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Isolation of Small RNAs using Biotinylated PNAs

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In this study, an RNA isolation method was developed using a biotinylated peptide nucleic acid (PNA) that is complementary to the target RNA. Using the biotinylated PNA method, we successfully isolated several RNAs from *Escherichia coli* and from human total RNA in pure form. Damage to the RNA appears to be negligible by this method because the method is rapid and does not require a high temperature treatment to facilitate RNA–PNA binding.

Key words: non-coding RNA, peptide nucleic acid, ribosomal RNA, RNA isolation, transfer RNA.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; PNA, peptide nucleic acid.

Recent transcriptome analyses have revealed that numerous RNAs that have no protein-coding potential exist in living organisms. Because the non-coding RNAs regulate cellular processes in many different ways (*1*, *2*), their molecular characterization is urgently needed. *In vitro* RNA transcripts can be used as substitutes for native RNAs to investigate the structures and functions of these RNAs. However, most of the native RNAs have post-transcriptional modifications (*3*, *4*), and some of the modifications are quite important for their structure and function. Thus, isolation of intact RNAs from living organisms is essential for studying non-coding RNAs. Traditional RNA isolation methods are based on successive column chromatography steps. However, application of such methods to a wide range of RNAs is difficult because the most effective combination of columns varies depending on the RNA. To overcome such difficulties, a method to isolate RNAs by sequence-specific selection using solid-supported DNAs has been devised (*5*). In this method, one RNA species can be purified from an RNA mixture in a single step. Furthermore, this strategy has been extended to the parallel isolation of multiple RNA species by Miyauchi *et al*. (*6*). Although the DNA-based method is easier than methods based on sequential column chromatography, RNA isolation using a complementary DNA can be difficult when the target sequence is in the stem region because the RNA–RNA hybrid is more stable than a DNA–RNA hybrid (*7*). Furthermore, the DNA-based method requires a high temperature treatment for RNA unfolding and DNA–RNA hybridization, which may cause undesired hydrolysis of RNA strand and damage heat labile modifications [for example, the conversion of m^1A to m^6A (*8*) seems to be promoted by high temperature].

Peptide nucleic acid (PNA)–RNA hybrids are more stable than DNA–RNA hybrids (*9*) and RNA–RNA hybrids. PNA–RNA hybrids are easily formed without any high temperature treatment. Previous efforts to use PNAs for RNA capture (*10*, *11*) demonstrated efficient binding of PNA toward RNAs, but these studies did not show the procedure to collect the captured RNA without the hybridized PNA. Locked nucleic acid (LNA) is also a general and versatile tool for specific, high-affinity recognition of RNA (*12*). However, it was shown that the PNA–RNA hybrid is stronger than the LNA–RNA hybrid (*13*). Thus, we developed an RNA isolation method using biotinylated PNA.

The biotinylated PNAs for RNA isolation were designed to hybridize to sequences near the 3′-end of the target RNAs. Purine-rich sequences were avoided in the biotinylated PNAs. Biotinylated PNAs were manually synthesized using a solid-phase Fmoc method as described (*14*). Fmoc-protected PNA monomers were purchased from Applied Biosystems (US). The sequences of PNAs used in this study are: PNA(tL), *N*-biotin-(O6 linker)$_2$-PNA(TACCGAGGAC)-KK-NH_2-C; PNA(5S), *N*-biotin-(O6 linker)$_2$-PNA(CCT GGCAGTT)-KK-NH_2-C; PNA(5.8S), *N*-biotin-(O6 linker)$_2$-PNA(GCGACGCTCA)-KK-NH_2-C; PNA(II52), *N*-biotin-(O6 linker)$_2$-PNA(CCTCAGCGTA)-KK-NH_2–C. K indicates a lysine residue and 'O6 linker' indicates a long spacer consisting of six ethylene glycol units (NeOMPS, France). Each N-terminus was cross-linked to biotin (biotin-X, SE, Molecular Probes, US). The crude oligomers were purified by preparative HPLC and the purified oligomers were identified by MALDI-TOF mass spectroscopy.

RNA isolation was performed as follows. Total RNA (200 μg) was dissolved in 100 μl of a solution containing 1 nmol biotinylated PNA, 10 mM Tris–HCl (pH 7.5) and 10 mM EDTA. The RNA/PNA mixture was incubated at 40–55°C for 5 min, and then mixed with 400 μl of a slurry containing 50 μl streptavidin agarose (Novagen, Germany) in 10 mM Tris–HCl (pH 7.5). The slurry was gently mixed at room temperature (20–30°C) for 30 min. The slurry was loaded onto a centrifugal filter device (Ultrafree-MC, 0.45 μm, Millipore, US). The streptavidin agarose beads were washed five times at room temperature (20–30°C) on the filter device with 400 μl of wash

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buffer containing 10 mM Tris–HCl (pH 7.5) and 150 mM NaCl. The RNA bound to the PNA was eluted as follows: (i) the beads in the filter device were suspended with 200 µl of elution buffer containing 10 mM Tris–HCl (pH 7.5) and 5 M NaCl, (ii) the suspension was incubated at 37–55°C for 5 min, (iii) the elution buffer, which contained the eluted RNA, was separated from the beads using the filter device. The elution procedure was performed twice and the eluted RNA was collected by ethanol precipitation. The isolated RNA was confirmed by 10% denaturing PAGE.

