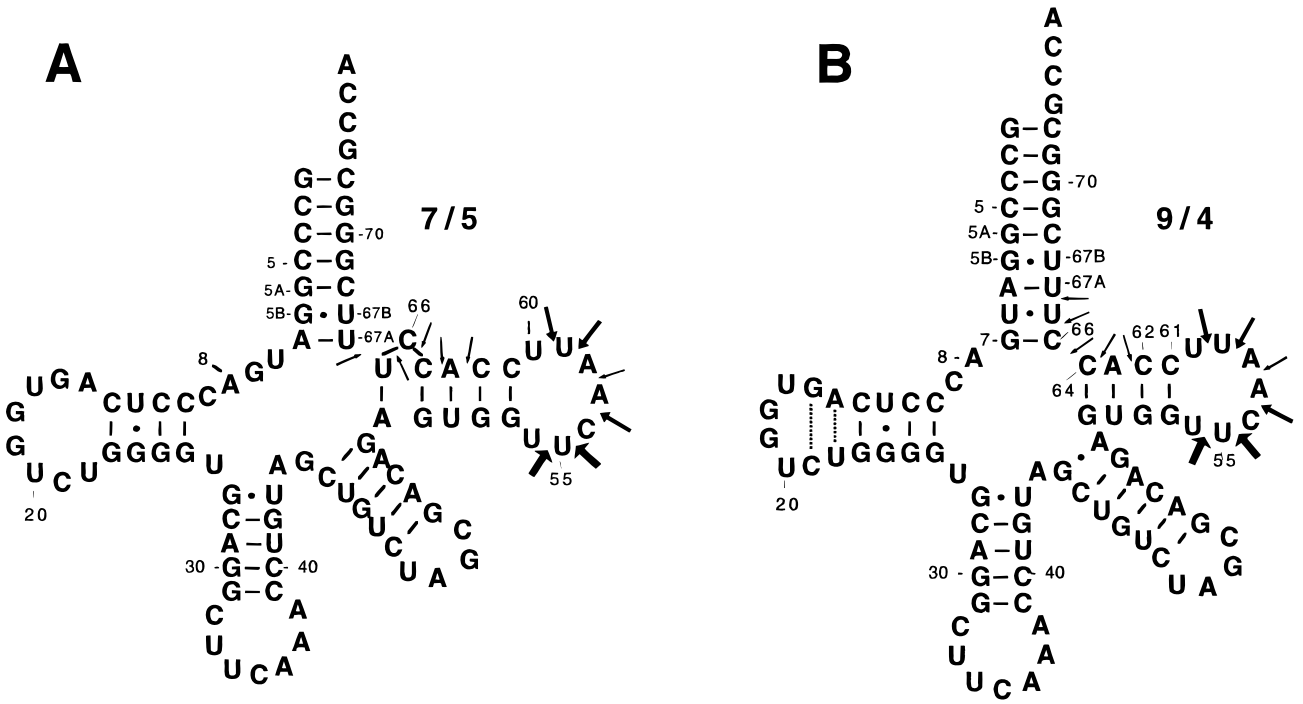

FIGURE 1. Two possible hand-folded secondary structure models for the *X. laevis* selenocysteine tRNA^{Sec}. **A:** The 7/5 model, from Diamond et al. (1981). **B:** The 9/4 folding, from Böck et al. (1991). Dotted line shows the additional base pairs in the D-stem that we found by structure probing (Sturchler et al., 1993). 7/5 and 9/4 stand for the lengths of the acceptor/T-stems. Arrows depict the phosphodiester bonds cleaved by Pb^{2+}, taken from the gel shown in Figure 2B. Intensities of cleavages are represented by the thickness of the arrows.

