Mapping tRNA structure in solution using double-strand-specific ribonuclease V₁ from cobra venom

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ABSTRACT

A method for mapping all base-paired stems in both elongation and initiator tRNAs is described using double-strand-specific ribonuclease V₁ from the venom of the cobra Naja naja oxiana. ³²P-end-labeled RNA is first partially digested with double-strand-specific V₁ nuclease under near physiological conditions, and the resultant fragments are then electrophoretically fractionated by size in adjacent lanes of a polyacrylamide gel run in 90% formamide. After autoradiography, the base-paired nucleotides are definitively located by comparing V₁ generated bands with fragments of known length produced by both Neurospora endonuclease and base-specific ribonucleases. Using the substrates yeast tRNA^{Phe} and E. coli tRNA^{Met} of known three-dimensional structure, we find V₁ nuclease to cleave entirely within every base-paired stem. Our studies also reveal that nuclease V₁ will digest paired nucleotides not hydrogen-bonded by standard Watson-Crick base-pairing. In yeast tRNA^{Phe} cleavage of both wobble base-pairs and nucleotides involved in tertiary base-base hydrogen bonding is demonstrated.

INTRODUCTION

Determination of the structure of RNA molecules in solution is important for our understanding of their biological role within a cell. Though considerable RNA structural information has recently been derived from both spectroscopic and X-ray crystallographic studies (1), as well as from chemical modification work (2-4); nuclease susceptibility offers many advantages including definitive identification of RNA cleavage sites using a structure-specific enzymatic probe in solution. In an RNA molecule, the accessibility of a specific region towards nucleolytic cleavage is dictated, in part, by its relative exposure to the surface of the molecule, and also by the size and structural specificity of the enzyme. Recent studies have shown that both single-strand-specific S₁ nuclease and T₁ RNase can be used successfully for mapping nucleotides known to be in single-stranded loops in yeast tRNA^{Phe} (5,6) and E. coli tRNA^{Met} (7,8). This structure mapping procedure by Wurst, et al. (5), employs ³²P-end-labeled RNA which is first partially digested with single-strand-specific nucleases under nondenaturing

conditions, and the resultant fragments then electrophoretically fractionated by size in adjacent lanes of a denaturing polyacrylamide gel. The procedure is limited, however, in allowing detection of only the unpaired nucleotides within a molecule. Recently, Favorova, et. al. (9) reported the use of "cobra venom ribonuclease" isolated from Naja naja oxiana venom for mapping double-stranded regions in ^{32}P -end-labeled yeast tRNA^{Phe} and tRNA^{Val}. However, this enzyme does not cleave either the D or T ψ stems in tRNA^{Phe} or the D stem of tRNA^{Val}, indicating a more limited application of this enzyme fraction in both RNA structure determination and mapping protein protected sites (9). In this paper we report the isolation of a double-strand-specific ribonuclease (V₁) also from the venom of the cobra Naja naja oxiana. We show that by using this enzyme as a structure probe, one can definitively localize all base-paired stems within a ^{32}P -end-labeled tRNA molecule after electrophoretic fractionation of the V₁ digest on polyacrylamide gels run in 90% formamide. We have also determined, using ^{32}P -end-labeled yeast tRNA^{Phe} as a substrate, that some nucleotides known to be involved in tertiary interactions are also recognized and cleaved by this enzyme.

