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Selective labeling and detection of specific RNAs in an RNA mixture

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Abstract

We report here a unique approach to selectively label and detect specific RNA in an RNA mixture (without separation or purification) using DNA polymerase, dNTP labels, and a short synthetic DNA template complementary to the 3′-terminus of the RNA. The detection sensitivity is high, at attomole level (10–18 mole). The selective principle was demonstrated by individually labeling and detecting RNAs in a RNA mixture when different templates were provided. By taking advantage of the template-directed selectivity, poly(A) tail-containing mRNA in total RNA was detected and labeled at the 3′-terminal on a poly(T) template. Nonradioactive labels, such as fluorophore and antigen labels, may also be used; this method can be applied in methodology for direct detection and quantification of viral RNAs.

Keywords: RNA selective labeling; Direct detection and quantification of viral RNA; DNA template; DNA polymerase

Nucleic acid detection methods for viral and bacterial real-time analysis, using reverse-transcription and polymerase chain reaction (PCR)[1] technologies [1–4], have significantly increased pathogen detection accuracy and shortened analysis time, which is especially useful in emergency. Based on a terminal RNA-labeling method, we demonstrated here a novel and simple approach to directly label and detect specific RNA molecules in a mixture without reverse transcription and PCR, which can further save analysis time.

Three methods are commonly used for RNA terminal labeling: 5′-labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP [5]; 3′-labeling with T4 RNA ligase and 3′, 5′-[5′-^{32}P]pCp [6]; and 3′-labeling with poly(A) polymerase and [α-^{32}P]cordycepin 5′-triphosphate (CoTP or 3′-deoxy-ATP) [7]. Since the enzymes recognize RNA substrates nonspecifically, all RNA substrates in an RNA mixture are labeled at either 5′- or 3′-termini. The nonspecific labeling feature of these methods provides an advantage when labeling and detection of all RNAs in an RNA mixture are desired. This advantage becomes a disadvantage, however, when labeling and detection of a specific RNA in an RNA mixture are desired [8], such as labeling and analysis of viral RNA, ribosomal RNA, or specific mRNA in a total RNA sample. In order to use the conventional methods in direct detection and analysis of a specific RNA, separation steps are needed to isolate the specific RNA from its mixture. Selective labeling and analysis of specific RNA in a mixture, without purification and RT-PCR, remained a challenge.

We have reported a novel method to label 3′-termini of RNA [9] by taking advantage of the natural function of DNA polymerases: elongation of RNA primers on DNA templates (Fig. 1). To investigate the possibility of developing a selective labeling and detection method, we further explored this enzymatic template effect. The ready availability of short synthetic DNA template allows any RNA of known 3′-terminal sequence to be selectively extended in a template-dependent manner at its 3′-end, which promises labeling and detection of a

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[1] *Abbreviations used:* PCR, polymerase chain reaction; CoTP, [α-^{32}P]cordycepin 5′-triphosphate, DTT, dithiothreitol.

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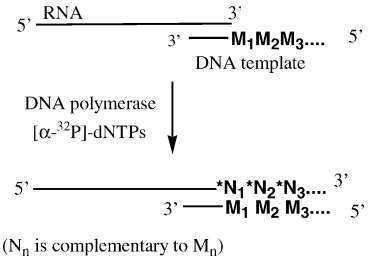

Fig. 1. 3′-Terminal labeling of RNA on a short DNA template.

specific RNA in an RNA mixture,without separation, purification, reverse transcription, and PCR.

Materials and methods

Oligonucleotides and enzymes

DNA11.1 (5′-TGGGTTGCTGG-3′), DNA12.1 (5′-GTGGGTTGCTGG-3′), DNA17.1 (5′-GTGGGTTGC TGGCACCG-3′), DNA17.2 (5′-GTGTAGTTGAATC AGCA-3′), and $(T)_{20}$ were synthesized chemically. RNA40 (5′-GGGUUGGGAAGAAACUGUGGCAC-UUCGG-UGCCAGCAACCC3′) and RNA50 (5′-GG AGAGUAUGCAGUAGUCAUCGCGACGU-AGCU AGAUGCUGAUUCAACUA C-3′) were prepared by in vitro transcription of synthetic oligodeoxynucleotide templates with T7 RNA polymerase. All DNAs and RNAs were purified by gel electrophoresis. *Escherichia coli* DNA polymerase I, the Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, T7 RNA polymerase, and M-MuLV reverse transcriptase were purchased from New England Biolabs; *Taq* DNA polymerase was purchased from United States Biochemical.

