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RNA secondary structure by chemical probing methods using DMS and CMCT

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Abstract

RNA folds into secondary structures that can serve in understanding various RNA functions **(1)**. Chemical probing is a method that enables the characterization of RNA secondary structures using chemical reagents that specifically modify RNA nucleotides that are located in single-stranded areas. In our protocol, we used Dimethyl Sulfate (DMS) and Cyclohexyl-3-(2-Morpholinoethyl) Carbodiimide metho-p-Toluene sulfonate (CMCT) that are both base-specific modifying reagents **(2)**. These modifications are mapped by primer extension arrests using 5' fluorescently labelled primers. In this protocol, we show a comprehensive method to identify RNA secondary structures *in vitro* using fluorescently labelled oligos. To demonstrate the efficiency of the method, we give an example of a structure we have designed which corresponds to a part of the 5' UTR regulatory element called Translation Inhibitory element (TIE) from Hox a3 mRNA **(3)**.

1. Introduction

RNA probing enables the characterization of RNA secondary structures by using specific ribonucleases or chemical reagents. Here, we focus on probing using chemical reagents. In fact, exhaustive study of RNA structures requires the knowledge of the reactivity of the four bases. This is essentially enabled using two chemical reagents, DMS and CMCT. DMS modifies unpaired A and C nucleotides at N1 and N3 respectively while CMCT modifies unpaired G and U nucleotides at N1 and N3 respectively (Fig. 1). Historically, these techniques have been set up with ³²P-radio-labelled DNA primers that were used for primer extension. The

detection of Reverse Transcriptase (RT) arrests (induced by the modification sites) was achieved by PAGE and phosphorimaging.

In this protocol, we describe technical developments of this approach. First, instead of using radioactive primers, we used 5' fluorescently labelled primers for reverse transcription (Fig. 2A). Second, the detection of the modification sites is achieved by capillary electrophoresis rather than classical PAGE analysis. With this novel approach, the detection and the quantification of the RT arrests are faster and more accurate. This allows the identification of nucleotides that are accessible as part of single-stranded regions or inaccessible when they are involved in double-stranded regions (Fig. 2B). We present here a detailed protocol to probe the RNA secondary structures of RNA transcripts. We used this protocol to probe a part of the 5' UTR regulatory element called Translation Inhibitory Element (TIE), which is known to inhibit cap-dependent translation (3). Regulatory elements in the 5' UTR can adopt secondary structures that are not unwound by classical helicases and thereby induce translation inhibition (4). To get further insight into the translation inhibition by TIE from Hox a3 mRNA, which is a 170 nucleotide-long element, we were interested in deciphering its secondary structure using our novel probing protocol.

2. Materials

Prepare buffers with autoclaved milliQ water at room temperature and keep them at -20°C for long-term storage (*see Notes 1 and 2*).

- TMSDT buffer 7X : 40 mM Tris-HCl (pH 8.1), 20 mM MgCl₂, 5 mM DTT (dithiothreitol), 0.01 % Triton-X100, 1 mM spermidine.
- DMS buffer 5X: 50 mM Na Cacodylate pH 7.5, 5 mM MgCl₂ and 100 mM KCl.
- CMCT buffer 5X: 50 mM Na borate, 5 mM MgCl₂, 100 mM KCl.
- AMV RT buffer 10X (Promega®): 100 mM Tris-HCl (pH 9.0), 500 mM KCl, 1% Triton® X-100.
- Two sets of 5' fluorescently labelled primers with Vic and Ned dyes.
- A 96-well sequencing plate for Applied Biosystems 3130xl genetic analyser.

3. Methods

3.1 In vitro transcription of RNA

Resuspend dry DNA pellet used as transcription template in 20 µl milliQ water. Add 13.4 µl TMSDT buffer 7X, 10 µl of each NTP (5 mM), 40 Units of RNase inhibitor (RNasin) Promega®, 0,2 mg/ml of T7 RNA polymerase and milliQ water until final volume is 100 µl.

