RTL-P: a sensitive approach for detecting sites of 2'-O-methylation in RNA molecules

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ABSTRACT

2'-O-methylation is present within various cellular RNAs and is essential to RNA biogenesis and functionality. Several methods have been developed for the identification and localization of 2'-O-methylated sites in RNAs; however, the detection of RNA modifications, especially in low-abundance RNAs and small non-coding RNAs with a 2'-O-methylation at the 3'-end, remains a difficult task. Here, we introduce a new method to detect 2'-O-methylated sites in diverse RNA species, referred to as RTL-P [Reverse Transcription at Low deoxyribonucleoside triphosphate (dNTP) concentrations followed by polymerase chain reaction (PCR)] that demonstrates precise mapping and superior sensitivity compared with previous techniques. The main procedures of RTL-P include a site-specific primer extension by reverse transcriptase at a low dNTP concentration and a semi-quantitative PCR amplification step. No radiolabeled or fluorescent primers are required. By designing specific RT primers, we used RTL-P to detect both previously identified and novel 2'-O-methylated sites in human and yeast ribosomal RNAs (rRNAs), as well as mouse piwi-interacting RNAs (piRNAs). These results demonstrate the powerful application of RTL-P for the systematic analysis of fully or partially methylated residues in diverse RNA species, including low-abundance RNAs or small non-coding RNAs such as piRNAs and microRNAs (miRNAs).

INTRODUCTION

The presence of post-transcriptional RNA modifications, which are catalyzed by numerous specific enzymes or ribonucleoprotein (RNP) particles, is a characteristic feature of most cellular RNAs (1). In ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs), 2'-O-methylation of the backbone ribose is the most common and conserved type of RNA modification (2). Human rRNAs contain approximately 105 2'-O-methylated sites (3), which are predominantly directed by box C/D small nucleolar RNAs (snoRNAs) through 1 or 2 10–21 nt antisense elements that base pair to the specific region of the rRNA (2). Most 2'-O-methylated sites are clustered around functionally important regions of rRNAs and may influence ribosome structure and function (4). Ribose 2'-O-methylation also occurs within the cap structure of mRNAs and provides a molecular signature for the distinction of self versus non-self mRNA by the RNA sensor Mda5 (5). Recently, several lines of evidence have shown that small RNAs, including piwi-interacting RNAs (piRNAs), endogenous small interfering RNAs (endo-siRNAs) and plant microRNAs (miRNAs), are 2'-O-methylated at their 3'-ends by methyltransferases (6). The 3'-terminal methylation in the small RNAs protects the molecules from uridylation (7) and degradation by exonucleases (8) and may also regulate specific RNA interference (RNAi) pathways. The detection of 2'-O-methylated nucleotides and the mechanistic study of this post-transcriptional modification are important for the understanding of RNA biogenesis and function as well as the mechanisms regulating gene expression.

Many biochemical approaches have been developed to detect and analyze nucleotide modifications including 2'-O-methylation. Liquid chromatography coupled with mass spectrometry (LC/MS) (9) and two-dimensional thin-layer chromatography (2D-TLC) approaches (10,11) can yield extensive information regarding the modifications in a given RNA; however, the procedures are complex, labor intensive and usually require a large amount of starting material or the purification of an individual RNA to a homogeneous state. An alternative to

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these techniques is primer extension by reverse transcriptase, which does not require RNA purification, as specificity for the RNA of interest is determined by annealing specific oligonucleotide DNA primers. Two types of methods based on reverse transcription (RT) have also been developed to study RNA modifications. The first exploits the tendency of 2'-O-methyl groups to impede reverse transcriptase at a low dNTP concentration, and the second utilizes the resistance of the phosphodiester bonds adjacent to the 2'-O-methyl groups to alkaline hydrolysis (3). These traditional RT-based methods are easily preformed but are laborious and time-consuming due to the required radioactive labeling, polyacrylamide gel electrophoresis and sequencing reaction steps. In some cases, a large amount of total RNA is also needed for primer extension due to the low sensitivity of the method. Recently, the detection sensitivity of RT-based methods has been improved by using fluorescent primers followed by capillary electrophoresis (12), or by using fluorescent dye in PCR (13). A site-specific method based on the resistance of 2'-O-methylated sites to cleavage by RNase H has also been developed (14,15). This assay is highly sensitive for the detection of methylated sites and particularly applicable for the analysis of low-abundance RNAs; however, this method also requires a radiolabeling step. Saikia et al. (16) also developed a ligation-based approach for the high-throughput analysis of RNA modifications using 32P-labeled oligonucleotides. Recently, a method using RNA-cleaving deoxyribozymes (DNAzymes) has been reported that is easily executed and requires no radioactive labeling to detect 2'-O-ribose methylations in rRNAs (17); however, this method is inapplicable for the analysis of modified residues in RNAs of low abundance or modifications near the ends of the RNA molecules.