MATERIALS

Lyophilized venom from the cobra Naja naja oxiana was purchased from Sigma Chemical Co. The RNase purification resins were obtained from the following sources: Sephadex G-75 (superfine) from Pharmacia Chemicals, and SP-Sephadex C-50-120 (sulfopropyl-sephadex) from the Sigma Chemical Co. [^3H]-uridine-labeled reoviral genome RNA (type 3) was a kind gift from Dr. Aaron J. Shatkin, Roche Institute of Molecular Biology. [^3H]-polyuridylylate (22 mCi/mmol) was purchased from Schwarz/Mann. Polyuridylic acid was obtained from P.L. Biochemicals Inc. Human placental initiator tRNA^{Met} was a kind gift from Dr. U.L. RajBhandary, MIT. Both E. coli tRNA^{Met} and yeast initiator tRNA^{Met} were kind gifts from Dr. Eric Ackerman, University of Chicago. Yeast phenylalanine tRNA was purchased from Boehringer-Mannheim. Both calf intestinal alkaline phosphatase and T₄ polynucleotide kinase were supplied by Boehringer-Mannheim and used without further purification. T₄ RNA ligase and Neurospora crassa endonuclease were purchased from P.L. Biochemicals, Inc. Highly purified S₁ nuclease was a kind gift from Dr. John Vournakis and Dr. George Pavlakis, Syracuse University. [γ - ^{32}P]-ATP (> 7,000 Ci/mmol) was prepared from carrier-free $^{32}\text{P}_1$ (New England Nuclear-NEX-053) by the procedure of Johnson and Walseth (10). [$5'$ - ^{32}P]Cp (2,900 Ci/mmol) was purchased from New England Nuclear, Inc.

METHODS

Preparation and Assay of Double-Strand-Specific Ribonuclease V₁ from *Naja naja oxiana* Cobra Venom

Ribonucleases present in the cobra venom *Naja naja oxiana* were prepared and fractionated by modifications of the procedure of Vasilenko and Rait (11). 1.5 g of powdered venom was dissolved in 15 ml of 0.05 M Tris-succinate, pH 5.6 (Buffer A) and centrifuged at 15,000 RPM for 30 min at 4°C. The supernatant was removed and dialyzed overnight at 4°C against 3 liters of Buffer A. The dialyzed solution of venom was immediately applied to a Sephadex G-75 (superfine) column (3 x 105 cm), and eluted with Buffer A at a flow rate of 4.0 ml/hr. Fractions of 3.5 ml were collected, absorbance at 280 nm was read, and each fraction was assayed for both RNA double- and single-stranded specificity.

RNA double-stranded specificity was assayed using [³H]-uridine-labeled reoviral genome RNA as a substrate. Five µl was removed from each column fraction and added to 5 µl of a cocktail containing 3,000 CPM of [³H]-reoviral double-stranded RNA (8,000 CPM/µg) in buffer containing 0.20 M NaCl, 10 mM MgCl₂, and 20 mM Tris-HCl pH 7.2. The reaction was incubated at 37° for 60 min after which 20 µl of carrier yeast tRNA (10 mg/ml in 0.2M NaCl) was added, followed by the addition of 75 µl of ethanol. The mixture was vortexed and allowed to precipitate at 4°C for 30 min. The precipitate was collected by centrifugation for 20 min at 15,000 RPM at 4°C, and 50 µl of supernatant was removed and counted in aqueous scintillant.

Single-strand-specific RNase activity was assayed using uniformly [³H]-labeled polyuridylic acid (2000 DPM/nmol) as a substrate. Five µl from each column fraction was added to 15 µl of a cocktail containing 30,000 DPM of [³H]-poly U in 25 mM Tris-HCl pH 7.2. The mixture was incubated at 37° for 30 min. The reaction was terminated by the addition of carrier yeast tRNA and likewise ethanol precipitated as in the double-stranded assay, and 50 µl of supernatant was then removed after centrifugation and counted in aqueous scintillant.

The four major peaks of double-stranded ribonuclease activity eluting from the G-75 column (see Fig. 1A) also contained single-stranded activity. Peak #4 was the only fraction which exhibited primarily double-stranded specificity when assayed under partial digestion conditions using ³²P-end-labeled tRNA as a substrate (see below). Fractions 84-94 of peak #4 were pooled and applied to a sulfopropyl-sephadex column (1 x 50 cm) equilibrated with 0.02 M of Buffer A and eluted with a 350 ml linear gradient of 0.02M

Buffer A and 0.02M Tris pH 10.1 containing 0.40M KCl (Buffer B). The flow rate of the column was 12 ml/hr and 1.8 ml fractions were collected. The absorbance at 280 nm was read and each fraction was assayed for both RNA double- and single-stranded specificity. Tubes #136-142 (see Fig. 1B) were the only fractions which exhibited entirely double-stranded activity in the absence of contaminating single-stranded activity. These fractions, venom RNase 1 (V_1) were individually dialyzed at 4°C against 500 ml of buffer containing 20 mM Tris-HCl, pH 7.4 and 60% glycerol, and could be stored indefinitely at -20°C in 50% glycerol without noticeable loss of activity.

