Synthesis of Small RNA Transcripts with Discrete 5' and 3' Ends

Generation of small RNA transcripts of defined sequence and length facilitates investigation of structure and base contributions of ribozymes, aptamers and naturally occurring small RNAs by eliminating ambiguity present in a heterogeneous RNA population. Interpretation of data from physical studies, chemical footprinting and nuclear magnetic resonance (NMR) spectra of small RNAs would also be simplified if the homogeneity of the sample could be guaranteed. Synthesis of small RNAs with T7 RNA polymerase often leads to 3'-end heterogeneity through both incomplete extension on the template and addition of extra, non-template encoded nucleotides (4). One method developed to circumvent this problem has been to synthesize ribozymes attached to the RNA transcripts. After self-cleavage, the RNA of interest has a discrete 3' end. Though effective, this approach requires specific recognition sequences exposed on both sides of the cleavage site. This constrains the sequence and structure of the RNA of interest. Also, most small ribozyme motifs generate a 2'-3' cyclic phosphate on the product, which may not be desirable in structural determinations or in 3'-end labeling.

A solution for producing RNA transcripts with discrete 5' and 3' termini has been devised by synthesizing a fusion transcript where an unconstrained insert RNA is attached to a 3' tRNA structure. The resulting fusion transcript is cleaved by exogenous RNase P ribozyme. RNase P recognizes only the downstream tRNA structure of the transcript as a substrate, thereby placing no constraints on the small RNA structure or sequence. Cleavage occurs at the 5' end of the tRNA producing a mature tRNA and the discrete insert RNA with a 3'-hydroxyl group.

It should be noted that this strategy for fusion transcripts has an additional advantage in RNA structure analysis. Detailed RNA footprinting by chemical sensitivity requires the RNA of interest to have an extra 3' sequence to serve as a primer binding site, allowing RNA cleavages and modifications to be detected by primer extension with reverse transcription. The tRNA structure provides such a site that folds into a tight, independent domain, thereby not interfering with the folding of the insert RNA of interest (5).

The construct used to create the small RNA-tRNA fusion is described in Figure 1. The basal plasmid construct (pSP64, Promega, Madison, WI,
USA) contains a modified *B. subtilis* tRNA<sup>Asp</sup> sequence with a unique *Bsp*120I restriction site embedded in the sequence encoding the 5' side of the aminoacyl stem. This plasmid is available upon request. An insert consisting of the T7 promoter fused to the desired small RNA (shown in italics) is ligated into the *Eco*RI and *Bsp*120I sites. The T7 promoter is contiguous with the RNA insert sequence to allow transcription to initiate with the first nucleotide of the RNA of interest. Efficient transcription requires a 5'-terminal guanosine in the coding region of the RNA of interest. Additional manipulation of the 5' sequence can also influence efficiency (4).

Templates for runoff transcription can be prepared either by cleaving the plasmid immediately past the tRNA sequence with *Bst*NI or by polymerase chain reaction (PCR) from primers A and B in Figure 1. Transcription is per-
formed using the following reaction conditions: 40 mM Tris, pH 8.1, 20 mM MgCl₂, 1 mM spermidine, 0.01% Triton® X-100, 20 mM dithiothreitol (DTT), 4 mM of each NTP, 0.3 μM template DNA and saturating levels of highly purified T7 RNA polymerase (prepared according to Reference 1 and D. Draper, Johns Hopkins University, personal communication). Saturating levels of T7 RNA polymerase must be determined by titration. The reaction is incubated at 37°C for 2–12 h. EDTA at 50 mM final concentration is added to the reaction to dissolve magnesium precipitates. The reaction is then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 2.5 volumes of 90% ethanol 0.3 M sodium acetate (NaOAc), pH 5.2. If the insert RNA-tRNA fusion is the desired product, it can be purified away from abortive transcripts by denaturing polyacrylamide gel electrophoresis and recovered as desired.

Generation of the discrete RNA of interest is accomplished by cleaving the insert RNA-tRNA fusion with the M1 ribozyme, the RNA component of E. coli RNase P (2). M1 RNA is synthesized from a PCR-derived template wherein the same T7 promoter shown in Figure 1 is fused to the mature coding sequence of M1 RNA (6). The 5' primer (5' TAATACGACTCACTATAGGAAGCTGACCAGACCTC 3'), which includes the T7 promoter, and the 3' primer (5' AGGTGAAACTGACCGATAAGC 3') are used to amplify the M1 gene from pDW27 (7) using PCR. The pDW27 plasmid is available upon request. The T7 promoter-M1 gene PCR product is then transcribed with T7 RNA polymerase, and the M1 transcripts can be purified on a denaturing 6% polyacrylamide gel or used directly after extraction and ethanol precipitation. Cleavage of the insert RNA-tRNA fusion with M1 is carried out in 1 M NaOAc and 25 mM MgCl₂. Typically, the reaction is assembled and the RNAs are refolded by heating to 70–90°C for 2–5 min and then incubated at 37°C for 2–24 h, depending on the extent of cleavage desired. If desired, 0.1% sodium dodecyl sulfate (SDS) can be added to the cleavage reaction to minimize any nuclelease contamination (3).

The titration of M1 ribozyme in Figure 2 indicates that a 1:10 molar ratio of gel-purified M1 ribozyme to substrate cleaves approximately 50% of the substrate after 2 h. Longer digestion times have resulted in near-complete cleavage. Supporting the notion that the tRNA domain is folded primarily into a single appropriate substrate structure. Digestion products are ethanol-precipitated and purified by denaturing polyacrylamide gel electrophoresis as described above.

This technique provides a means of generating RNAs with discrete termini without constraining either sequence or structure. Presently, our laboratory is utilizing both aspects of this technique to generate small RNA-tRNA fusions for primer-extension experiments and using M1-processed transcripts to ensure homogeneous populations of small RNAs for use in UV-melting curves and NMR studies.

REFERENCES


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