Here, we describe a new approach, RTL-P, for the detection of 2'-O-methylated sites in RNAs that uses reverse transcription at Low dNTP concentrations followed by PCR. By designing specific RT primers, RTL-P was successfully applied to identify both previously characterized and novel 2'-O-methylated sites in human rRNA, yeast rRNA and mouse piRNAs. In contrast to previously developed methods, RTL-P is highly sensitive and easy to perform. RTL-P is applicable for the high-throughput analysis of fully or partially methylated residues in diverse RNA species, including low-abundance RNAs or small non-coding RNAs. This new tool will help map the numerous post-transcriptional modifications in cellular RNAs.

MATERIALS AND METHODS

Sample preparation and RNA isolation

HEK293T (Human embryonic kidney 293T) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) and incubated at 37°C in a humidified chamber with 5% CO2. Total RNA was extracted from the HEK293T cells and adult C57BL/6 mouse testes using TRIzol (Invitrogen). The testes small RNA (<40 nt) was fractionated using the flashPAGE system (Ambion). Schizosaccharomyces pombe wild-type cells and the mutant haploid strain sp972 (ΔsnR61), which was constructed by our laboratory, were grown in rich yeast extract, peptone, dextrose (YPD) medium at 30°C. Total RNA was isolated from S. pombe as previously described (18). The RNA samples were treated with DNase I (Takara) to remove residual DNA contamination.

The detection of ribose-methylated nucleotides by RTL-P

For the detection of 2'-O-methylated sites in the target rRNAs, RT-PCR was performed according to conventional methods with the following modifications. RT was performed in 25 μl reaction mixture containing 2–200 ng of total RNA, 1 μl (10 mM) specific RT primers and a low (0.5–4 μM) or high (40 μM–1 mM) concentration of dNTPs. The primer/RNA mixture was denatured at 70°C for 5 min and then chilled on ice. Following an initial annealing step at 42°C for 10 min, 200 U of M-MLV reverse transcriptase (Promega) and 0.5 U RNasin Ribonuclease Inhibitor (Promega) were added. The reaction was incubated at 42°C for 1 h and then heated at 75°C for 15 min to deactivate the reverse transcriptase. The resulting yeast extract, peptone, dextrose (YPD) product was directly added to subsequent amplification reactions.

For the detection of methylated sites at the 3'-ends of mouse miRNAs or piRNAs, the small RNA fraction (<40 nt) was isolated using the flashPAGE™ Fractionator (Ambion) and ligated to a 3' RNA adapter using T4 RNA ligase (Takara). The ligation product was then reverse-transcribed into complementary DNA (cDNA) using a low (0.4 μM) or high (40 μM) dNTP concentration with or without anchored RT primers that were designed to anchor the modified nucleotide. The cDNA was subsequently amplified by PCR with specific primers under the same reaction conditions.

For the detection of partially methylated sites in RNA species, the RNA oligonucleotides with or without a 2'-O-methylated adenosine nucleotide were synthesized (Guangzhou RiboBio Co., Ltd) and mixed at different ratios in three experimental runs. RT was performed in 25 μl reaction mixture containing 2 ng of total RNA oligonucleotides, 1 μl (10 mM) specific RT primers and a low (0.5 μM) concentration of dNTPs. The cDNA was amplified by PCR with specific primers for 13 cycles.