C66 is bulged out of the helix as in the 7/5 model (Fig. 1A) or base paired to G7 to give rise to the 9/4 model. We provided data at the time that C66 cannot be bulged out because N3-C66 was unreactive to dimethylsulfate (DMS) under native conditions. We have repeated this experiment, which is shown in Figure 2A. The presence of the modification is reflected on the gel by a pause of the reverse transcriptase one nucleotide prior to the modified base. Again, one can see that C66 (pointed by the arrow in Fig. 2A) is reactive to DMS only under semi-denaturing and denaturing conditions, showing the same reactivity as N3-C61 and N3-C62, which pair to G53 and G52, respectively, and N3-C68, which pairs to G5 (Fig. 2A, lanes 4 and 6). To further strengthen the argument, we made use of lead acetate probing. This chemical probe has been used with a variety of different RNAs by several authors as a single-strand-specific probe (Brunel et al., 1990; Hüttenhofer et al., 1996; Walczak et al., 1996). In addition, bulges in helices are exquisitely susceptible to this chemical (Hüttenhofer et al., 1996), especially when flanked by two pyrimidines (Ciesiolka et al., 1998), which is precisely the case in the tRNA^{Sec} examined. This molecule was submitted to lead acetate cleavage and no strong cleavages appeared in the C64/C66 area (Fig. 2B). Cleavages were indeed observed between U67A and C62, but they are of an extremely low and evenly distributed intensity and, in addition, localized in the C64-C62 base paired region that should not be cut whether it is the 7/5 or 9/4 model (see Fig. 1A and B for a summary of the cleavages). Therefore, these can be considered background cleavages. Instead, a much stronger intensity of cuts should have been expected at the level of U66-C64 if C66 were bulged. As anticipated, the T-loop was cleaved efficiently by Pb^{2+} (Figs. 1, 2B).

These two experiments establish that C66 is not bulged, therefore strongly arguing in favor of the 9/4 model.

The tRNA^{Sec} of the Archae *M. jannaschii* can only adopt the 9/4 folding

Elucidation of the complete genomic sequence of the Archae *M. jannaschii* led the authors to the finding that the transcription and translation machineries in eukaryotes and this archae are very much alike (Bult et al., 1996). When it comes to the tRNA^{Sec} secondary structure of this organism, we found that the sequence constraints absolutely preclude a folding according to the 7/5 model, allowing only the 9/4 possibility (Fig. 3). The complete similarity of the archaeal tRNA^{Sec} secondary structure to that of eukaryotes cannot be taken as a fortuitous argument for two reasons.

The first reason arises from the findings of Wilting et al. (1997) that selenoprotein mRNAs, in *M. jannaschii*, contain SECIS-like elements in their 3′UTR

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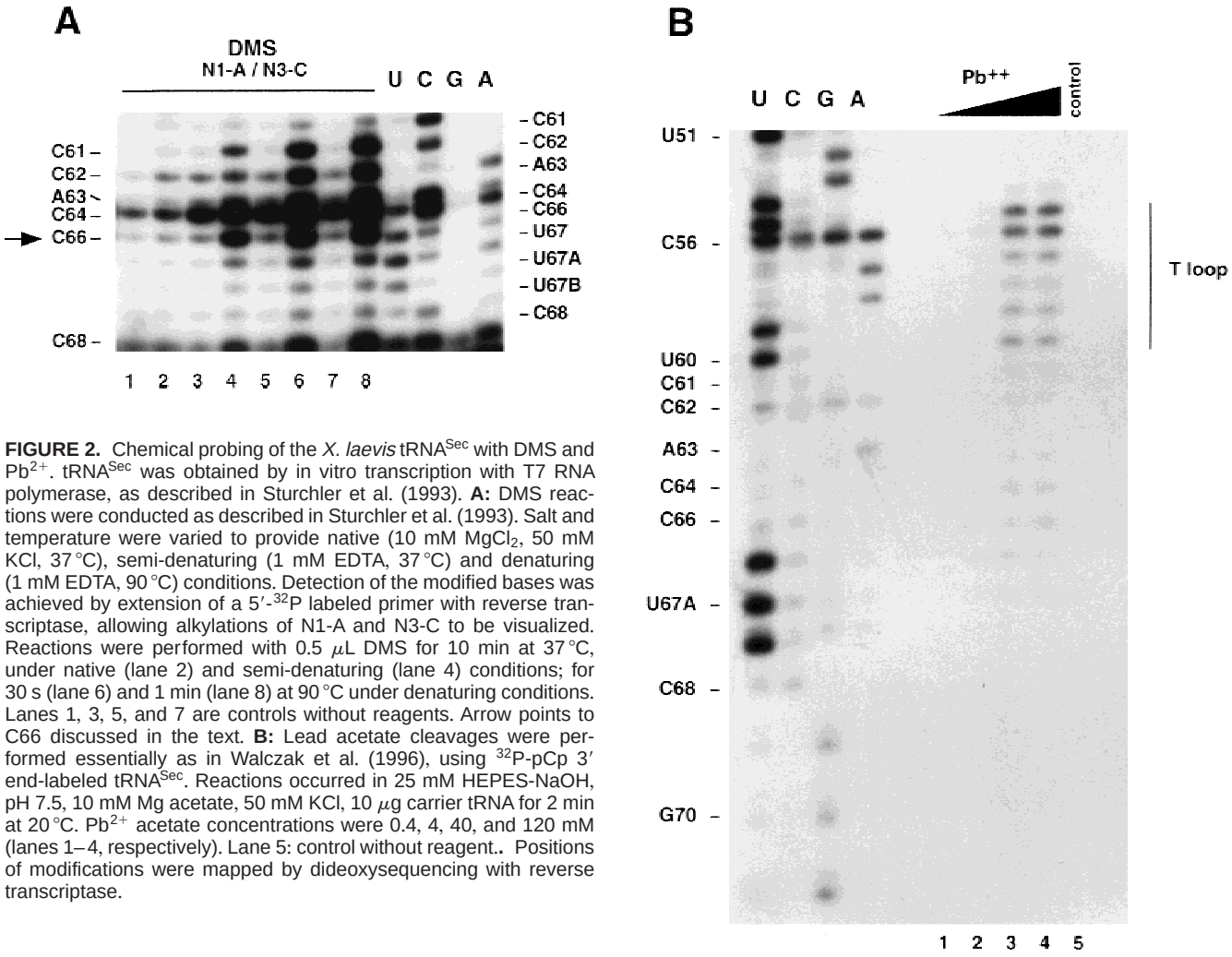