One unit of nuclease activity is defined as the amount of enzyme that solubilizes 10 μ g of double-stranded reoviral RNA in 30 min at 37°C in 20 mM Tris-HCl, pH 7.2, buffer containing 0.20 M NaCl and 10 mM $MgCl_2$.

Preparation of [5'- ^{32}P]-Labeled and [3'- ^{32}P]-Labeled tRNA

5'- ^{32}P -end-labeled tRNA was prepared as described by Silberklang et al. (12). For 3'- ^{32}P -end-labeling, 0.10 A_{260} units of either yeast tRNA^{Phe}, *E. coli* tRNA^{Met}, or human tRNA^{Met} were reacted with 200 pmols of [5'- ^{32}P]Cp (2,900 Ci/mmol) in a 10 μ l reaction mixture containing 50 mM Hepes-KOH, pH 8.3, 10 mM $MgCl_2$, 3.3 mM DTT, 10% glycerol, 5 μ M ATP and 6 units of T_4 RNA ligase for 60 min at 37°C. Calf intestinal alkaline phosphatase was then added to a final concentration of 0.5 units/ml and incubated for an additional 45 min at 37°C to remove all terminal 3'-phosphates from the 3'- ^{32}P -labeled tRNA molecules. The reaction was terminated by the addition of 0.20 M EDTA to a final concentration of 20 mM and 40 μ l of loading solution, and then layered onto a 15% polyacrylamide gel. The gel was electrophoresed at 30°C until the xylene cyanol migrated 20 cm, and the 3'- ^{32}P -labeled tRNA was localized by autoradiography, excised, recovered by electrophoretic elution, and ethanol precipitated as previously described (13).

Digestions with S_1 Nuclease and T_1 RNase for Secondary Structure Analysis

Partial digestion of ^{32}P -end-labeled tRNA with single-strand specific S_1 nuclease and T_1 RNase were carried out as previously detailed (14).

Digestion with V_1 Nuclease for Secondary Structure Analysis

Partial digestions with V_1 nuclease were performed in 5 μ l reactions containing 25 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 0.20 M NaCl at enzyme-to-substrate ratios of 1×10^{-5} - 10^{-6} units/ μ g RNA. Samples were preincubated for 10 min at 37°C prior to addition of enzyme, and digested with V_1 nuclease at 37°C for 1-10 min as indicated. The reactions were stopped with an equal volume of STOP solution (9M urea, 20 mM EDTA, 0.5 μ g/ml tRNA carrier, 0.05% XC and 0.05% BPB) and the digests were stored at -80°C until

analyzed by electrophoresis.

Digestions for Sequence Analysis

Partial enzymatic digestion of ^{32}P -end-labeled tRNA with T_1 RNase was performed as previously described (13,14), except the digestion time was limited to 10 min at 70°C at an enzyme-to-substrate ratio of 5×10^{-2} units/ μg RNA. Controlled acid hydrolysis (H^+) was performed by boiling ^{32}P -end-labeled tRNA with 1 $\mu\text{g}/\text{ml}$ of tRNA carrier in 10 mM H_2SO_4 for 1 min as previously described (15).

Electrophoresis of Partial Digests on Formamide Polyacrylamide Gels

Both structure and sequence reactions were fractionated in adjacent lanes of 15% polyacrylamide slab gels (0.40 mm thick x 33 cm wide x 80 cm long) run in 90% formamide as a denaturant.

