Mass spectrometric analysis of 3’-terminal nucleosides of non-coding RNAs

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Method Article

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Abstract

Introduction

Non-coding RNAs \(ncRNAs) have emerged as important regulatory elements of gene expression in a wide variety of biological processes. RNA molecules mature through various post-transcriptional processing events in a spatiotemporal manner. Post-transcriptional modifications \(or RNA editing) are characteristic structural features of RNA molecules and are required for their proper functioning. Most of these modifications have been found in abundant RNA molecules, such as tRNAs, rRNAs and/or UsnRNAs. It has recently been reported that even small RNAs are modified. In mammals, certain populations of miRNAs \(~6%) contain inosine \(I)[1,2]. In plants, the 3'-termini of miRNAs are modified by 2'methylation[3], and this modification is required for normal maturation of miRNAs[4]. Recently, we found that mouse Piwi-interacting RNAs \(piRNAs) also have 3'-terminal 2'methylations[5]. Direct analysis of RNA molecules by mass spectrometry provides qualitative information about the nature of the modifications embedded in RNA molecules, such as base modifications and terminal chemical structures. Here, we describe a detailed protocol for the mass spectrometric analysis of the 3' termini in ncRNAs.

Reagents

1) Ribonuclease T_2 \(RNase T_2) \(Invitrogen, Cat. No. 18031-013)) 2) Distilled water, HPLC grade \(Wako Pure Chemical Industries, Cat. No. 042-16973) 3) Ammonium acetate, HPLC grade \(J. T. Baker, Cat. No. 0599-08) 4) Acetic acid, LC/MS grade \(Wako Pure Chemical Industries, Cat. No. 018-20061) **CAUTION** This reagent is corrosive and flammable. Handle with caution according to MSDS instructions. 5) Acetonitrile, HPLC grade \(Nacalai Tesque, Cat. No. 00430-83) **CAUTION** This reagent is toxic and flammable. Handle with caution according to MSDS instructions. 6) Yeast $tRNA^{Phe}$ \(Sigma, Cat. No. R4018). This tRNA molecule is used as a positive control in the method described below. 7) Mouse piRNAs is obtained from mouse testes as described[5]. Mouse piRNAs have 2'--nucleosides \(Nm) at their 3'-termini. h3. Reagent set up For dissolving or diluting reagents, only use the distilled water mentioned in the Reagent section. 1) 500 mM ammonium acetate buffer \(pH 5.3); Prepare by titrating ammonium acetate solution with acetic acid. 2) HPLC Solvent A, 5 mM ammonium acetate \(pH 5.3); Prepare by diluting with the buffer mentioned in 1). 3) HPLC Solvent B, 60 % acetonitrile \(v/v). After preparing the solutions, degas them for 15 min.

Equipment

1) LCQ^{Duo} ion-trap mass spectrometer \(Thermo Fisher Scientific) equipped with an electrospray ionization \(ESI) source. 2) HP1100 liquid chromatography system \(Agilent Technologies) 3) Inertsil ODS-3 column \(5 µm particle, 2.1 x 250 mm, GL Science) 4) Guard column cartridge \(ODS-3, 3 x 10 mm, GL Science) h3. Equipment setup 1) The HP 1100 system is run with a UV detector set to 254 nm \(absorbance), a flow rate of 150 µl/min, and a multistep linear gradient sequence with the following

parameters; 1-35% B from 0-35 min, 35-99% B from 35-40 min, 99% B from 40-50 min, 99-1% B from 50-50.1 min and 1% B from 50.1-60 min. 2) An ODS-3 column with a guard column cartridge is equilibrated with 1% B after washing the column once or twice with the gradient cycle described above. 3) LCQ^{Duo} is set with a scan range covering an _m/z_ of 103-900 in positive polarity mode. The parameters of the LCQ^{Duo} are adjusted by using an appropriate sample according to the manufacturer's instructions. We have regularly described and updated the conditions for the liquid chromatography / mass spectrometry \(LC/MS) system in our recent publications[6-8]. Therefore, we recommend referring to them for more detailed information before performing this analysis.

Procedure

The following procedure describes the determination of 3' terminal nucleosides in yeast tRNA^{Phe} and mouse piRNAs as described previously[5]. This method can be applied to any RNA molecule that does not possess a 3'-phosphate at its 3' terminus. **RNA hydrolysis with RNase T_2** 1) Dissolve >0.8 µg of yeast tRNA^{Phe} or mouse piRNAs in 13.4 µl of Milli-Q water. 2) Add 1.6 µl of 500 mM ammonium acetate buffer \(pH 5.3) and mix gently 3) Add 5 µl of 5 units/ml RNase T_2 and mix gently 4) Incubate the mixture at 37 °C for 3 hours. **LC/MS analysis of the hydrolysate** 5) Analyze the hydrolysate by using the LC/MS system

Timing

3 hours for RNA hydrolysis \(steps 1-4) 1 hour for LC/MS measurements \(step 5)

