Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4955–4959, May 1994
Biochemistry

Visualization of RNA tertiary structure by RNA-EDTA·Fe(II) autocleavage: Analysis of $tRNA^{Phe}$ with uridine-EDTA·Fe(II) at position 47

(affinity cleaving/yeast $tRNA^{Phe}$/cleavage of RNA)

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Contributed by Peter B. Dervan, February 24, 1993

ABSTRACT **To test whether intramolecular autocleavage of RNA labeled at a single nucleotide position with EDTA·Fe(II) is a useful approach for the investigation of tertiary structures of RNAs, yeast phenylalanine tRNA was synthesized with uridine-EDTA (*U) at position U47. Autocleavage of [*U47]$tRNA^{Phe}$ in the presence of $Fe(NH_4)_2(SO_4)_2$ and dithiothreitol produced a set of cleavage fragments which are in general agreement with the three-dimensional structure derived from x-ray analysis.**

RNA molecules require specifically folded tertiary structures for their biological and chemical functions (1, 2). Although RNA secondary structures can be determined by chemical and enzymatic modification reagents and comparative sequence analysis, significantly less is known about the tertiary structure of RNAs. At present, the tertiary structures of only some small RNAs have been determined, by high-resolution x-ray crystallographic and NMR analysis (3–13). In the absence of such high-resolution studies, there is a need for new methods for analyzing the folded structure of RNA in solution.

Attachment of EDTA·Fe(II) to small molecules, proteins, and oligonucleotides has proven useful for studying the structures of such molecules in complex with nucleic acids (14–21; and H.H. and P.B.D., unpublished data). Upon addition of a reducing agent such as dithiothreitol or sodium ascorbate, the EDTA·Fe(II) moiety generates a nonspecific diffusible oxidant, most likely hydroxyl radical. Cleavage at several nucleotide positions proximal in space to the unique location of the bound EDTA·Fe(II) affords DNA or RNA fragments of different size and amount. Hence, structural information regarding the nucleotides neighboring the discretely bound ligand-EDTA·Fe(II) can be determined by electrophoretic separation of nucleic acid cleavage products in a high-resolution polyacrylamide gel. The size and amount of cleaved fragments relate to the distance in the three-dimensional structure.

The question arises whether self-cleavage of a folded RNA structure with EDTA·Fe(II) at a unique base position would be useful for analyzing nearest neighbors by cleavage pattern analysis. Yeast $tRNA^{Phe}$ is an attractive candidate for initial studies because its three-dimensional structure has been well characterized by x-ray crystallography and the solution structure of the fully unmodified yeast $tRNA^{Phe}$ has been shown to be similar to that of the native yeast $tRNA^{Phe}$ (3, 23–25). The correlation of autocleavage data with those from the crystal structure of the native tRNA would establish the potential of autocleavage for analyzing tertiary structures of RNAs. tRNA-EDTA suitable for intramolecular autocleavage studies was constructed by the chemical incorporation of uridine-EDTA (*U) into the 3′ half fragment of tRNA at position U47 followed by enzymatic ligation to the 5′ half of the [*U47]tRNA by T4 DNA ligase (Fig. 1) (22, 26). Lead-specific cleavage of [*U47]$tRNA^{Phe}$ indicates that the folded structure is not disrupted by the presence of EDTA at the U47 position (see Table 1 and Fig. 2) (9, 27). The autocleavage pattern of [*U47]$tRNA^{Phe}$·Fe(II) correlates reasonably well with the high-resolution x-ray crystal structure, leading to the conclusion that autocleavage may be applicable for studies of tertiary structures of RNAs.

MATERIALS AND METHODS

General. Adenosine 5′-triphosphate and DNase I were purchased from Pharmacia, and T4 polynucleotide kinase and T4 RNA ligase were obtained from New England Biolabs. T4 DNA ligase was purchased from United States Biochemical. [γ-^{32}P]ATP (≈6000 Ci/mmol; 1 Ci = 37 GBq) and [5′-^{32}P]pCp (≈3000 Ci/mmol) were obtained from Amersham and New England Nuclear, respectively. Phosphoramidites were purchased from Applied Biosystems (dA, dG, dC, and dT) and from BioGenex Laboratories (San Ramon, CA) (rA, rG, rC, and rU).