Reagent-grade formamide (Fisher Scientific) was repeatedly deionized by mixing with Rexyn-I-300 (Fisher Scientific) ion-exchange resin until the pH was 4.0-4.5. Deionized formamide was stored at -20° until use. For preparation of one gel, to 216 ml of deionized formamide (prewarmed to 30°C) 12 ml of 40X TBE buffer (2 M Tris-base - 2 M boric acid - 0.04 M EDTA, pH 8.3) was added to give a final concentration of 2 X TBE. For a 15% gel, 34.2 g acrylamide - 1.8 g of bis-acrylamide was added, and for a 10% gel, 22.8 g acrylamide - 1.2 g of bis-acrylamide was added. After the ingredients were thoroughly dissolved, the solution was quickly filtered through a Whatman #1 filter-disc, but was not degassed. Two hundred milligrams of ammonium persulfate, predissolved in 200 μl of H_2O was immediately added, followed by 700 μl of TEMED (N, N, N', N'-tetramethylethylene-diamine). The gel solution was immediately injected into the gel mold from the top using a 50 ml syringe fitted with an 18-gauge needle. Gels were used within 12 hrs after polymerization and were pre-electrophoresed at 4000 volts for 3-4 hr with 2X TBE running buffer prior to loading the samples. Gels 80 and 100 cm long were run between 4000-5000 volts to generate enough heat to ensure complete denaturation of RNA fragments.

RESULTS

Isolation of Double-Strand Specific Ribonuclease V_1 from Cobra Venom

When dialyzed venom is completely fractionated on a Sephadex-G75 column and assayed for double-strand ribonuclease activity using [^3H]-labeled reoviral genome RNA as a substrate as shown in Figure 1A, four major peaks of activity are resolved rather than two as previously reported (11). These same fractions when assayed for single-strand ribonuclease

FIGURE 1. (A) Sephadex G-75 column chromatography of dialyzed *Naja naja oxiana* cobra venom.

(●—●) Absorbance at 280 nm. (○---○) Double-stranded RNase activity expressed as ethanol soluble 3H -CPMs generated after digestion of [3H]-uridine-labeled reoviral genome RNA (see METHODS).

(B) Sulfopropyl-sephadex column chromatography of "pooled" fractions from G-75 column. (●—●) Absorbance at 280 nm. (○---○) Double-stranded RNase activity. (x--x). Single-stranded RNase activity expressed as ethanol soluble 3H -CPMs generated after digestion of [3H]-polyuridylic acid (see METHODS). V_1 indicates fractions containing entirely double-stranded activity.

activity using either [3H]-labeled poly U, poly C, or poly A as substrates, generated a similar profile (data not shown), indicating that nuclease activity which would degrade single-stranded RNA copurified with the double-stranded activity. When fractions within peaks #3 and #4 of the G-75 column were further assayed for their ability to specifically cleave base-paired regions in ^{32}P -end-labeled yeast tRNA^{Phe} (see METHODS), only peak #4 was found to be enriched for the double-stranded activity. Fractions within peak #3 displayed neither sequence nor structural specificity in such an assay under a variety of nondenaturing and denaturing conditions. Peaks #1 and #2 were not assayed with ^{32}P -end-labeled tRNA^{Phe} due to an excessive amount of phosphatase copurifying with these fractions.

Fractions exhibiting double-strand-specific ribonuclease activity

were pooled from peak #4 and were further purified from contaminating single-strand activity by sulfopropyl-sephadex column chromatography as shown in Figure 1B. Fractions exhibiting double-strand-specific activity eluted as two defined peaks; one major peak (fractions 145-165) preceded by a shoulder peak (fractions 135-145). When these fractions were similarly assayed with [^3H]-labeled Poly U, only the shoulder fractions were free of single-strand-specific ribonuclease activity. A comparison of the nucleolytic activities of both the shoulder fractions, venom ribonuclease 1 (V_1), and single-strand-specific S_1 nuclease is shown in Figure 2. V_1 nuclease exhibits no detectable single-stranded activity using either [^3H]-poly U as a substrate (Figure 2A) or poly C and poly A (data not shown). Alternatively, as indicated in Figure 2B, V_1 nuclease actively hydrolyzed double-stranded [^3H]-reoviral genomic RNA unlike S_1 nuclease, underscoring the definitive structural specificity of V_1 's enzymatic activity.