General conditions for RNA labeling and detection via Klenow extension

Klenow-labeling reactions (5 μL) were generally carried out at 37 °C for 1 h, unless noted otherwise, in buffer [10 mM Tris-HCl (pH 7.5), 17.5 mM DTT, and 5 mM $MgCl_2$], with RNA (0.001–500 nM), DNA template (1 nM–10 μM), Klenow (0.5 U/μL), and 0.1–1 μL of [α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/mL; NEN). Electrophoresis on polyacrylamide gels was used to separate oligonucleotides by size, and radioactively labeled oligonucleotides were visualized by autoradiography.

Results

Polymerase screening and optimization of RNA 3′-labeling

During dsDNA replication in cells, Okazaki fragments [10] are synthesized by DNA polymerase III, via extension of RNA primers on a DNA strand to allow 3′–5′ DNA lagging synthesis. Based on this RNA primer extension principle, we have demonstrated previously that DNA polymerase is able to extend RNA substrate with dNTPs on a short DNA template (Fig. 1). When [α-^{32}P]dNTPs are used in the extension, the RNA substrate is radioactively labeled using this approach.

A variety of DNA and RNA polymerases have been screened and examined for the RNA 3′-extension on a DNA template. Enzymes, including *E. coli* DNA polymerase I, the Klenow fragment of *E. coli* DNA polymerase I [11], T4 DNA polymerase, T7 DNA polymerase, T7 RNA polymerase, M-MuLV reverse transcriptase, and *Taq* DNA polymerase were tested by incubating with a 5′-^{32}P-labeled RNA, dNTPs, and a DNA template (Fig. 2). Like DNA polymerase III extension of RNA with dNTPs on DNA template in vivo, many other DNA polymerases are able to extend RNA with dNTPs on DNA template in vitro. Both Klenow and T7 DNA polymerase effectively extended RNA; the other polymerases either performed less efficiently or caused degradation. Under more stringent conditions, such as lower RNA substrate and DNA template concentrations, the Klenow fragment showed higher extension efficiency than T7 DNA polymerase (Fig. 3). Therefore, the Klenow fragment was chosen for further characterization and optimization of the 3′-labeling reaction.

Studies of time course and detection sensitivity

In order to optimize the reaction time (Fig. 4A), RNA40 (5 nM) was labeled for different periods of incubation time. The extension reaction reached half-maximal level in approximately 30 min and its maximum level in 2 h. The labeled product was stable and remained unchanged for many hours. Under the optimum conditions, the detection limit of this method was investigated (Fig. 4B). The results indicated that more RNA was labeled when its concentration was high, and that RNA even at low concentration (pM level) was also labeled. As the reaction volume was small (only 5 μL), the RNA detection sensitivity was high, at attomole levels (10^{-18} mol). The effective labeling may be due to

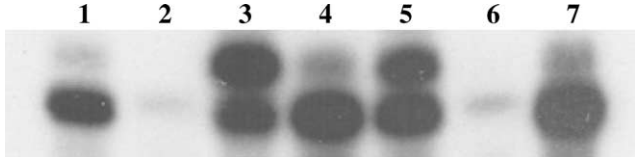

Fig. 2. Screening of polymerases. 5′-^{32}P-labled RNA40 (500 nM) as substrate and DNA11.1 (5 μM) as template. Lane 1, RNA substrate; lane 2, extension using DNA polymerase I; lane 3, Klenow; lane 4, T4 DNA polymerase; lane 5, T7 DNA polymerase; lane 6 *Taq* DNA polymerase; lane 7, M-MuLV reverse transcriptase.

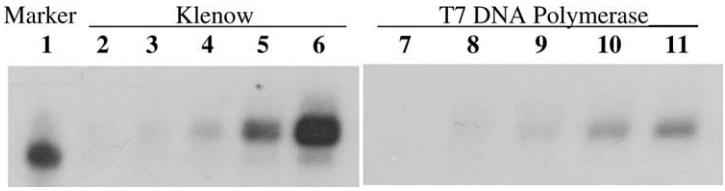

Fig. 3. Labeling of RNA substrate at different template concentrations using Klenow fragment and T7 DNA polymerase. Labeling reactions were performed with RNA40 (50 nM), and Klenow (lanes 2–6) or T7 DNA polymerase (lanes 7–11). DNA12.1 concentration: lane 2, 0 μM; lane 3, 0.1 μM; lane 4, 1 μM; lane 5, 10 μM; lane 6, 100 μM; lane 7, 0 μM; lane 8, 0.1 μM; lane 9, 1 μM; lane 10, 10 μM; lane 11, 100 μM.