Preparation of tRNA and [*U47]$tRNA^{Phe}$. Oligonucleotides were synthesized by standard automated solid-support chemistry with an Applied Biosystems model 394 DNA/RNA synthesizer. RNA oligonucleotides with EDTA at a single uridine position were prepared as described (22). RNA was 5′-end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP (28). RNA was 3′-end labeled by using T4 RNA ligase and [5′-^{32}P]pCp (29). Prior to the ligation with T4 DNA ligase, 3′-half RNA was phosphorylated at the 5′ end with T4 polynucleotide kinase and ATP (28). The 5′-^{32}P-end-labeled tRNA was prepared by ligation of 5′-^{32}P-labeled 5′-half tRNA (nt 1–36) to 3′-half tRNA (nt 37–76) using a 50-base DNA template (nt 10–59) complementary to the 3′ end of 5′-half tRNA (27 nt) and the 5′ end of 3′-half tRNA (23 nt) (Fig. 1) (26). The 3′-^{32}P-end-labeled tRNA was prepared by using 5′-half tRNA and 3′-^{32}P-labeled 3′-half tRNA. 5′-Half tRNA (50 pmol) and 3′-half tRNA (50 pmol) were added to the DNA template (50 pmol) in 20 μl of hybridization buffer (10 mM Tris·HCl, pH 7.6/10 mM KCl), heated at 90°C for 1 min, and cooled to room temperature over 1 hr. After hybridization, 2.5 μl of 10× ligase buffer (660 mM Tris·HCl, pH 7.6/660 μM ATP/66 mM $MgCl_2$/100 mM dithiothreitol and 2.5 μl of T4 DNA ligase solution (10 units/μl) were added. After a 6-hr incubation at 16°C, the reaction mixture was treated with 1 μl of DNase I solution (10 units/μl) for 30 min at 37°C. Samples were ethanol precipitated, purified by electrophoresis in a 20% polyacrylamide/7 M urea gel (0.8 mm thick), and eluted in 250 μl of elution buffer (0.3 M sodium acetate) at room temperature. Eluted RNAs were ethanol precipitated, dried, and stored at −20°C until use.

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Abbreviation: *U, uridine-EDTA.