Use of Ribonuclease Fraction V_1 as a Probe for Mapping RNA Higher-Order Structure in Solution

Yeast tRNA^{Phe}

Previous studies have shown that both single-strand-specific S_1 nuclease and T_1 RNase can be used successfully as structure probes for mapping single-stranded regions in yeast tRNA^{Phe} (5,6) and other tRNAs in solution (7,8), as well as for localizing unpaired nucleotides in 5S and 5.8S rRNA (17,18) and in α and β globin mRNA (14). Figure 3 shows a structure map

FIGURE 2. Comparison of the rates of nucleolytic cleavage of (A) single-stranded [^3H]-polyuridylic acid, and (B) double-stranded [^3H]-reoviral genome RNA with both S_1 nuclease ($0.3 \text{ U}/\lambda$) and V_1 nuclease ($1 \times 10^{-4} \text{ U}/\lambda$) (See METHODS).

FIGURE 3. Autoradiogram of partial digests on 5'-³²P-end-labeled yeast tRNA^{Phe} electrophoresed on a 15% polyacrylamide slab gel in 90% formamide. Gel dimensions were 0.40 mm thick x 33 cm wide x 80 cm long. Partial digests contained the following amounts of enzyme per µg of RNA. From left to right: (-) minus enzyme; (S₁)S₁ nuclease, 0.06 U-1 min, 0.06 U-10 min, 0.006 U-1 min, 0.006 U-10 min; (N.E.) Neurospora crassa endonuclease, 5 x 10⁻⁴ U, (V₁)V₁ nuclease, 1 x 10⁻⁵ U-1 min, 1 x 10⁻⁵ U-10 min, 1 x 10⁻⁵ U-10 min + 20 mM EDTA, 1 x 10⁻⁶ U-1 min, 1 x 10⁻⁶ U-10 min; (T₁s)T₁ RNase under nondenaturing conditions, 3 x 10⁻⁵ U, and 3 x 10⁻⁴ U; (H⁺) controlled acid hydrolysis; (T₁)T₁ RNase under denaturing conditions, 0.005 U, 0.0005 U. Bracketed nucleotide sequences at left indicate regions digested by nuclease V₁.

of 5'-³²P-end-labeled yeast tRNA^{Phe}. Double-strand-specific V₁ cleavages are indicated along with single-strand-specific S₁ and T₁ nuclease digestions. V₁ nuclease cleaves all the stem regions of this molecule as summarized in Figure 6A using both 5'- and 3'-³²P-end-labeled RNA. Primary V₁ cleavage sites were determined from digests in which less than 3% of the molecules were cleaved (1 x 10⁻⁶ units/μg RNA for 1 min at 37°C in 20 mM

Tris-HCl pH 7.2, 10 mM MgCl₂, and 0.2 M NaCl). No difference in the cleavage pattern was noted when the Mg⁺⁺ concentration of the incubation mixture was varied from 1-10 mM. However, the addition of 20 mM EDTA at zero time, as shown in Figure 3, resulted in greater than 90% inhibition of the enzyme, indicating that V₁ nuclease requires divalent cations for activity. Since the pH of the reaction mixture when varied from pH 4-9 had relatively little effect on the enzymatic activity, our studies were carried out under essentially physiological conditions. V₁ nuclease like S₁ nuclease generates fragments with 3' hydroxyls and 5'-phosphates at the site of cleavage. We found that a complete ladder of fragments with 3' hydroxyls can easily be generated upon partial digestion of ³²P-end-labeled RNA with Neurospora crassa endonuclease. When loaded onto a lane between both the S₁ and V₁ partial digests, the Neurospora digest facilitates simultaneous interpretation of both single- and double-strand-specific cleavages.