We first isolated $tRNA^{Leu}_{GAG}$ (Fig. 1A) from an *Escherichia coli* tRNA mixture (Sigma-Aldrich, US). The PNA_tL/tRNA hybrid was formed at room temperature (see Fig. 1B, Lane 6), but a heat treatment of 40–55°C for 5 min improved the selectivity of the RNA isolation. Elution of the RNA from PNA_tL supported on SA beads is difficult because the RNA binds too tightly to PNA_tL (see Fig. 1B Lanes 5 and 6; the RNA bound to PNA_tL even in a denaturing polyacrylamide gel). We could not elute the RNA from the SA beads with a low salt solution (100 mM NaCl) even at high temperatures (60–75°C). Additionally, we could not efficiently elute the RNA using a 10 mM Tris–HCl (pH 7.5) buffer containing 8 M urea, 8 M guanidine–HCl, 80% ethanol or 50% phenol/chloroform. However, we found that the RNA could easily be eluted using buffer containing 5 M NaCl, without high temperature treatment. The tRNA was isolated using 8-mer, 9-mer, 10-mer or 12-mer biotinylated PNAs, which are complementary to the 3′-end sequence of the tRNA, except for the CCA end, and the best results were obtained using the 9- or 10-mer PNAs. The amount of $tRNA^{Leu}_{GAG}$ obtained using the 8-mer PNA was lower than that obtained using other PNAs, and many impurities were associated with the RNA obtained using the 12-mer PNA (data not shown). Thus, we used 10-mer PNAs in the following experiments. The results of $tRNA^{Leu}_{GAG}$ isolation with the 10-mer biotinylated PNA (PNA_tL) are shown in Fig. 1B. After the purification procedure, the eluate contained a single major RNA component having the same length as the synthetic $tRNA^{Leu}_{GAG}$ (Fig. 1B, Lanes 4 and 5). The mobility of the tRNA eluted from the beads (Lane 4) is same as the synthetic $tRNA^{Leu}_{GAG}$ (Lane 5) but differs from that of the tRNA/PNA complex (Lane 6), indicating that the tRNA but not the tRNA/PNA complex was eluted by this procedure. The purified tRNA was sequenced by the Donis-Keller method (*15*), and was confirmed to be the *E. coli* $tRNA^{Leu}_{GAG}$ (data not shown).

Purification of *E. coli* $tRNA^{Leu}_{GAG}$ ($tRNA^{Leu2}$) by DNA-based methods was relatively difficult compared to other *E. coli* tRNAs (*6*). To compare the PNA-based method with the DNA-based method, we also purified the tRNA using 10-mer and 30-mer biotinylated DNAs (Fig. 1A). The $tRNA^{Leu}_{GAG}$ could not be isolated using the biotinylated DNAs with the PNA protocol. Thus, the method described in the Supplementary Material, which is basically the method of Wakita *et al*. (*16*), was used. The amount of $tRNA^{Leu}_{GAG}$ isolated using the 10-mer DNA was much less than that isolated with the 10-mer PNA, and the purity of the $tRNA^{Leu}_{GAG}$ obtained using the 30-mer DNA was lower than that obtained using the