The PCR reaction mixtures contained 1.25 U ExTaq DNA polymerase (Takara), 0.5 μl of dNTPs (10 mM each), 0.5 μl of forward and reverse primers (10 mM), 20 μl 1 × PCR buffer and 2 μl of the cDNA template. PCR reactions were performed as follows: one cycle of 94°C for 4 min followed by cycles at 94°C for 20–30 s and at 60°C for 20–30 s. The number of PCR cycles performed was determined based on the template concentration. Generally, an optimum range of cycle number should be carefully checked by performing PCR assays with a serial of cycles. The PCR products were then equally loaded and separated on 1.5–2.5% agarose gels, stained with GelRed dye (Biotium) and visualized by UV
The detection of ribose-methylated nucleotides by primer extension

Total RNA from *S. pombe* was subjected to primer extension at varying concentrations of dNTPs to detect 2'-O-methylation as previously described (19,20). Briefly, ~30 μg of total yeast RNA was mixed with the snR61-RT primer labeled with [γ-32P]ATP at the 5'-end in 20μl of 1 × RT buffer (Promega). After a denaturation step at 65°C for 5 min, hybridization was performed at 42°C for 10 min. Primer extension with 200 U M-MLV reverse transcriptase (Promega) was performed on two aliquots of the RNA in parallel in the presence of either 4μM or 0.15 mM dNTPs at 42°C for 1 h. The *S. pombe* 25S ribosomal DNA (rDNA) was cloned into the pMD-18T vector (Takara). The plasmid was subjected to alkaline hydrolysis and sequenced with the corresponding primer used in the RT reaction. The cDNA products were separated on 8% polyacrylamide-8M urea gels with sequenced 25S rDNA in parallel and analyzed by a Storm 820 Phosphorimager (Molecular Dynamics).

Primers and oligonucleotides

The sequences of the primers and oligonucleotides used in this study are listed in Supplementary Table S1.

RESULTS

The principle of RTL-P for the detection of 2'-O-methylated RNA nucleotides

Reverse transcriptase pauses immediately preceding a 2'-O-methylated nucleotide and tends to terminate cDNA synthesis at a low dNTP concentration (19,21). Based on this principle, we developed a novel strategy to detect specific 2'-O-methylated sites in RNA by combining primer extension by reverse transcriptase at a low or high dNTP concentration with a subsequent PCR amplification (Figure 1).

A specific set of designed RT-PCR primers for RTL-P is shown in Figure 1A. The primer used in the RT reaction was downstream of the predicted 2'-O-methylated site. Two forward primers for the subsequent PCR amplification were designed near each other and located either downstream (FD) or upstream (FU) of the predicted 2'-O-methylated site. The reverse PCR primer (R) was downstream of the FD.

The principle of the RTL-P method is depicted in Figure 1B and C. DNase-treated RNA samples were reverse-transcribed into cDNA with the RT primer at either a low or high dNTP concentration. The modified residues should induce a pause in the RT profile at a low dNTP concentration; therefore, the RT reaction with a low dNTP concentration should produce shorter cDNA products than the RT reaction with a high dNTP concentration. Each RT product was then analyzed by PCR with the designed PCR primer pairs. During the PCR, the R/FU primer pair should only amplify the long cDNA products, whereas the R/FD primer pair should amplify both the long and short cDNA products. When the RT products that had been generated using a low dNTP concentration were used as the DNA template for the PCR, the quantity of the PCR product produced using the R/FU primer pair should be less than the quantity produced by the R/FD primer pair. In contrast, when the RT products that had been generated using a high dNTP concentration were used as the DNA template, the quantity of the PCR products should be equal for both primer sets. Finally, equal volumes of the R/FU PCR product and the R/FD PCR product from the RT templates produced with a low or high dNTP concentration, respectively, are mixed. The PCR product yield and quality is then analyzed by gel electrophoresis. If the ribose 2'-OH of the residue in the target RNA is methylated, the PCR band intensity of the longer product produced by the R/FU primer pair should be weaker than the shorter product produced by the R/FD primer pair in the lane containing DNA amplified from the RT with a low dNTP concentration. In contrast, the intensity of the longer product will be slightly stronger than the shorter product in the lane containing DNA amplified from the RT with a high dNTP concentration due to the larger incorporation of dye within a longer template (Figure 1B). However, if the ribose 2'-OH is unmodified, the band intensity of the R/FU PCR product will be stronger than that of the R/FD product with both of the RT reactions produced with a low or high dNTP concentration (Figure 1C).