It is apparent from the digestion pattern of yeast tRNA^{Phe} that not all base-paired nucleotides are digested by V₁ nuclease even at longer digestion times. Differences in the intensity of the cleavages also exist. The acceptor stem appears to be asymmetrically cleaved (G₄-A₅ and U₆₉-G₇₂) while more symmetrical cutting occurs within the D stem (C₁₁-C₁₃ and G₂₂-G₂₄), the anti-codon stem (C₂₇-A₂₉ and ψ₃₉-G₄₂), and the Tψ stem (m⁵C₄₉-U₅₀ and A₆₃-G₆₆). The strongest cleavage within the entire yeast tRNA^{Phe} molecule occurs after C₂₈ on the 5' side of the anticodon stem. The nucleotide at this position is not only cleaved strongly within this elongation tRNA, but is, likewise, very accessible in both prokaryotic and eukaryotic initiator tRNAs (see below). One particularly interesting cleavage site is a weak scission after G₄ and a corresponding strong cleavage at U₆₉ in the acceptor stem. Refinement of the electron density map at a resolution of 2.5 angstroms has confirmed the wobble-type of base-pairing between G₄ and U₆₉ (16). Nuclease V₁, therefore, appears to cleave after nucleotides in helical regions which are not hydrogen-bonded by standard Watson-Crick base-pairing. We have found V₁ to also cleave after two nucleotides known to be involved in tertiary interactions. The most profound of which occurs after m²G₂₆ which forms a "propeller" base-pair with A₄₄ (16). A weak cleavage after C₄₈ is also evident. C₄₈ is involved in trans base-pairing with G₁₅ by two hydrogen bonds which appear to stabilize the joining of the D stem with the Tψ stem by stacking (15).

Single-strand-specific cleavages by S₁ nuclease are identical to those previously reported for yeast tRNA^{Phe} by Wurster et al. (5). Particularly

strong cleavage are observed at the -C-C-A end and in the anticodon loop after A₃₅ and A₃₆ within 1 min and U₃₃ after 10 min of digestion at 37°C. Single-strand-specific T₁ RNase cleavages are also the same as previously reported by Wrede et al. (6). Weak cleavages after G₃ and G₄ of the acceptor stem were also noted after long digestion periods. It is quite possible that these scissions are a product of secondary digestion and unrelated to the G₄-U₆₉ wobble base-pair, since nuclease V₁ also cleaves G₄. Alternatively, it is conceivable that a region within a helical domain that is weakly base-paired can be cleaved by both single- and double-strand specific enzymatic probes. We have found G-U wobble base-pairs in *E. coli* 5S RNA similarly cleaved by both S₁ and V₁ nucleases (unpublished experiments).

E. coli tRNA^{Met}_f

Figure 4 shows a structure map of 3'-³²P-end-labeled *E. coli* initiator tRNA^{Met}_f using both single- and double-strand-specific enzymatic probes. Our results summarized in Figure 6B from studies with both 5'- and 3'-³²P-end-labeled RNA indicate that V₁ nuclease again cleaves all stem regions within this tRNA. The acceptor stem is more symmetrically cleaved (G₂-G₅ and C₆₆-C₇₁) than in yeast tRNA^{Phe}, with a preponderance of strong scissions on the C-rich 3'-end strand. A marked reduction in cleavage is observed in the D stem (A₁₁-G₁₂ and C₂₃-U₂₄) and also within the Tψ stem (C₅₁-G₅₂ and C₆₁-C₆₂) both in the number and intensity of cleavage sites, possibly due to a more compact structure than seen in yeast tRNA^{Phe} (19). Cleavage of the anticodon stem (U₂₇-G₂₉) and (C₄₀-A₄₃) was, however, strikingly similar to that of yeast tRNA^{Phe} with a strong scission at C₂₈ (the numbering system according to Gauss et al. (20)). Since the three-dimensional structure of *E. coli* tRNA^{Met}_f has been determined from X-ray crystallographic studies to a level of only 3.5 angstroms (19), precise identification of the tertiary interactions cannot yet be made. Neither G₂₆ nor C₄₈ was found to be cleaved, possibly suggesting some differences from yeast tRNA^{Phe} in both the hydrogen bonding and stacking interactions associated with these nucleotides.