FIGURE 2. Chemical probing of the *X. laevis* tRNA^{Sec} with DMS and Pb^{2+}. tRNA^{Sec} was obtained by in vitro transcription with T7 RNA polymerase, as described in Sturchler et al. (1993). **A:** DMS reactions were conducted as described in Sturchler et al. (1993). Salt and temperature were varied to provide native (10 mM $MgCl_2$, 50 mM KCl, 37 °C), semi-denaturing (1 mM EDTA, 37 °C) and denaturing (1 mM EDTA, 90 °C) conditions. Detection of the modified bases was achieved by extension of a 5′-^{32}P labeled primer with reverse transcriptase, allowing alkylations of N1-A and N3-C to be visualized. Reactions were performed with 0.5 μL DMS for 10 min at 37 °C, under native (lane 2) and semi-denaturing (lane 4) conditions; for 30 s (lane 6) and 1 min (lane 8) at 90 °C under denaturing conditions. Lanes 1, 3, 5, and 7 are controls without reagents. Arrow points to C66 discussed in the text. **B:** Lead acetate cleavages were performed essentially as in Walczak et al. (1996), using ^{32}P-pCp 3′ end-labeled tRNA^{Sec}. Reactions occurred in 25 mM HEPES-NaOH, pH 7.5, 10 mM Mg acetate, 50 mM KCl, 10 μg carrier tRNA for 2 min at 20 °C. Pb^{2+} acetate concentrations were 0.4, 4, 40, and 120 mM (lanes 1–4, respectively). Lane 5: control without reagent.. Positions of modifications were mapped by dideoxysequencing with reverse transcriptase.

(SECIS elements are required for decoding the UGA selenocysteine codon) in much the same way as eukaryotes, but in contrast to bacteria, where these elements are adjacent to the UGA selenocysteine codon. It is therefore very likely that, paralleling the classical translation machinery (see above), the selenocysteine machinery functions similarly in eukaryotes and Archae, even though the sequences of some of the molecular actors differ in the two kingdoms.