The detection of 2'-O-methylated nucleotides in rRNAs by RTL-P

To test the feasibility of our method, we first examined two cases, including a region of the human 28S rRNA with multiple methylation sites (Cm2338, Am2350, Cm2352, Am2388, Um2402, Cm2409 and Gm2411) and a single 2'-O-methylated site (Gm1490) in the human 18S rRNA from HEK293T cells. As shown in Figure 2A, we designed the 28S-RT primer downstream of the 2'-O-methylated nucleotides for the RT reaction as well as one reverse primer (28S-R primer) and five forward primers (28S-FD1, 28S-FD2, 28S-FU1, 28S-FU2 and 28S-FU3) for the semi-quantitative PCR. The 28S-FI1, 28S-FI2 and 28S-FI3 primers were located upstream of the various 2'-O-methylated sites, and the 28S-FD1 and 28S-FD2 primers were located downstream of the last 2'-O-methylated site, Gm2411. No 2'-O-methylated sites were located in the region between the binding sites of the 28S-FD1 and 28S-FD2 primers. The results of the semi-quantitative RT-PCR at varying dNTP concentrations (from 2μM to 1 mM) are shown in Figure 2B. As expected, the tendency of the 2'-O-methyl groups to impede RT at a low dNTP concentration (<40μM) was clearly detected. More 2'-O-methylated sites led to a reduced quantity of RT-PCR products at lower dNTP concentrations. No obvious differences between band intensities were observed between the RT-PCR products generated from the 28S-FD1 and 28S-FD2 primers as no
2′-O-methylated sites were located in the binding regions of these two primers. These results demonstrate that RTL-P can be applied for the detection of 2′-O-methylation in rRNA.

Next, we tested the sensitivity of this method by attempting to detect a single known 2′-O-methylated site, Gm1490, in the human 18S rRNA. As shown in Figure 2C, Gm1490 is between the binding sites of the 18S-FD1 or 18S-FD2 primers, and no 2′-O-methylated sites were located in the region between the 18S-FD1 and 18S-FD2 primer-binding sites. Figure 2D shows the results of RTL-P detection of the 2′-O-methylation at Gm1490 using 200 ng, 20 ng or 2 ng of total RNA in each RT reaction at a low (0.5–1 μM) or high (40 μM–1 mM) dNTP concentration and the comparison of the RT-PCR products using three different PCR cycles. In each assay, the band intensity of the 18S-FU RT-PCR product was obviously weaker (signal ratio <1) than the 18S-FD1 RT-PCR product at a low dNTP concentration and stronger (signal ratio >1) with a high dNTP concentration. Since no 2′-O-methylation sites were located between the binding sites of the 18S-FD1 and 18S-FD2 primers, the signal intensity of the 4. Electrophoresis and image

An alternative RTL-P strategy for determining the exact location of a 2′-O-methylated site within RNA

Although the method described above is sensitive and easily performed, it can only be used to detect the presence or absence of a 2′-O-methylated site within the region between the PCR primer-binding sites and cannot be used to determine which specific site within that region...
was 2'-O-methylated. To solve this problem, we developed a new RTL-P based strategy that used different RT primer sets to anchor the methylated site (Figure 3).

For this method, two RT primers, a methylated site unanchored primer (MeUA-RT) and a methylated site anchored primer (MeA-RT), are designed to the +1 and 0 nt downstream of the predicted methylated residue. The forward and reverse primers used for the PCR are located upstream and downstream of the methylated site (Figure 3A). The RNA samples are reverse-transcribed into cDNAs with the MeUA-RT or MeA-RT primers at a low or high dNTP concentration. At the low dNTP concentration, the extension of the MeUA-RT primer by reverse transcriptase pauses at the site with 2'-O-methylation, whereas the extension of the MeA-RT primer does not. This results in different concentrations of the RT products, which can be further amplified by PCR. In contrast, at a high dNTP concentration, the methylated residue usually does not affect the MeUA-RT primer extension; therefore, no significant concentration differences are seen between the RT or PCR products generated by the MeUA-RT and MeA-RT primer extension. If the
The exact location of the 2′-O-methylation site could be accurately determined by comparing the RT-PCR products generated by the MeUA-RT and MeA-RT primers at the low dNTP concentration.