1. Incubate the reaction mixture at 37°C for 1 h.
2. Add 2 µl of pyrophosphatase (Roche®) and Incubate at 37°C for 30 min.
3. Add 2 µl of DNase I RNase-free (New England Biolabs®) and incubate for 1 h at 37°C.
4. To check RNA integrity, mix an aliquot (0.5 µl) of transcription product with 4.5 µl Formamide Dye and load on 4% denaturing polyacrylamide gel.
5. To eliminate unincorporated nucleotides, load the remaining of RNA

sample on a gel filtration Sephadex G25-column (Pharmacia Fine Chemicals).

6. Perform phenol-chloroform extraction on retained sample (200 μ l) and precipitate with 0.25 M of NaCl and 3 volumes of ethanol 100%.
7. Store the sample overnight at -20°C.
8. Centrifuge the sample at 13,000 G for 10 min. Discard supernatant. Wash the pellet with 500 μ l ethanol 80%. Centrifuge and dry the pellet.
9. Resuspend the pellet in 20 μ l milliQ water.

3.2 Modification by DMS or CMCT

Modifications by DMS or CMCT are performed on 2 pmoles of a3 TIE RNA. For such type of probing, two sets of samples (+) and (-) are considered for the RNA of interest. The (+) set corresponds to the RNA incubated in the presence of DMS/CMCT modification reagent, in this RNA sample the accessible nucleotides are modified and the (-) set contains the same RNA without modifications, The comparison of (+) and (-) will enable the detection of reverse transcription stops that are specifically induced by the chemical reagent. Triplicates of each set are considered for significant results.

Modification by DMS

1. For both sets, add 2 pmoles of RNA in 4 μ L of DMS buffer 5X and 1 μ g of yeast total tRNA (1 mg/mL). Add water until 18 μ L.
2. Incubate tubes for 10 min at 20°C.
3. Add 1.25% of DMS (diluted in ethanol 100%) to the (+) set and equivalent volume of water to the (-) set (*see Note 3*). Total reaction volume should be

20 μ l.

4. Incubate 10 min at 20°C (*see Note 4*).
5. Place tubes on ice.

Modification by CMCT

1. For both (+) and (-) sets, add 2 pmoles of RNA in 4 μ L of CMCT buffer 5X and 1 μ g of yeast total tRNA. Add water until 15 μ L.
2. Incubate tubes for 10 min at 20°C.
3. Add 5 μ L of CMCT at 42 mg/mL (final concentration is 10.5 mg/mL) to the (+) set and equivalent volume of water to (-) set. Total reaction volume is 20 μ l (*see Note 3*).
4. Incubate 15-20 min at 20°C (*see Note 4*).
5. Place tubes on ice.

After modification by DMS or CMCT:

6. Add 80 μ L of water and precipitate samples with 0.25 M NaCl, 0.1 mg/mL glycogen and 3 volumes of ethanol 100%.
7. Store the samples overnight at -20°C.
8. Centrifuge samples at 13,000xg for 10 min at 4°C.
9. Wash the samples with ethanol 80% and dry the pellet.
10. Resuspend the pellet in 7 μ L water.

3.3 Primer extension using fluorescent primers

Primer extension is carried out in 20 μ L reaction volume with 2 pmoles of RNA (modified (+) or not (-)) and 0.9 pmoles of 5' fluorescently labelled primers. We

use Vic and Ned primers of same sequence for all primer extension reactions on a3 TIE RNA, which are complementary of the 5'UTR β -globin from nts 6 to 37:

5' GGTTGCTAGTGAACACAGTTGTGTCAGAAGC 3'.

1. Add 1 μ L of Vic-labelled fluorescent primers at 2 μ M to both sets. After this step, all manipulations should be carried out in a darker zone (*see Note 5*).
2. Incubate for 2 min at 95°C (denaturation), 2 min at 65°C (annealing) then keep on ice for 2 min.
3. Add 2 μ L of AMV RT buffer 10X.
4. Incubate 10 min at 20°C.

- In the meantime, prepare a master mix depending on the number of reactions (*see Note 6*).

5. Primer extension of one sample is performed in a mix containing: 2 μ L of AMV RT buffer 10X (Promega®), 0.75 mM of each of the four deoxynucleotides (dNTP) and 1 unit of Avian Myoblastosis Virus reverse transcriptase (AMV RT) (Promega®). Add water till 10 μ L.
6. Add 10 μ L of the mix to the (+) and (-) tubes.
7. Transfer the mixture to PCR tubes.
8. Place the tubes in a thermocycler and use the following program: 42°C for 2 min, then 50°C for 30 min and finally 65°C for 5 min.