The second reason is supplied by evolutionary considerations. The recent publication of the complete genome sequence of the hyperthermophilic bacterium *Aquifex aeolicus* (Deckert et al., 1998) allowed us to fold the tRNA^{Sec} sequence into the 8/5 secondary structure model as for the other bacterial tRNAs^{Sec} (Fig. 3). Several pieces of evidence point to the conclusion that the Archae and Eukarya share a common evolutionary trajectory independent of the lineage of bacteria (Woese et al., 1990; Bult et al., 1996; Pace, 1997). Also, the kinship degree between the bacterium *A. aeolicus* and the Archae *M. jannaschii* is far from being close (reviewed in Pennisi, 1998). Thus, the congruence of the 8/5 versus 9/4 tRNA^{Sec} classification with the bacterial versus archaeal/eukaryal phylogenetic kingdom classification provides strong evidence for the 9/4 tRNA^{Sec} structure. Indeed, a 7/5 structure model for eukaryotes would lead to three different secondary structure models for the three different kingdoms instead of only two (Fig. 3). Therefore, the 8/5 and 9/4 tRNA^{Sec} classification that we posited is relevant, thus providing additional evidence for the merits of the archaeal tRNA^{Sec} argument.

CONCLUSIONS

We think that both the previous data presented in Sturchler et al. (1993) and the new data presented here provide further compelling evidence in favor of the 9/4 model for the eukaryotic tRNA^{Sec}. Without replying point by point to the arguments developed by Steinberg et al. (1998), we would like to comment on a few specific aspects of their analysis.

1. Their reinvestigation of our experimentally derived model originated from their assumption that the tRNA^{Sec}

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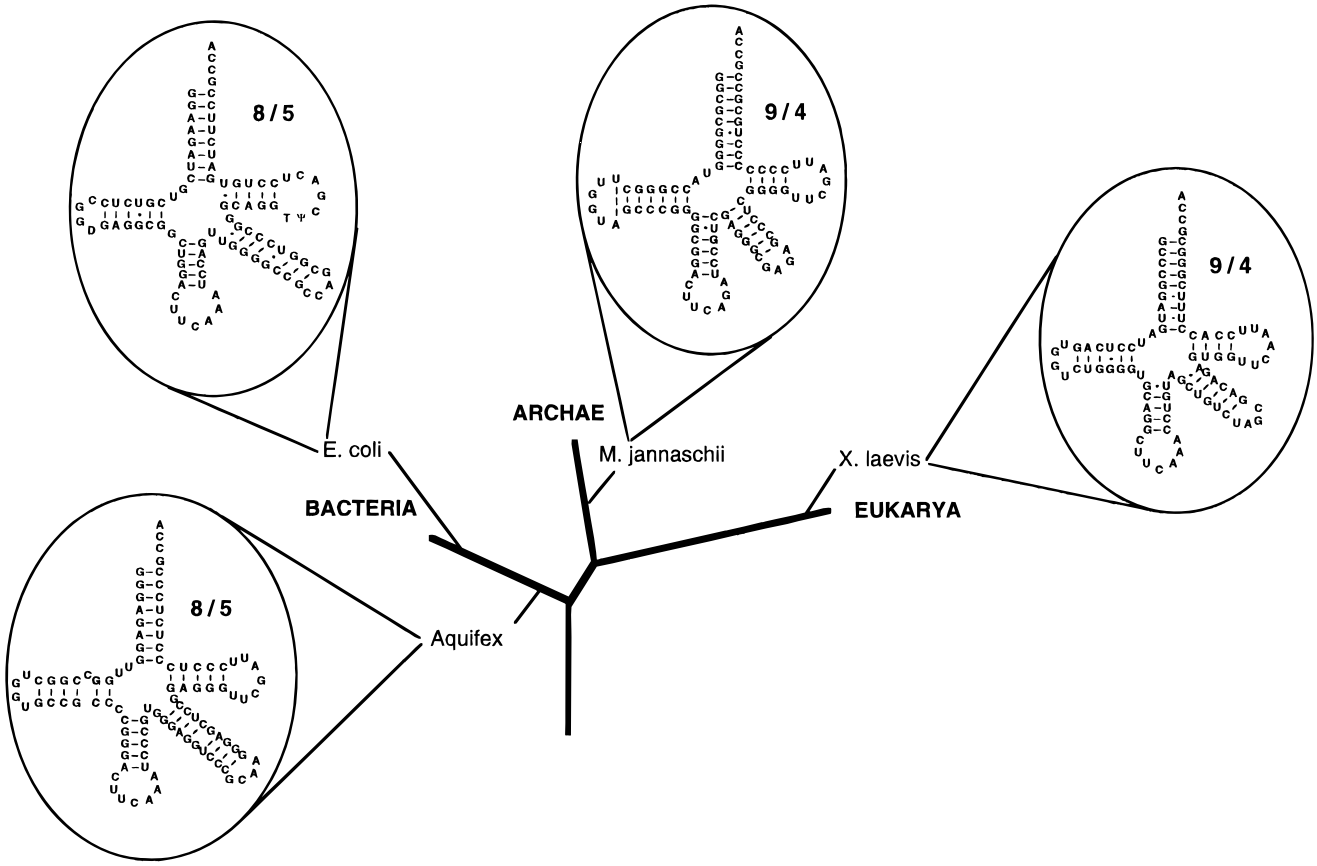