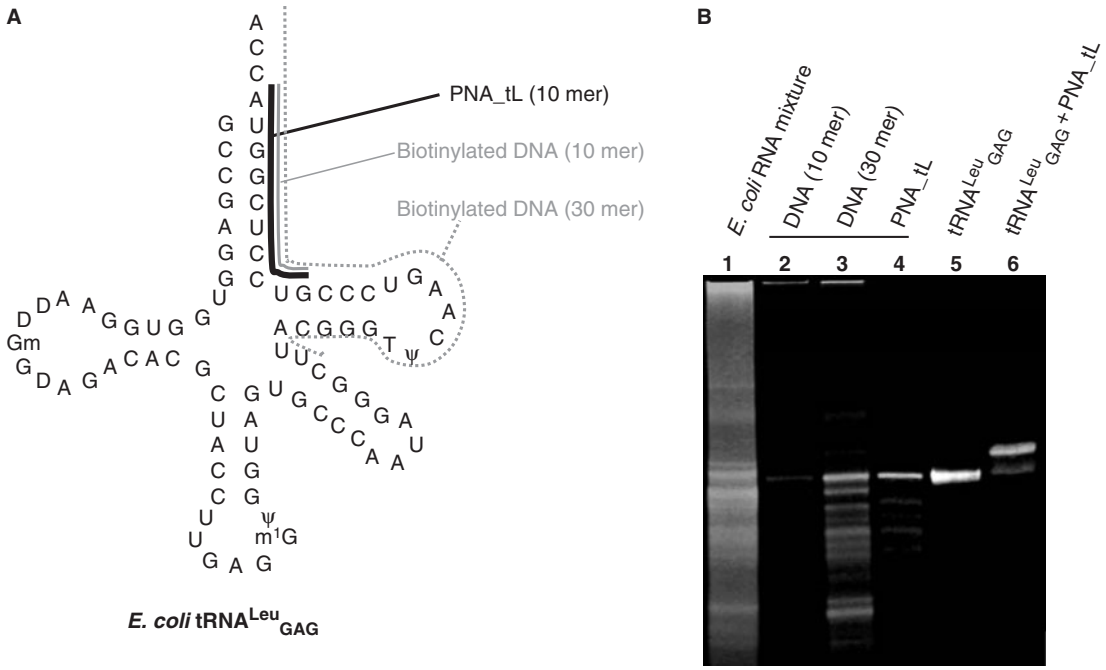

Fig. 1. **Isolation of *E. coli* $tRNA^{Leu}_{GAG}$.** (A) Secondary structure of the *E. coli* $tRNA^{Leu}_{GAG}$. The regions complementary to biotinylated PNA_tL and the biotinylated DNAs (10-mer and 30-mer) are shown by bold, thin and dotted lines, respectively. (B) A 10% denaturing PAGE analysis of purified RNAs. RNAs were visualized by ethidium bromide staining. Lane 1: 4 µg of *E. coli* tRNA mixture. Lane 2, 3 and 4: RNAs obtained by the purification from 200 µg of an *E. coli* tRNA mixture using 1 nmol of 10-mer biotinylated DNA, 30-mer biotinylated DNA or PNA_tL, respectively. Lane 5: 0.2 µg synthetic *E. coli* $tRNA^{Leu}_{GAG}$. Lane 6: 0.2 µg synthetic *E. coli* $tRNA^{Leu}_{GAG}$ and 20 pmol PNA_tL. The synthetic tRNA having an unmodified $tRNA^{Leu}_{GAG}$ sequence was prepared by T7 transcription.

10-mer PNA (Fig. 1B). The yields of $tRNA^{Leu}_{GAG}$ roughly estimated from its band intensities were 0.1 μg for the 30-mer DNA and 10-mer PNA, and 0.01 μg for the 10-mer DNA. The purities of the $tRNA^{Leu}_{GAG}$ were roughly estimated as 40, 15 and 65% for the 10-mer DNA, 30-mer DNA and 10-mer PNA, respectively.

To estimate the tRNA recovery, the $tRNA^{Leu}_{GAG}$ was purified using 1 nmol of PNA_tL from a mixture containing 4.0 μg each of yeast $tRNA^{Phe}$, bovine mitochondrial $tRNA^{Phe}$ and *E. coli* $tRNA^{Leu}_{GAG}$, all of which were synthesized using an *in vitro* transcription system. No $tRNA^{Leu}_{GAG}$ was detected in the wash fractions, indicating that the tRNA bound quantitatively to the streptavidin beads through the biotinylated PNA (Fig. 2A). The amount of $tRNA^{Leu}_{GAG}$ isolated from the tRNA mixture was estimated to be 2.8 μg from its band intensity using ImageJ Ver.1.34s (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). Therefore, the recovery was ~70%. Figure 2A shows that $tRNA^{Leu}$ was not degraded by this method, indicating that the RNA fragments that are smaller than the tRNA in lane 4 of Fig. 1B are not degradation products of $tRNA^{Leu}$ generated during the RNA isolation procedure. Northern analyses showed that the small fragments in lane 4 of Fig. 1B were degradation products of $tRNA^{Leu}$ present in the total RNA (data not shown).