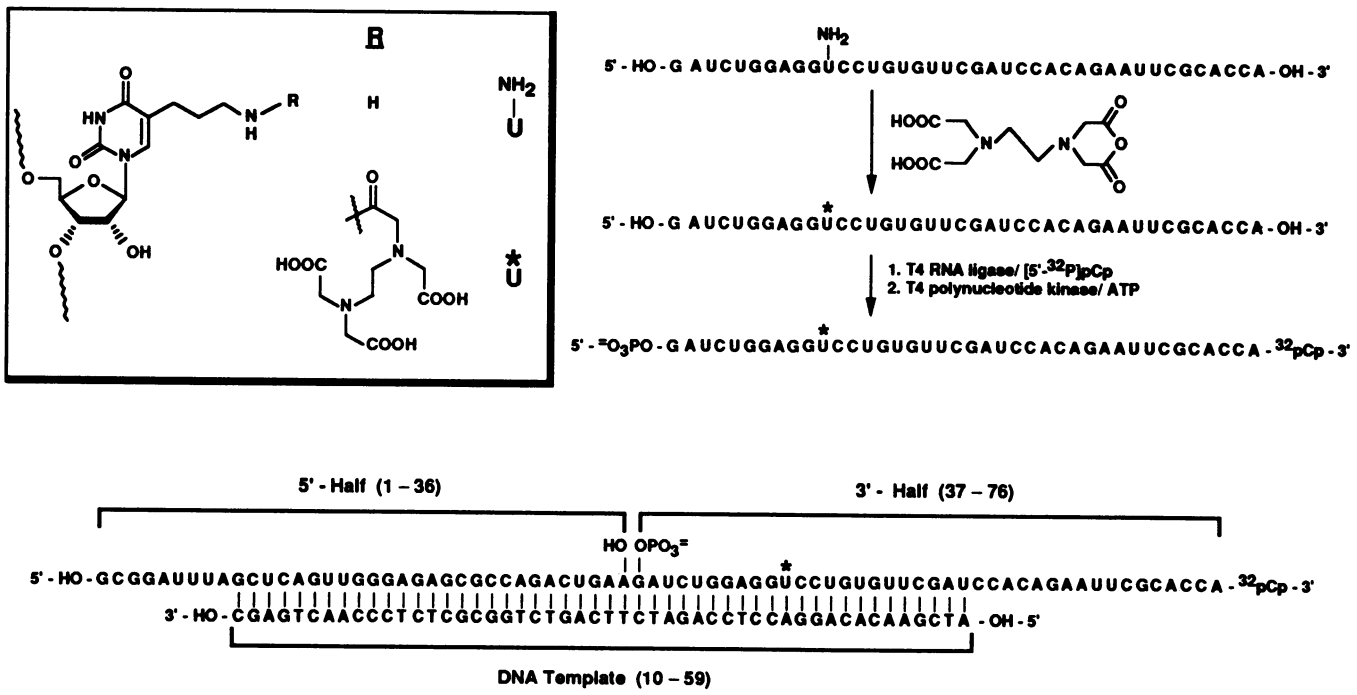

FIG. 1. (*Upper Left*) Uridine-amine (U^{NH2}) and *U. (*Upper Right*) Scheme for the synthesis of 3'-^{32}P-labeled 3'-half tRNA. (*Lower*) Sequences of oligonucleotides, 5'-half and 3'-half, and DNA template used to construct 3'-^{32}P-labeled tRNA-EDTA by ligation with T4 DNA ligase. Star indicates *U that is incorporated at U47. A 3'-hydroxyl and a 5'-phosphate are shown in the ligation junction at which phosphodiester bond formation is catalyzed by T4 DNA ligase.

Cleavage Reactions. All tRNAs were renatured by heating at 70°C for 1 min in 10 mM Tris·HCl (pH 7.0) and slow cooling to room temperature prior to use. A typical 50-μl reaction mixture contained 2 μM tRNA (≈200,000 cpm), 20 mM Tris·HCl (pH 7.0), 10 mM $MgCl_2$, and 20 mM NaCl. The lead cleavage reaction was carried out by addition of $Pb(OAc)_2$ (200 μM) at 25°C, and at appropriate intervals 5-μl aliquots were removed, added to 5 μl of stop buffer (7 M urea/50 mM EDTA/dyes), and analyzed by electrophoresis in a 15% polyacrylamide/7 M urea gel (27). Autocleavage reactions for [*U47]tRNA^{Phe} were performed by incubation of [*U47]tRNA^{Phe} with $Fe(NH_4)_2(SO_4)_2$ (1 μM) for 2 hr at 25°C followed by addition of dithiothreitol (4 mM). After 8 hr at 25°C, the cleavage reactions were analyzed by loading 20,000 cpm of the sample on a 20% polyacrylamide/7 M urea wedge gel (31.0 cm wide, 38.5 cm long, 0.4 mm thick at top, and 1.2 mm thick at bottom). The product fragments were quantitated by PhosphorImager analysis (Molecular Dynamics) (30).