Single-strand-specific cleavages by S₁ nuclease are essentially identical to those previously reported by Wrede and Rich (8). Only two strong scissions are observed after C₃₄ and A₃₅ of the anticodon loop, different from the more extensive S₁ digestion pattern observed within the anticodon loops of elongation tRNAs (7). Longer digestion times result in weak cleavages after U₃₆ as well as C₅₆ and A₅₇ of the Tψ loop. Two strong single-strand-specific T₁ RNase cleavages are detected after G₁₈ and G₁₉ in the D loop.

FIGURE 4. Autoradiogram of partial digests on 3'-³²P-end-labeled *E. coli* tRNA^{Met} electrophoresed on a 15% polyacrylamide slab gel in 90% formamide. Gel dimensions were 0.40 mm thick x 33 cm wide x 80 cm long. Partial digestion conditions were the same as in Fig.3. Bracketed nucleotide sequences at left indicate regions digested by nuclease V1.

Human tRNA^{Met}

Figure 5 shows a structure map of 3'-³²P-end-labeled human placental initiator tRNA^{Met} with both single- and double-strand-specific probes. A summary of all cleavage sites using both 5'- and 3'-³²P-end-labeled RNA is shown in Figure 6C. The V₁ digestion pattern shows similarities with both yeast tRNA^{Phe} and *E. coli* tRNA^{Met} in cutting within all stems. The acceptor

FIGURE 5. Autoradiogram of partial digests on 3'-³²P-end-labeled human tRNA^{Met} electrophoresed on a 15% polyacrylamide slab gel in 90% formamide. Gel dimensions were 0.40 mm thick x 33 cm wide x 80 cm long. Partial digestion conditions were the same as in Fig.3. Bracketed nucleotide sequences at left indicate regions digested by nuclease V₁.

stem is asymmetrically cleaved (A₄-G₅ and U₆₇-C₇₁) as in yeast tRNA^{Phe}. Limited digestion, however, occurs within the Tψ stem (A₅₀-G₅₂ and A₆₃-C₆₅) similar to that of *E. coli* tRNA^{Met}, while the intensity and number of cleavages is greater within the D-stem (C₁₁-C₁₃ and C₂₃-G₂₄). Again, cleavage of the anticodon stem (C₂₇-G₂₉ and C₄₀-G₄₃) is strikingly similar in all three of these tRNAs with nucleotide #28 again being the strongest cleavage in the anticodon stem. As with *E. coli* tRNA^{Met}, no cleavage of unpaired nucleotides is observed. Neither m²G₂₆ nor m⁵C₄₈ is cleaved, possibly suggesting similarities with *E. coli* tRNA^{Met} in both the hydrogen bonding and stacking interactions associated with these nucleotides. As with the prokaryotic ini-

tiator tRNA^{Met}, two relatively strong cleavages with S₁ nuclease are observed after C₃₄ and A₃₅ in the anticodon loop, with a much weaker scission at U₃₆ only after longer digestion times. Strong T₁ RNase cleavages are detected after G₁₅, G₁₈ and G₁₉ with a weaker scission after G₅₇ (7).