- In parallel, perform sequencing reactions:

For each set of (+) and (-) tubes, one sequencing reaction is required.

1. Use 4 pmoles of RNA in a total of 14 μ L.
2. Add 2 μ L of Ned-labelled fluorescent primers at 2 μ M to sequencing tubes. After this step, all manipulations should be carried out in a darker zone (*see Note 5*).

3. Incubate for 2 min at 95°C (denaturation), 2 min at 65°C (annealing) then keep on ice for 2 min.
 4. Add 4 µl of AMV RT buffer 10X.
 5. Incubate 10 min at 20°C.
- Prepare master mix depending on the number of sequencing reactions required (*see Note 6*).
6. One sequencing reaction is performed using a mix of 24 µl containing: 4 µL of AMV RT buffer 10X, 12 µL of pre-mixed deoxyribonucleosides triphosphates T10 mix containing 0.25 µM of dTTP (*see Note 7*) and 1 µM of each of dCTP, dATP and dGTP, 4 µL of dideoxythymidine triphosphate (ddTTP) at 100 µM, and 1 unit of AMV RT (Promega®). Add water until 24 µL.
 7. Add the 24 µL mix to each sequencing tube. Total volume should be 44 µL.
 8. Transfer the mixture to PCR tubes.
 9. Incubate, as previously mentioned, at: 42°C for 2 min, then 50°C for 30 min and finally 65°C for 5 min.
 10. After reverse transcription, keep samples on ice.
 11. Split half of the same sequencing tube (22 µL) in the (+) and half in the (-) set. Add water until 100 µL and perform two-step phenol-chloroform extraction on the synthesized cDNA.
 12. Precipitate in 0.25 M NaCl and 3 volumes of ethanol 100% and store overnight at -20°C.
 13. Centrifuge at 13,000xg for 10 min at 4°C.
 14. Wash with ethanol 80%, dry the pellet and resuspend in 10 µL highly deionized formamide dye (*see Note 8*).
 15. Incubate at 95°C for 2 min then on ice for 2 min.

16. Centrifuge at 13,000xg for 30 min.

3.4 Capillary electrophoresis and reactivity analysis

The samples were loaded on a 96-well sequencing plate on an Applied Biosystems 3130xl genetic analyser and run for around 1 hr.

The resulting electropherograms were analysed using QuSHAPE software **(5)**, which aligns signal within and across capillaries, as well as to the dideoxy references of nucleotide at specific position and corrects for signal decay (Fig. 3A). The surface of the peaks is integrated with the corresponding background signal and sequencing panels.

Normalized reactivities for each nucleotide range from 0 to 2, with 1 to 2 being the range of highly reactive nucleotides. After obtaining reactivity values from three independent experiments, we calculated the average of reactivity for each nucleotide in TIE a3 sequence (Fig. 3B). The secondary structure model prediction was initiated using Mfold **(6)** to find putative base pairs and then edited and refined according to our reactivity values. As an example, we show a part of our model, which encompasses a long 5' proximal stem-loop structure spanning nucleotides 1-60 (Fig. 4). With this model, we were able to get further insights into the translational inhibition by TIE a3 and discover the structure-function interplay of such 5' UTR regulatory element.

3.5 Notes

1. Avoid performing the modifications with more than two sets of tubes (maximum of 6), since most steps require fast manipulations and transfer of tubes.
2. Before starting the experiment, defreeze all reagents and buffers.
3. Work with DMS and CMCT under the hood and prepare them fresh each time at room temperature.
4. Adjust the incubation time with DMS/CMCT to the length of the RNA of interest. For very long RNA, incubate longer (up to 20 min).
5. Upon addition of fluorescently labelled primers, all tubes should be kept in dark from this step on, due to light sensitivity. For easier manipulations, place the tubes in a box and work in a darker zone. When incubating them at a certain temperature, place a cover above them.
6. Master mixes for the primer extension step are highly preferred, to avoid pipetting errors.
7. The choice of ddNTP as a dideoxy reference is adapted to the abundance of certain nucleotides among the sequence of the RNA of interest. In the case of A-rich RNA, ddTTP is the best choice for sequencing.
8. Resuspend the cDNA pellet with the deionized formamide dye under the hood.