To evaluate the versatility of this method, we purified *E. coli* 5S rRNA, human 5.8S rRNA and human HBII-52 RNA using the PNA method. HBII-52 is a human small nucleolar RNA (snoRNA), which is specifically expressed in brain (*17*) and regulates alternative splicing of the serotonin receptor 2C (*18*). *Escherichia coli* 5S rRNA was purified from *E. coli* total RNA, which was prepared from *E. coli* pellets using the TRIzol reagant (Invitrogen, US). Human 5.8S rRNA and HBII-52 were purified from human brain total RNA (Ambion, US). The RNAs isolated by this method were analysed on denaturing polyacrylamide gels. In the 5S rRNA and 5.8S rRNA isolations, a single RNA band was observed migrating at the approximate size of each synthetic unmodified RNA, as shown in Fig. 2B. In the HBII-52 isolation, two RNA bands were observed. To confirm that purified RNAs were the target RNAs, the RNA bands indicated by the arrowheads in Fig. 2B were excised from the gels and the RNAs were extracted and analysed by RT–PCR. DNA fragments of the expected sizes were obtained by RT–PCR, and DNA sequencing of the RT–PCR products indicated that the isolated RNAs were the target RNAs

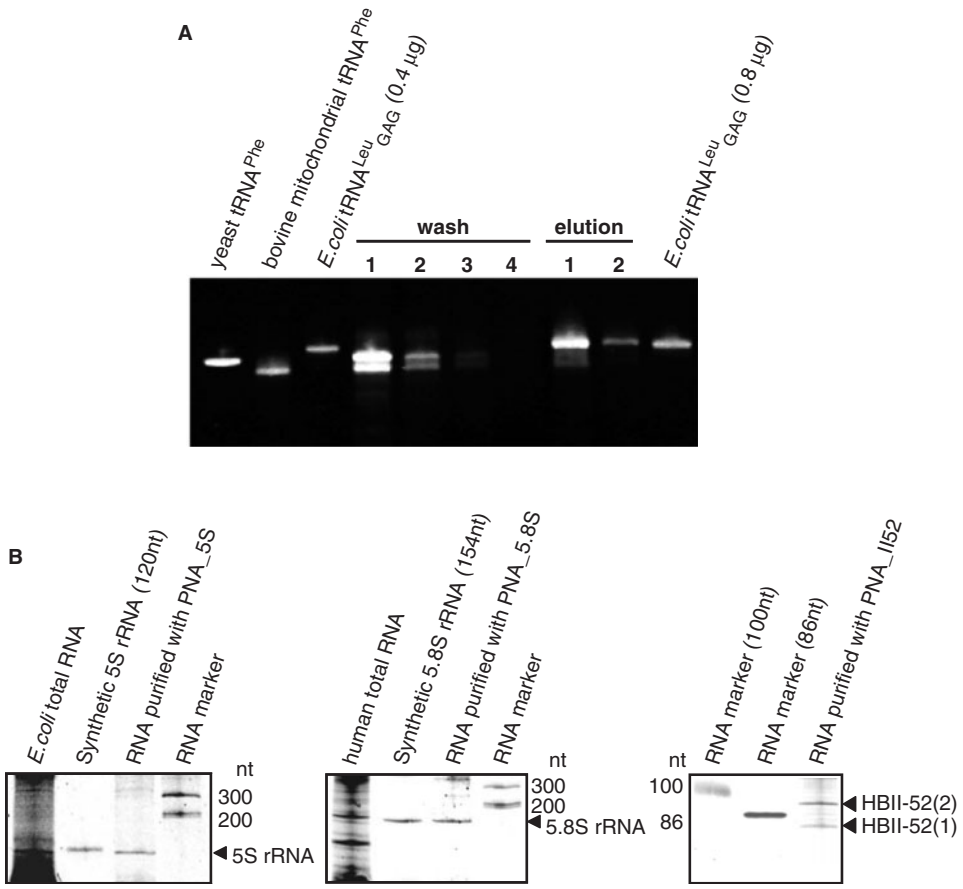

Fig. 2. (A) **Estimation of the yield of isolated $tRNA^{Leu}_{GAG}$.** PAGE analysis of wash and elution fractions of the PNA-based $tRNA^{Leu}_{GAG}$-isolation procedure using a mixture of 4 μg each of yeast $tRNA^{Phe}$, bovine mitochondrial $tRNA^{Phe}$ and *E. coli* $tRNA^{Leu}_{GAG}$. The gel was stained with ethidium bromide. (B) Isolation of *E. coli* 5S rRNA, human 5.8S rRNA and human HBII-52 RNA. Synthetic RNAs prepared by *in vitro* transcription were unmodified. The gels were stained with GelStar (Lonza). The RNA bands shown by the arrowheads were excised from the gel and analysed by RT–PCR.

(data not shown). Two bands around 82 nt were also detected in total brain RNA by northern analyses using the anti-HBII-52 probe (*17*), suggesting that the HBII-52(*1*) and HBII-52(*2*) bands in Fig. 2B (right) are processing variants that contain the HBII-52 sequence.

In this study, we successfully isolated several small RNAs using biotinylated PNAs. Damage to the RNA in the procedure appears to be negligible by this method because the method is rapid and does not require a high temperature treatment to facilitate RNA–PNA binding. The extension of this method for the parallel isolation of multiple RNA species, such as achieved by the RCC method (*6*), will enable genome-wide RNA analysis.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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