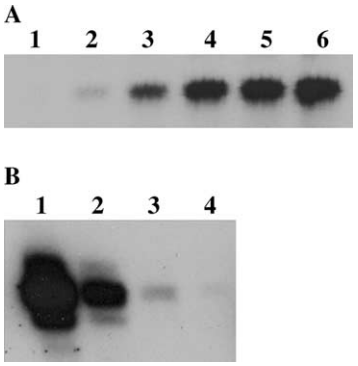

Fig. 4. (A) Time course of the labeling reaction. Substrate RNA40 (5 nM, 5 μL) was labeled in the presence of template DNA17.1 (100 nM), Klenow (0.5 U/μl), and 0.1 μL of [α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml). Lane 1, 0 min; lane 2, 4 min; lane 3, 15 min; lane 4, 45 min, lane 5, 1.5 h, lane 6, 3 h. (B) RNA detection sensitivity. Labeling reaction (5 μL) was conducted with Klenow (1 U/μL), DNA17.2 (100 eq to RNA50), and 1 μL of [α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/mL). RNA50 concentration: Lane 1, 1 nM; lane 2, 100 pM; lane 3, 10 pM; lane 4, 1 pM.

high affinity of DNA polymerase for DNA template/RNA substrate complex [12,13]. This behavior with high affinity is analogous to *Taq* DNA polymerase binding and amplifying DNA molecules at extreme low levels. The sensitivity of this method can potentially be further enhanced by increasing the length of the overhang sequence of the DNA template, allowing incorporation of more labeling nucleotides.

Selective detection and labeling of RNA at 3′-termini

We reported earlier that a dinucleotide-overhang template with a noncomplementary 5′-terminal nucleotide (e.g., 5′-AT, 5′-CT, or 5′-GT) resulted in rapid and efficient incorporation of a single nucleotide (e.g., dA in this case), and that this mono-nucleotide incorporation was 20-fold greater than that on a mononucleotide-overhang template [9]. Therefore, two DNA templates with dinucleotide overhang, where the terminal nucleotide was noncomplementary to the incoming dNTP, and 15-nucleotide-overlaping sequences were designed to demonstrate selectivity of the labeling and detection. Because of the template-dependent nature of the RNA 3′-terminal labeling, RNA 1 (RNA40) or RNA 2 (RNA50) in an RNA mixture was selectively labeled and detected when complementary template 1 or template 2 was added (Fig. 5). In the presence of both templates, both RNA 1 and RNA 2 were labeled and detected simultaneously. Interestingly, these selective labeling and detection reactions were independent events as individual labeling and detection efficiency was not compromised by the presence of the other RNA and/or template.

Selective labeling of mRNAs on poly$(T)_{20}$

Selectivity and noninterference features of this method make it possible to label and detect specific RNA molecules in a RNA mixture without purification. These features provide a solid foundation for detection of specific RNA in a RNA mixture, e.g., a viral RNA or bacterial RNA in a total RNA mixture. We have demonstrated this by specifically labeling poly(A) tail-containing mRNA using a total RNA sample extracted from human leukemia T cells. Since 5′-termini of mRNAs are capped, it is difficult to label mRNA with the conventional T4 kinase method at 5′-termini. Likewise, using the conventional 3′-labeling methods, it is also difficult to just label mRNAs in a total RNA sample. By taking advantage of the 3′-poly(A) tail of mRNAs, however, mRNAs in the total RNA sample were specifically labeled on oligo(T) template using this 3′-labeling method (Fig. 6). The mRNAs were not labeled in the presence of a control template, or [α-^{32}P]dCTP; but they were labeled and detected only in the presence of both [α-^{32}P]dATP and $(T)_{20}$ template, indicating this terminal extension was specific, selective, and template dependent.

Discussion

We have successfully demonstrated a simple RNA selective labeling and direct detection approach for RNA analysis without reverse transcription and PCR, which can greatly shorten time for detection and analysis. This method is extremely sensitive, allowing detection of RNA at 10^{-18} mole level; the detection sensitivity can be further increased by extending the overhang sequence of the DNA template. The selectivity