Acknowledgments

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References

(1) Weeks K. M. (2010). Advances in RNA structure analysis by chemical probing. *Current opinion in structural biology*, 20(3), 295–304

(2) Behm-Ansmant, I., Helm, M., & Motorin, Y. (2011). Use of specific chemical reagents for detection of modified nucleotides in RNA. *Journal of nucleic acids*, 2011, 408053.

(3) Xue, S., Tian, S., Fujii, K., Kladwang, W., Das, R., & Barna, M. (2015). RNA regulons in Hox 5' UTRs confer ribosome specificity to gene regulation. *Nature*, 517(7532), 33–38.

(4) Pestova, T. V., & Kolupaeva, V. G. (2002). The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. *Genes & development*, 16(22), 2906–2922.

(5) Karabiber, F., McGinnis, J. L., Favorov, O. V., & Weeks, K. M. (2013). QuShape: rapid, accurate, and best-practices quantification of nucleic acid probing information, resolved by capillary electrophoresis. *RNA (New York, N.Y.)*, 19(1), 63–73.

(6) Zuker M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic acids research*, 31(13), 3406–3415.

Figure legends

Figure 1: Site of base-specific modifications by DMS and CMCT. The red arrows refer to the N atom, which is modified by the reagents. To the left, DMS specifically modifies N₁ of adenine and N₃ of cytidine. To the right, CMCT modifies N₁ of guanine and N₃ of uracil.

Figure 2: Principle of detection of the modification site by reverse transcription. A. Detection of RNA modifications by RT. To probe TIE element of Hox a3, we used a construct with TIE and 5' UTR of β -globin. Using a 5' fluorescently labelled Vic/Ned primer complementary to the 5' UTR of β -globin, we performed reverse transcription. This reverse transcriptase is stopped by the presence of a modified nucleotide and induces an RT arrest that will generate a cDNA. The position of the modification is mapped by determination of the lengths of the synthesized cDNAs. **B. Examples of modifications in a stem-loop structure.** To the left, DMS modifies accessible As and Cs involved located in single-stranded regions. To the right, CMCT modifies accessible Gs and Us located in single-stranded regions. The strength of reactivity depends on the position of accessible nucleotides.

Figure 3: Analysis of electrophoregrams by QuSHAPE software. A. The electrophoregram includes four panels. The red panel represents reactivity of bases in the (+) set of RNA, which is modified by DMS and the blue panel represents the reactivities of unmodified nucleotides in the (-) set of RNA which corresponds the background reactivity. The green and violet panels are duplicates of sequencing reactions in the (+) and (-) sets respectively and correspond to A nucleotides (in this case, ddTTP was used as dideoxy reference). The software calculates a reactivity value and background signal value for every nucleotide at specific position defined in the sequencing panel. **B. Example of the average of reactivities by DMS in a subdomain of TIE a3.** The averages of DMS reactivities for each nucleotide (1-60) in TIE a3 were calculated from three independent experiments.

Figure 4: Subdomain of the secondary structure model of TIE element from Hox a3. The 5' proximal stem-loop structure was modelled based on the modification patterns obtained with DMS and CMCT. The reactivities are shown as average reactivities from three independent experiments. A representation of reactivities is assigned and represented according to a color code depending on a range of values as shown in the figure legend on the right.