DISCUSSION

In conjunction with the structure mapping procedure of Wurst et al. (5), we show how one can definitively localize all base-paired nucleotides in a ³²P-end-labeled tRNA molecule by partial digestion with double-strand-specific ribonuclease V₁ from cobra venom. Using tRNA substrates of known three-dimensional structure, we have found V₁ nuclease to cleave within all base-paired stems. It is apparent from digestion patterns of these tRNAs that not all base-paired nucleotides are cleaved, and that variation in the intensity of cleavages within a stem also prevails. For example, as summarized in Figure 6 the acceptor stems of both yeast tRNA^{Phe} and human tRNA^{Met} are asymmetrically digested, while more symmetrical cleavage in both strands of this stem occurs with *E. coli* tRNA^{Met}. Also, some cleavage sites are so weak, e.g., C₆₁-C₆₂ of *E. coli* tRNA^{Met} and A₆₃-C₆₅ of human tRNA^{Met}, that they are likely due to secondary cutting and are only discernible after relatively long exposures of the polyacrylamide gels. It is presently unclear whether such variations are due in part to differences in accessibility of regions of these helical domains in these tRNAs, or are due to the yet undefined sequence and precise structural requirements of this enzyme. Only after examining a wide spectrum of substrates will we be able to distinguish more clearly between these alternatives.

A consistently strong cleavage in all three tRNAs studied here is nucleotide #28 on the 5'-side of the anticodon stem irregardless of what base is present. In yeast tRNA^{Met} we have found A₂₈, likewise, to be a very strong cleavage in this molecule. The very similar cleavage pattern in all anticodon stems examined thus far, suggests that both eukaryotic elongation and initiator tRNAs, as well as prokaryotic initiators, might have a similar helical structure in this region. Why nucleotide #28 in these tRNAs is so readily recognized and cleaved by V₁ is possibly related to the precise structural specificity of the enzyme.

It has become increasingly clear from the tRNA substrates studied thus far, that V₁ nuclease unlike the previously characterized "cobra venom ribonuclease" (9), has the ability to cleave nucleotides which are not hydrogen-bonded by standard Watson-Crick base-pairing. For example, the wobble-base-

FIGURE 6. Complete structure maps of (A) yeast tRNA^{Phe}, (B) *E. coli* tRNA^{Met}, and (C) human tRNA^{Met} using both single-strand-specific nucleases S_1 and T_1 , and double-strand-specific V_1 nuclease as structure probes.

pair G₄-U₆₉ in the acceptor stem of yeast tRNA^{Phe} is readily recognized and cleaved. Likewise, we have found wobble base-pairs in *E. coli* 5S RNA cleaved by V₁ (unpublished data). More interesting, is the cleavage of two nucleotides within yeast tRNA^{Phe} which are known to be involved in tertiary interactions. m²G₂₆, which forms the "propeller" base-pair with A₄₄ (16), is easily digested by V₁ in both yeast tRNA^{Phe} and yeast tRNA^{Met} (unpublished experiments). However, G₂₆ of *E. coli* tRNA^{Met} and m²G₂₆ of human tRNA^{Met} are not cut. C₄₈ which forms a trans base-pair with G₁₅ in yeast tRNA^{Phe} is also cleaved, but not within any other tRNA currently examined. Whether these nuances are a reflection of subtle conformational differences between the tRNA molecules is difficult to ascertain as yet.

The method reported here for electrophoretic fractionation of enzymatic digests on thin polyacrylamide gels run in 90% formamide, rather than the more commonly used denaturant 8M urea, has many advantages. Not only will 90% formamide completely denature and allow resolution of V₁ generated fragments from highly stable base-paired RNA helices, but also will eliminate band contraction during sequence analysis of very G:C rich DNA restriction fragments (unpublished results), underscoring the general application of this method in both RNA and DNA sequence analysis. It is noteworthy that gels less than 10% in polyacrylamide appear not to polymerize adequately in 90% formamide and as a result give poor resolution of very large RNA and DNA fragments.

Finally, the method described here for localizing base-paired nucleotides within an RNA molecule in solution, is general and can complement other procedures currently available (21-24). This method, however, does not allow identification of which nucleotides are base-paired to which nucleotides, for which either psoralen crosslinking (22), or direct isolation of base-paired regions might be used (24). However, in conjunction with these other procedures, can be a powerful tool to ultimately define the architecture of important biological molecules in solution.

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