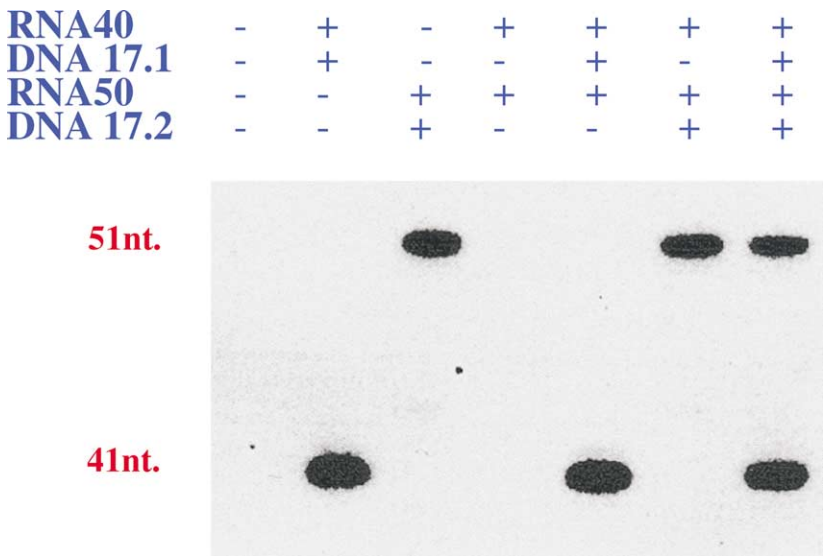

Fig. 5. Selective labeling of RNAs. Reactions (5 μL) were conducted under the following conditions: RNA40 (50 nM), RNA50 (50 nM), DNA17.1 (1 μM), DNA17.2 (1 μM), Klenow (0.5 U/μL), and 0.1 μL of [α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml).

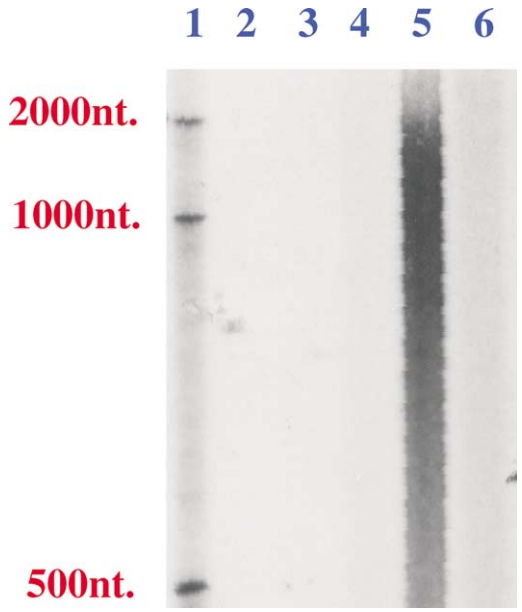

Fig. 6. Labeling of total mRNA from human leukemia T cells. The labeling reactions were performed in the presence of Klenow (1 U/μl) and 0.1 μL of [α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml) or 0.1 μL of [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml). Lane 1, marker; lane 2, blank; lane 3, Klenow, no $(T)_{20}$; lane 4, Klenow with $(T)_{20}$ and [α-^{32}P]dCTP; lane 5, Klenow with $(T)_{20}$ and [α-^{32}P]dATP; lane 6, Klenow with control template (5′-GGCTACAGGAAGGCCAGACG-3′) and [α-^{32}P]dATP.

and sensitivity of this approach have paved a new way for labeling, detection, and even quantification of a viral RNA in an RNA sample, or total mRNAs and rRNAs in a total RNA sample. It is important to be able to rapidly and accurately identify viruses and bacteria in diagnosis and food contamination examination. A rapid analytical method will be especially useful in emergency, such as disease outbreak. Direct analysis and quantification of total mRNA expression level can provide information for aging and disease analysis based on the change of overall mRNA expression levels [14,15]. Likewise, based on the rRNA 3′-terminal labeling and analysis [16], direct labeling and analysis of rRNA expression level can also provide valuable insights on aging and diseases [15,17,18]. In addition, direct analysis of specific mRNA, hard to accomplish by DNA microarray technology [19], can be achieved using the terminal selective labeling approach via RNaseH digestion [20] of the 3′-region common sequences, such as 3′-poly(A) tail and 3′-UTR.

As RNA may form 3′-terminal secondary structure or intramolecular duplex, RNA 3′-termini may be inaccessible for the conventional 3′-labeling methods. The DNA template used in this method competes, however, with intermolecular and/or intramolecular duplexes to free up RNA 3′-termini. The labeling and detection can also be performed using nonradioactive labels (e.g., fluorophores) [21] or via ELISA (e.g., conjugated alkaline phosphatase or horseradish peroxidase to catalyze chemiluminescence reactions) [4]. This novel strategy for RNA labeling, detection, and analysis has great potential in directly detecting and quantifying viruses and other pathogens, in monitoring metabolic processes in disease states, and in directly profiling gene expression.

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