Figure 1

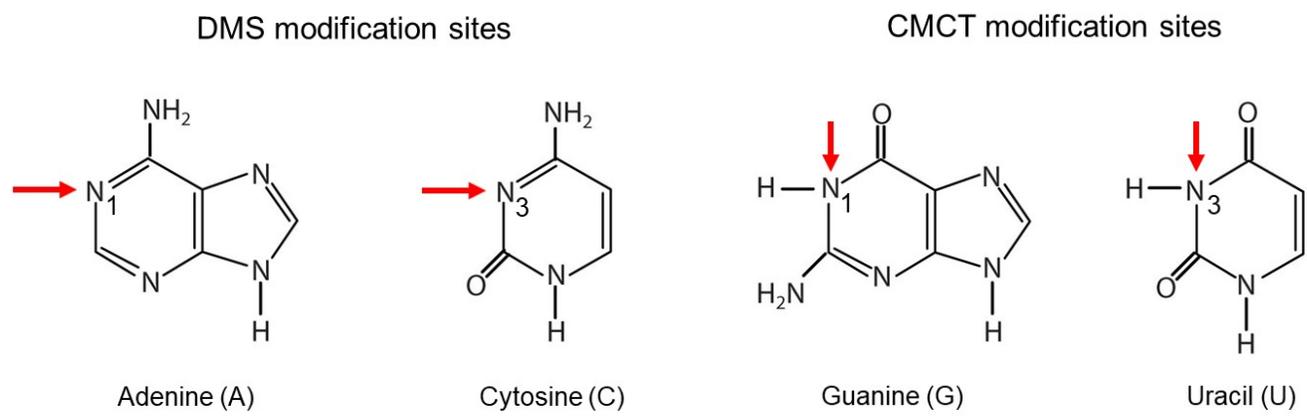
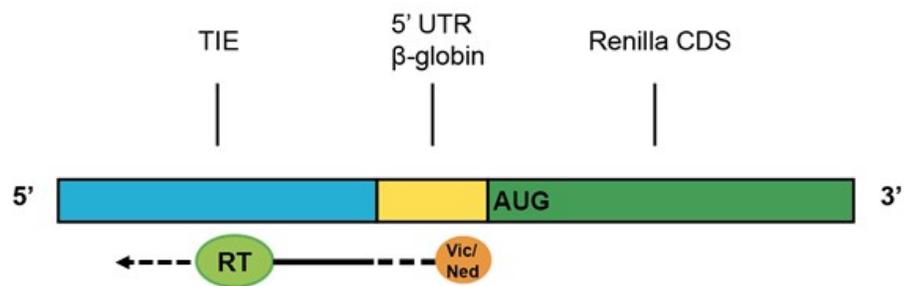


Figure 2

A.



B.

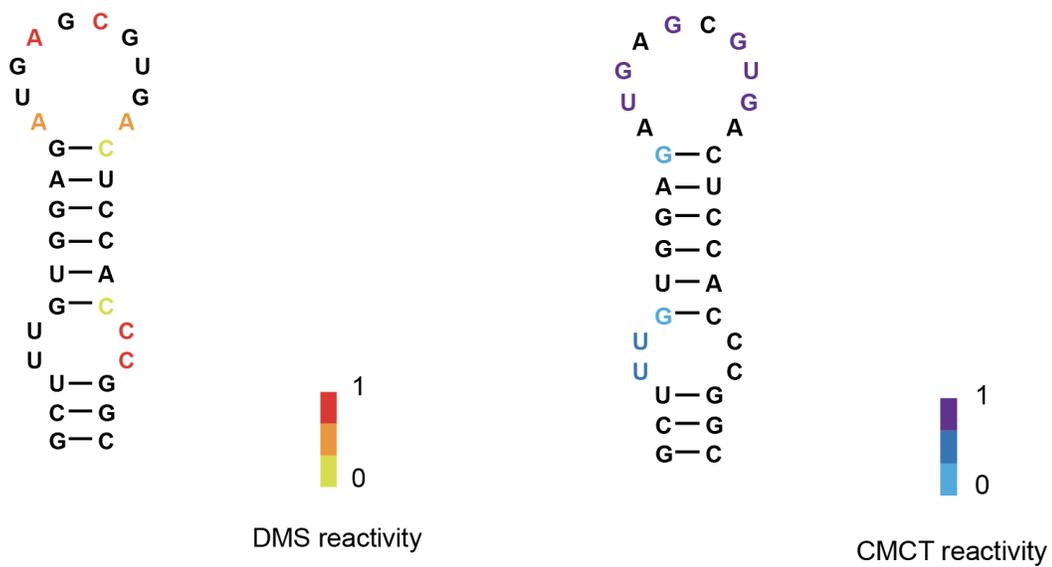


Figure 4