RESULTS AND DISCUSSION

Site for the Incorporation of *U. Examination of the high-resolution x-ray crystal structure of yeast tRNA^{Phe} and of mutant studies suggests that nucleotide position U47 of tRNA is a reasonable choice for EDTA·Fe(II) attachment (3–6, 27, 31–33). The presence of a modified base, 3-(3-amino-3-carboxypropyl)uridine, located in the loop, occurs naturally at U47 of *Escherichia coli* tRNA^{Phe} (34).

tRNA-EDTA Preparation. 5'-Half tRNA, 36 nt in length, and 3'-half tRNA, 40 nt in length, were synthesized by automated methods using 2-cyanoethyl phosphoramidite chemistry (35). 3'-Half tRNA with *U at the U47 position was prepared by the incorporation of phosphoramidites of the functionalizable uridine amine into oligoribonucleotides, followed by subsequent modification with activated EDTA derivatives as described (Fig. 1) (22). RNA oligonucleotides containing *U with the attached EDTA moiety displayed a reduced electrophoretic mobility and could be purified by electrophoresis in a denaturing 20% polyacrylamide gel of 0.8 mm thickness (22). A 5–10% yield of purified RNA-EDTA was obtained on a 1-μmol-scale oligonucleotide synthesis. A DNA template with 50 bases complementary to 5'-half and 3'-half tRNA was used to juxtapose the ligation substrates. Full-length tRNAs, 76 nt in length, were constructed by ligation of 5'-half tRNA to 3'-half tRNA using T4 DNA ligase (Fig. 1) (26). The yield after ligation was approximately 10–15% for both tRNA and tRNA-EDTA.

Lead Cleavage Assay. The lead cleavage reaction was carried out to examine the correct folding of [*U47]tRNA^{Phe}. Analysis of the Pb(II) cleavage rates of the yeast tRNA^{Phe} mutants shows that the rate of cleavage is sensitive to the proper tertiary structure (27). The major lead-specific cleavage sites of [*U47]tRNA^{Phe} in 20 mM Tris·HCl, pH 7.0/10 mM $MgCl_2$/20 mM NaCl/2 μM tRNA/200 μM $Pb(OAc)_2$ were located between U17 and G18 in the D loop (Fig. 2 *Upper*). Under the conditions used here, [*U47]tRNA^{Phe} exhibits a cleavage rate similar to that of unmodified tRNA^{Phe}, whose rate is half that of native yeast tRNA^{Phe} (Table 1; see Fig. 2). This is consistent with previous results (27). The site and rate of lead cleavage observed for [*U47]tRNA^{Phe} indicate that the presence of EDTA at position U47 does not significantly alter the folding of tRNA, confirming that [*U47]tRNA^{Phe} can adopt a conformation similar to that of the native folded tRNAs.

Autocleavage. The sites of autocleavage in tRNA-EDTA·Fe(II) with *U at position U47 were determined by using 5'- or 3'-^{32}P-end-labeled [*U47]tRNA^{Phe}·Fe(II) (Fig. 3). Autocleavage was performed under conditions similar to those used for Pb(II) cleavage. The labeled [*U47]tRNA^{Phe} was renatured, equilibrated with Mg^{2+} for 20 min, and treated with Fe(II). After 2 hr at room temperature, autocleavage reactions were initiated by addition of dithiothreitol and incubated for 8 hr at 25°C. Cleavage sites between nucleotides 1 and 40 were mapped with 5'-end-labeled [*U47]tRNA^{Phe} and those between 41 and 76 with 3'-end-