Precise mapping of a new 2′-O-methylated site in *S. pombe* rRNA by RTL-P

The 2′-O-methylation of Am1164 in the 25S rRNA of *S. pombe* was predicted to be guided by a novel box C/D snoRNA, snR61 (accession number AJ617330), which possesses an 11-nt-long antisense stretch complementary to the 25S rRNA (Figure 4A). To test whether this methylation was dependent on snR61, we designed a set of RT primers, including two MeUA-RT (MeUA-RT-1/2) and two MeA-RT (MeA-RT-1/2) primers as well as PCR primers to detect the modification site (Figure 4B). The experiment could have been performed using only the two RT primers (MeUA-RT-2 and MeA-RT-1) instead of the four RT primers; nevertheless, the use of the other two primers (MeUA-RT-1 and MeA-RT-2) proved useful for interpretation of the results. The RT primer extension was performed using either 20 ng (Figure 4D) or 2 ng (Figure 4E) total RNA per RT reaction, which was followed by PCR using the RT products as DNA templates. As expected, the MeUA-RT PCR products were obviously less than the MeA-RT products at low dNTP concentrations (<4 μM) in wild type *S. pombe*; however, the band intensities of the RT-PCR products were approximately equal when the RT products were generated using high dNTP concentrations (>40 μM). The same experiment was then performed on the *AsnR61* *S. pombe* mutant strain, which is deficient for the Am1164 methylation of the 25S rRNA. As expected, no significantly different intensities of the RT-PCR products generated from the MeUA-RT or MeA-RT primers were found in the low or high dNTP lanes (Figure 4E). The traditional RT sequencing method was also performed to verify the methylation site in the 25S rRNA of *S. pombe* with 30 μg total RNA (Figure 4C). These results show that our novel method can be applied to accurately detect modification sites in rRNAs.

The detection of a 2′-O-methylated site in the 3′-end of small RNAs

The 3′-end of small RNAs, including animal piRNAs, endo-siRNAs and plant miRNAs, are fully methylated. To test the capability of RTL-P to detect methylation on endo-siRNAs, we assayed three individual mouse piRNAs (piRNA-1, piRNA-2 and piRNA-3) as well as two miRNAs from mouse testis as controls (miR-449 and miR-34 b). The mouse piRNA-1 [corresponding to piR-131190 (22)] and the other piRNAs have been verified to be 2′-O-methylated at their 3′-terminal nucleotide (23,24). The experimental RTL-P procedure was modified slightly to allow for RT-primer binding (Figure 5A). The small RNAs analyzed were first ligated to a RNA adapter, which provided a binding site for the RT primer. The results of the modified RTL-P showed the piRNAs were methylated at the 3′-termini (Figure 5B). In contrast, RTL-P did not detect the modification at the
3'-termini of the control mouse miRNAs, miR-449 and miR-34b (Figure 5B), which is consistent with the data that demonstrates animal miRNAs may not be modified unlike their counterparts in plants (25).

DISCUSSION

We have developed a novel approach, RTL-P, to detect 2'-O-methylated sites in RNA molecules. RTL-P does not require expensive reagents, sophisticated procedures or dangerous radioactive labeling and can be performed in any molecular biology laboratory with standard equipment. By designing specific RT primers, the approach can be applied not only to screen transcripts to detect the presence or absence of methylated sites but also to detect the exact location of the modified sites in the RNAs of interest.