Anticipated Results

RNase T_2 is known to cleave the 3' side of phosphodiester bonds in RNA molecules. Since mature tRNA and piRNA molecules possess 5'-monophosphate and 3'-hydroxy groups, RNase T_2 hydrolysates consist of 3',5'-diphosphonucleotides derived from 5' terminal residues \(pNp in schemes 1 and 2), 3'-monophosphonucleotides derived from internal residues \(Np in scheme 1 and 2) and nucleosides derived from 3' terminal residues \(N_{OH} in scheme 1, Nm in scheme 2). Thus, only 3'-terminal residues are converted to nucleosides in this analysis. Nucleosides can be easily discriminated from various nucleotides because of their characteristic molecular weights. See figure in Figures section. As shown in Figure 1, RNase T_2 hydrolysates of yeast tRNA^{Phe} yield four major mononucleotides \(Cp, Up, Gp and Ap) and six minor modified nucleotides \(Dp, Ψp, m^5Cp, m^7Gp, m^5Up and m^2Gp)[7]. It has been noted that certain modifications produce dinucleotides because of their resistance to RNase T_2 treatment \(CmpUp, GmpAp and m^2_2GpCp). Some internal modified nucleotides and 5' terminal nucleotide were not observed under the conditions mentioned above. The 3'-terminal adenosine \(A) was detected as a unique nucleoside in this analysis. In the case of mouse piRNAs, four kinds of 2'–methyl nucleosides \(Cm, Um, Gm and Am) were clearly detected as described previously[5].

References

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Figures

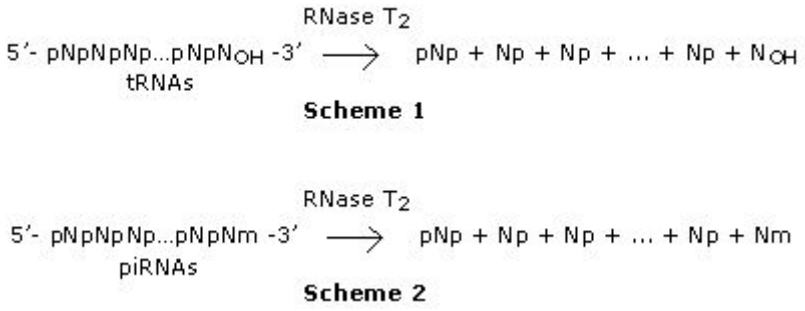

Figure 1

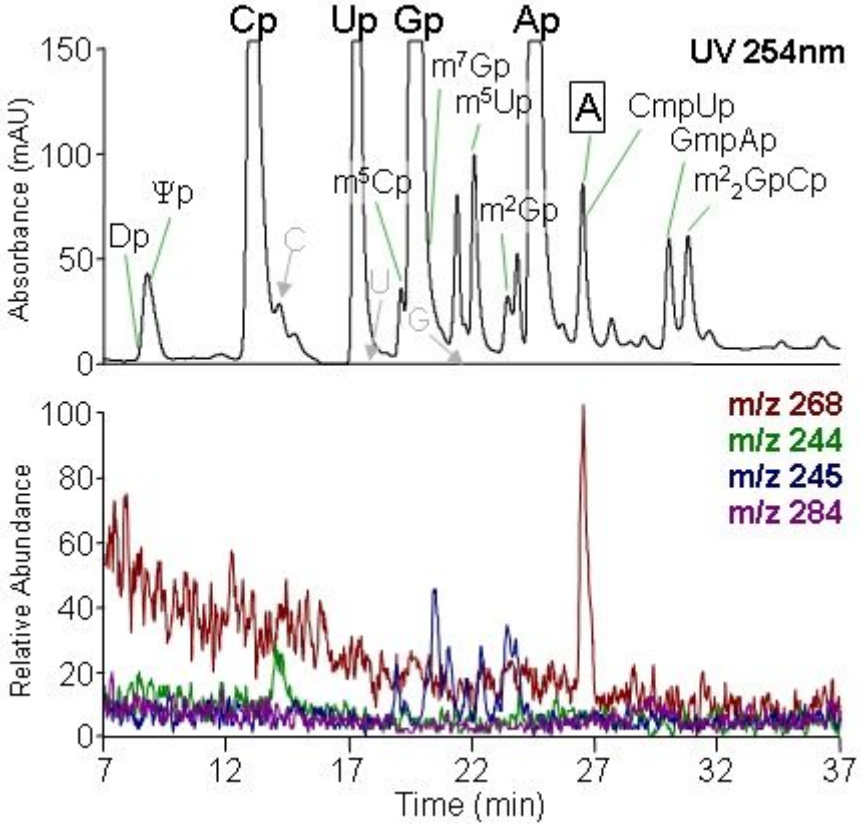

Figure 2

Figure 1 LC/MS analysis of RNase T~2~ digests of yeast tRNA^Phe^ _Top panel_: The UV chromatogram (absorbance at 254 nm) showing the elution profiles of internal nucleotides and 3’ terminal nucleosides. Modifications are represented as follows; dihydrouridine (D), pseudouridine (Ψ), 5-methylcytidine (m^5^C), 7-methylguanosine (m^7^G), 5-methyluridine (m^5^U), N^2^-methylguanosine (m^2^G), 2'-O-methylcytidine (Cm), 2'-O-methylguanosine (Gm) and N^2^,N^2^-dimethylguanosine (m^2^~2~G). “p” stands for a phosphate group. The predicted elution positions of C, U and G nucleosides are indicated by arrows. Other non-labeled peaks remain unidentified. _Bottom panel_: Mass chromatograms for proton adducts of A (_m/z_ 268, brown line), C (_m/z_ 244, green line), U (_m/z_ 245, navy line) and G (_m/z_ 284, purple line).