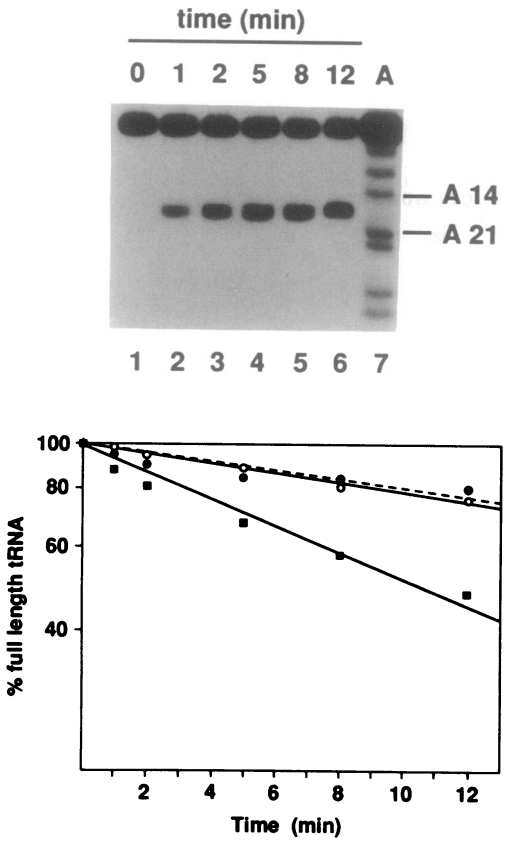

FIG. 2. (*Upper*) Autoradiogram of a denaturing 15% polyacrylamide gel showing lead cleavage reactions of a 3′-^{32}P-labeled [*U47]tRNA^{Phe}. Lanes 1–6, cleavage products produced from [*U47]tRNA^{Phe}, which were allowed to react for 0, 1, 2, 5, 8, and 12 min, respectively; lane 7, adenosine-specific chemical sequencing (36). (*Lower*) Cleavage kinetics of native yeast tRNA^{Phe} (■), unmodified tRNA^{Phe} (●), and [*U47]tRNA^{Phe} (○).

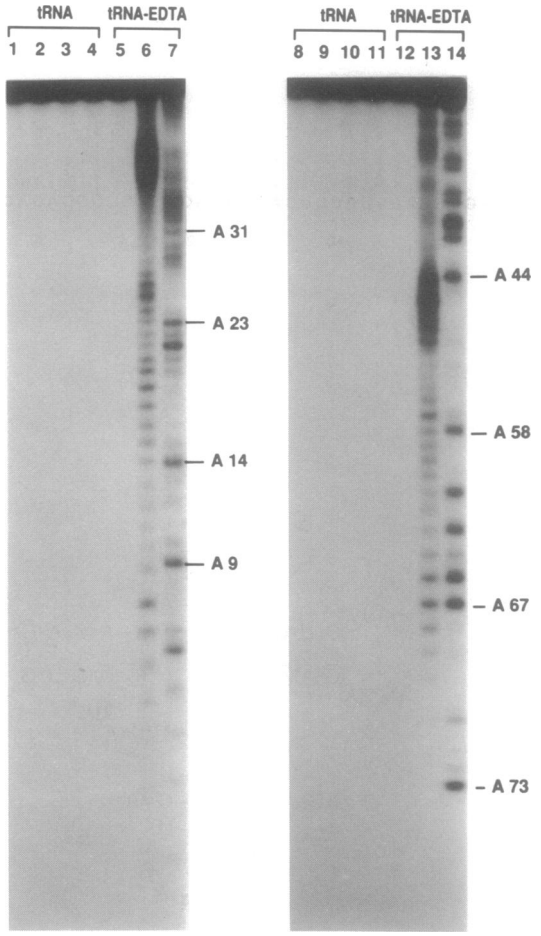

FIG. 3. Autoradiogram of a denaturing 20% polyacrylamide wedge gel showing affinity cleavage reactions of unmodified tRNA^{Phe} and [*U47]tRNA^{Phe} labeled with ^{32}P at the 5′ end (lanes 1–7) and at the 3′ end (lanes 8–14). Lanes 1–6 and 8–13, cleavage products of unmodified tRNA^{Phe} (lanes 1–4 and 8–11) and [*U47]tRNA^{Phe} (lanes 5, 6, 12, and 13) obtained after incubation under the conditions of autocleavage reactions in the absence of Fe(II) (lanes 1, 5, 8, and 12) and in the presence of EDTA·Fe(II) (lanes 2 and 9), propane-EDTA·Fe(II) (lanes 3 and 10), and Fe(II) (lanes 4, 6, 11, and 13); lanes 7 and 14, adenosine-specific chemical sequencing (22).

labeled [*U47]tRNA^{Phe}. Comparison of autocleavage products from [*U47]tRNA^{Phe} in the absence and presence of Fe(II) reveals cleavage sites and efficiencies (Fig. 3, lanes 5 and 12 and lanes 6 and 13). Very low levels of nonspecific cleavage were observed when unmodified tRNA^{Phe} was subjected to cleavage with EDTA·Fe(II), propane-EDTA·Fe(II), and Fe(II) at final concentrations of 1 μM, demonstrating that the observed autocleavage was generated by EDTA·Fe(II) tethered to tRNA at *U47 (Fig. 3, lanes 2–4 and lanes 9–11).