Compared with previously reported methods, RTL-P is easier to perform and more sensitive. Although traditional RT-based methods enhance their sensitivity by utilizing radiolabeled probes, large amounts of starting material (~2–20 μg total RNA) is usually required for the primer
extension reaction depending on the concentration of the RNA of interest (1). In contrast, RTL-P employs PCR to amplify the target cDNAs to increase the detection sensitivity. With the RTL-P method, the cDNA product generated from 2 ng of total RNA was enough to detect the 2'-O-methylated residue in the human 18S rRNA (Figure 2). This result demonstrated that the sensitivity of RTL-P is nearly 1000 times greater than traditional RT-based methods. Compared with two other RT based approaches (12,13) that apply fluorescent primers or fluorscent dye such as SYBR green to increase the detection sensitivity, RTL-P needs no sophisticated procedures such as capillary electrophoresis or expensive reagents including fluorescent dye. Moreover, RTL-P can be used to effectively detect the partially 2'-O-methylated site at a given position (Figure 6). Our data revealed that the extent of methylation has a direct impact on the efficiency of RT reaction. Partial modification with more than 50% of 2'-O-methylation at a given position of RNA species can significantly impede the RT reaction at a low dNTP concentration.

In the RTL-P assay, different PCR products (i.e. R/Fu and R/FD products in the Figure 2D) mixed together before the ratio of their intensity signals were analyzed.
by gel electrophoresis. Alternatively, it might render the RTL-P approach easier and reduce the experiment error if the multiplex PCR was performed in a single tube [Reverse Transcription at Low dNTP concentrations followed by Multiplex PCR (RTL-MP)] in which different PCR primers were mixed together (Supplementary Figure S1). However, multiple primers used in a single tube increase the possibility of primer–dimer formation. Researchers have to perform the ‘pre-PCR’ to mitigate this problem by a laborious primer design and validation process before using RTL-MP.

RTL-P serves as a powerful tool for the localization of modified residues in low-abundance cellular RNAs, including small non-coding RNAs. 2′-O-methylation is an important characteristic for certain small RNAs, including animal piRNAs, endo-siRNAs and plant microRNAs. Therefore, identification of 2′-O-methyl groups in the 3′-RNA termini helps to distinguish the different types of small RNAs. However, modifications located too close to the RNA termini, including RNAs shorter than 40 nt, such as piRNAs and microRNAs, are difficult to detect with traditional RT-based approaches, which require annealing an oligonucleotide primer to the RNA 3′-termini. In this study, we solved this issue by ligating a RNA adapter to the 3′-end of the small RNAs, which allowed the successful application of RTL-P to detect the modified nucleotides at the 3′-end of piRNAs. These results suggest that RTL-P can be applied to the other small RNA species for the detection of 2′-O-methylation. Additionally, with this ligation strategy, the RNA adapter could also serve as a universal binding site for the RT primer allowing for the use of RTL-P for the high-throughput analysis of 2′-O-methylation in small RNAs.

Similar to traditional RT-based approaches, RTL-P requires that the region of the RNA targeted by the RT primers is accessible and not hidden within a stable secondary structure, such as a stem loop. These secondary structures and some sequences can also induce RT pauses, which could cause the identification of false positive modifications. In some cases, performing the RT reaction at ≥42°C temperatures may serve as a solution to this problem. Therefore, other reverse transcriptases, such as the avian Mieloblastosis virus (AMV) reverse transcriptase, would be more applicable than the Moloney murine leukemia virus (M-MLV) reverse transcriptase used in this study due to its higher thermostability (up to 70°C) (26) and decreased sensitivity to RNA secondary structures (27). It is noted that the number of PCR cycles greatly influences the RT-PCR profile. In general, the less starting templates are used for subsequent PCR, the more PCR cycles will be required for an optimal ratio of PCR signal intensities (Figure 2D). Although the ratio of PCR signal intensity correlated with the PCR cycles (Figure 2E), the linear range of signal ratios versus cycle number is limited. No differences in product quantity can be detected if the PCR reaction is allowed to reach the plateau phase during later cycles. Therefore, an optimum range of cycle number should be carefully determined by performing PCR assays with a serial of cycles to find the optimal ratio. Comparing the quantity of the RT-PCR products generated under different experimental conditions, such as varied dNTP concentrations in the RT reaction and different cycles of PCR, generally yields quite reliable results. However, it should be noted that several 2′-O-methylation sites in rRNAs do not cause any pauses (19), which cannot be detected by the RT-based methods or by RTL-P. The use of other complementary RNA analysis techniques, including OH-cleavage or 2′-OH reactivity, LC/MS and 2D-TLC, is useful to confirm the modifications detected by the RTL-P method and to detect novel methylated sites.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figure 1.

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