FIGURE 3. Sketched phylogenetic tree, according to the present knowledge (Woese et al., 1990; Pace, 1997), showing distribution of the tRNA^{Sec} secondary structure models. The tRNA^{Sec} secondary structure of the Archae *M. jannaschii* (sequence from Bult et al., 1996) folds into the 9/4 model. Dotted line represents a putative U-A base pair. The tRNA^{Sec} secondary structure of the hyperthermophilic bacterium *A. aeolicus* (sequence from Deckert et al., 1998) folds into the 8/5 model, as do the other bacterial tRNAs^{Sec}. An alternate 2D structure can be proposed for the D-stem with a C.C pairing. The folding is 8/5 in bacteria (*E. coli* and *A. aeolicus*), 9/4 in Archaea (*M. jannaschii*) and Eukarya (*X. laevis*). The *M. jannaschii* tRNA^{Sec} sequence was extracted from the TIGR mjdatabase (positions 111766–111855). The *A. aeolicus* tRNA^{Sec} sequence was found at NCBI, accession number AE000720 (positions 8711–8809). The *E. coli* and *X. laevis* tRNAs^{Sec} sequences were from Tormay et al. (1994) and Sturchler et al. (1993), respectively.

fulfills the structural criteria derived from comparisons of mitochondrial tRNA 2D structures (Steinberg et al., 1997).

2. Nowhere in their publication did the authors mention the fact that our probing data (Sturchler et al., 1993) established that N3-C66 is protected against DMS alkylation under native conditions. This protection precludes C66 to be bulged, as it is in the 7/5 model. Protection experiments at N3-C66 were redone here and the same conclusion was reached.

3. The 9/4 structure does provide for a normal D/T loop interaction, in contrast to what was claimed by Steinberg et al. (1998). This is well attested by protection of N3-C56 against DMS and the 3D model we derived (Sturchler et al., 1993). Very recently, protection of N3-C56 was also observed by Heckl et al. (1998) in the human tRNA^{Sec}.

4. Because of what they called inconsistencies in some of our probing experiments, Steinberg et al. (1998) had reservations about the applicability of the structure probing approach, which, they claimed, could not distinguish unambiguously between the two alternate structures. These arguments can be rebutted as follows.

A. Heckl et al. (1998) recently and independently obtained chemical probing data similar to ours with the human tRNA^{Sec}.

B. The RNase V1 cleavage between U60 and C61 is consistent with a requirement by this enzyme for helical phosphodiester bonds to cleave, which is precisely the case between these two bases (Sturchler et al., 1993).

C. It has been shown that kethoxal possesses a denaturing effect, leading to a shift of the melting curve of an RNA toward lower temperatures (Jaeger et al., 1993). This takes care of G50, G52, and G53 being reactive toward this probe. The reactivities of N3-U6 and N3-U12 toward CMCT (carbodiimide) is not an unprecedented observation in RNAs when monitoring non-Watson–Crick base pairs. The literature describing the many chemically probed RNAs provides examples in this respect.

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D. It is frequently observed that N1-As (here N1-A63) react rapidly with DMS, even when base paired with a U, sometimes leading to the wrong interpretation that an A could be single stranded. This reactivity is very likely due to the small size of the reagent and breathing of the helix, providing enough transient accessibility of the N1-A for the chemical to react. However, in the gel provided in Figure 2A, it is obvious that N1-A63 is protected under native conditions, thus base paired to U51.

E. A secondary structure model obtained by enzymatic and chemical probing arises from the interpretation of an ensemble of results generated by a combination of several probes that eventually provide the model. Ambiguities remain when one focuses on single pieces of data obtained with a single probe, instead of trying to derive a global picture from a large number of probes.

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