Six regions of specific cleavage were identified: nt 7–9, 14–21, 22–27, 44–51, 56–61, and 66–68 (Fig. 4*A*). Strongest cleavage was observed at nt 44–51, which are adjacent to *U47, corresponding to 5% of the intact [*U47]tRNA^{Phe}. The sites of autocleavage are represented by circles on the secondary structure diagram (Fig. 4*B*). Five regions with weaker cleavage were mapped on the tertiary structure of tRNA^{Phe} (Fig. 4*C*) (6). The five medium-cleavage sites centered at positions U8, G18, G26, G57, and A67 are estimated to be within 11–24 Å from carbon-5 of *U47 in the tertiary structure. An intensity ratio, θ, defined as autocleavage at the given nucleotide to the average autocleavage in the region 44–51, was compared with the distance (*d*) measured between the 5-carbon of *U47 and the 4′-carbons of the nucleotides. For positions U8 and A67, which are at 11 and 14 Å from *U47, the cleavage intensity (θ) is 0.3 and 0.4, respectively. For G18, G26, and G57, which are at 24 Å, θ is 0.4. This reveals that θ is insensitive to fine tuning distances in the range 11–24 Å, most likely because not every ribose at fixed distance has identical reactivity or because the tethered EDTA·Fe(II) moiety may have preferred positions to "rut" on the folded molecule. The lack of autocleavage ($\theta \leq 0.04$) at the acceptor stem and 3′ end (d = 38 Å for A73) and the anticodon stem and loop (d = 50 Å for G34) is also consistent with the three-dimensional structure, since these regions are distant from *U47.

Table 1. Rate of lead cleavage for tRNA

	k_{obs}, $s^{-1} \times 10^4$	Relative k_{obs}
Yeast tRNA^{Phe}	7.9 ± 1.2	1
Unmodified tRNA^{Phe}	3.5 ± 0.4	0.44
[*U47]tRNA^{Phe}	3.4 ± 0.5	0.43

Unmodified tRNA^{Phe} was prepared by using T4 DNA ligase to ligate the synthetic 5′-half tRNA to the synthetic 3′-half tRNA containing uridine at position 47.

Implications and Limitations. Although there is overall agreement between the autocleavage data and the crystal structure for the tRNA study here, there are limitations which may be relevant to the general applicability of autocleavage for studying tertiary structures of large RNA molecules. The cleavage data correlate with three-dimensional distance only in broad categories: adjacent to *U (strong cleavage), proximal to *U in the range 11–24 Å (medium

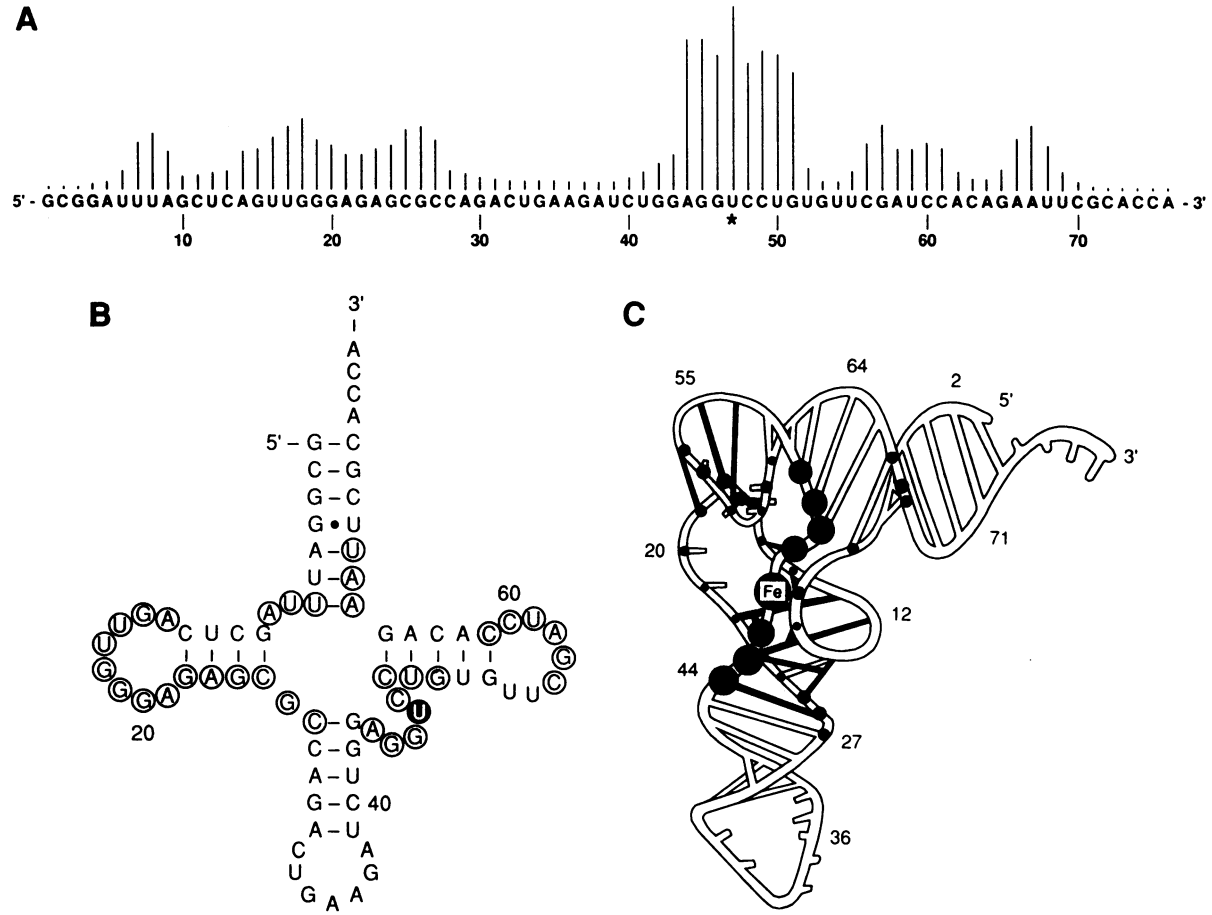

FIG. 4. (*A*) Histogram of the cleavage data derived from autoradiogram in Fig. 3. The heights of the bars represent the relative cleavage intensities at the indicated bases. Six cleavage regions—nt 7–9, 14–21, 22–27, 44–51, 56–61, and 66–68—were mapped on the secondary and tertiary structures shown below. (*B*) [*U47]tRNA^{Phe} autocleavage sites mapped on the secondary structure of tRNA^{Phe}. The position of *U47 is indicated by the filled circle. (*C*) [*U47]tRNA^{Phe} autocleavage sites mapped on a ribbon diagram of the tertiary structure adapted from the crystal structure of yeast tRNA^{Phe} (6). Filled circles indicate points of cleavage along the phosphodiester backbone, with size corresponding to relative cleavage intensity. The position of *U47 is indicated by the filled circle with Fe.

cleavage), and distal to *U in the range > 40 Å (very weak cleavage). For large RNA structures, this low-resolution information may be sufficient to distinguish between different hypothetical models. In addition, this approach may be useful for studying tRNA–ribosome interactions as well as tertiary structures of larger RNAs.

We are grateful to the National Institutes of Health for grant support.

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