As experimental methods in molecular biology have rapidly progressed, the need for simple methodology to create RNA size markers has become increasingly apparent. Ribosomal RNAs are commonly used as size markers for larger RNAs in denaturing agarose gels (9); however, RNA markers are not commercially available for the characterization of <100 nucleotide (nt) RNAs by electrophoresis. This fact, as well as the inherent instability of RNA vs. DNA, prompted the use of DNA size markers in early research. However, denatured DNA strands migrate faster than RNA, introducing a potential source of error in RNA size determination (11,14). For these reasons, several research groups have recently worked to find improved methods for generating RNA size markers of varied lengths.

RNA size estimation has been carried out by visualization of three or four mRNAs of known length using Northern blotting techniques (12) or by using the products of transcription of several DNA templates containing the appropriate promoter sequence using a radioactive nucleotide (8,10). These methods are relatively labor intensive and require considerable manipulation to generate new length markers. A more recent report used a single plasmid, linearized by 13 readily available restriction enzymes to produce 13 DNA templates (1). Transcription provided three sets of RNA markers (148–3621 nt) corresponding to low, medium and high size ranges that provide for significant flexibility in application. However, none of these approaches offers either markers for small RNAs (<100 nt) or a means for generating regularly spaced ladders.

A different method for production of RNA size markers has made use of the inherent cleavage activity of tandem, cis-cleaving hammerhead and hairpin ribozymes to segment a large RNA transcript (2). Transcription of a plasmid that encodes these ribozymes produces RNA that, after ribozyme cleavage, contains discrete RNAs ranging from 83–244 nt. This approach uses only a single plasmid to create six fragments; however, the size range of RNAs produced from this system is relatively small, and ribozyme size variation was not explored.

The above work has made RNA markers more accessible than they once were; however, there remains a need for developing even simpler, more generally useful methods. RNA ladders with regular band spacing would be particularly useful because they would facilitate more accurate size estimation by giving a greater number of markers, thus allowing for better interpolation between markers. Our approach to the synthesis of RNA size markers, like those of previous workers (2), uses ribozyme activity to create a ladder of RNA size markers. However, the method we use to synthesize the RNAs is, to our knowledge, unique. While previous approaches have involved the synthesis of RNA transcripts from a linearized plasmid DNA template, our ribozyme RNAs are produced by rolling circle transcription (RCT) of single-stranded DNA (ssDNA) circles (Figure 1). We have previously shown that RCT can be used to generate both hammerhead (3) and hairpin (5) ribozymes. We now demonstrate that regularly spaced RNA ladders can be obtained by transcription of DNA circles of various sizes (63–83 nt) encoding these ribozyme motifs.

To demonstrate a range of marker sizes, we constructed four differently sized circular ssDNAs, altering domains not required for ribozyme activity. Two of these DNA circles encode hairpin ribozymes (67TRSV and 73TRSV), and two encode hammerhead ribozymes (63ASBV and 83ASBV). These sequences are subsets (or close variants) of known viroids (15) and virusoids (6). A simple “one-pot” cyclization scheme using T4 DNA ligase and short synthetic DNA splints is used for preparation of ssDNA circles (4). Briefly, each ligation proceeds from two short linear DNAs (precursor segments) that are intermoleculary joined to give a linear full-length pre-circle using a templating oligo-
cleotide (splint) to juxtapose reactive ends for the enzymatic ligation. This full-length linear DNA is then intramolecularly ligated to form a circular DNA. Each DNA circle is constructed from crude DNA oligonucleotides in only approximately 16 h and is then purified by gel electrophoresis. A 1-µmol scale oligonucleotide synthesis can yield dozens of nanomoles of circular ssDNA by this approach. Considering each transcription reaction requires only 15 pmol of template, this allows each circle synthesis to provide template for thousands of reactions. The synthesis of these circular templates is not expected to be any more costly or time-consuming to prepare than a plasmid linearized by several restriction enzymes.

### Cyclization Precursors

Synthetic oligodeoxynucleotide precursors for cyclization were as follows; 63ASBV, 5'-pGAG GAC TTG ATC ACT TCG TCT CTT CAG CA-3' and 5'-pAAA TAT GTT TTG GAC CGT TGG TTT CGA CTT GTG A-3'. 67TRSV, 5'-pACA ACG TGT GTT TCT CTG GTT GAC TTC TCT GTG CC-3' and 5'-pTTG CAG GAC TGT CAG GAG GTA CCA GGT AAT ATA CC-3'. 73TRSV, 5'-pTTG AAA CAG GAC TGT CAG GAG GTA CCA GGT AAT ATA CC-3' and 5'-pACA ACG TGT GTT TCT CTG GTT GAC TTG TCT GC-3'. 83ASBV, 5'-pAGA AGG ATC TCT TGA TCA CTT CGT CTC TTC AGG GAA AGA TGG-3' and 5'-pGAG ATG TTC CGA CTT TCC GAC TCT GAG TTT CGA CTT GTG AG-3'. The linear precursor segments listed above and their corresponding 20-nt splints were synthesized on a Model 392 DNA Synthesizer (PE Applied Biosystems, Foster City, CA, USA) using the standard DNA cycle. They were 5'-phosphorylated using Chemical Phosphorylating Reagent (Glen Research, Sterling, VA, USA) (7) and deprotected with ammonium hydroxide (Mallinckrodt Chemical, Chesterfield, MO, USA). After lyophilization, the crude DNAs were used in the ligation-cyclization reactions in which the two precursor segments were twice joined in two successive steps. First-step ligation conditions were as fol-

---

**Figure 1.** Schematic of RCT of synthetic ssDNA circular templates. (A) RCT of synthetic circular ssDNA templates by *E. coli* RNA polymerase produces multimeric repeating RNAs that contain ribozyme cleavage sites. These ribozyme cleavage sites allow for self-processing to give concatemers of linear oligoribonucleotides. (B) Synthetic ssDNA circular templates. The DNA circles are each synthesized starting with approximately half-length precursor segments and with splint oligomers that are 20-nt long and complementary to 10 nt on each of the ends to be ligated.
Benchmarks

follows: 60 µM each precursor segment, 73 µM splint one, 3 U/µL T4 DNA Ligase (New England Biolabs, Beverly, MA, USA), in a 50 mM Tris-HCl, pH 7.5 buffer containing 10 mM MgCl₂, 10 mM dithiothreitol (DTT) and 100 µM ATP. The reaction was incubated at room temperature for 4–6 h. The step 1 solutions were carried on to step 2 (cyclization) without isolation or purification. The conditions for cyclization (step 2) are analogous to those for linear ligation with the following modifications: 1 µM linear precursor (taken directly from step 1 solution), 3 µM splint two (1.2 µM splint one remaining from step 1) and 0.33 U/µL freshly added ligase, with incubation at room temperature overnight. The circular products were isolated by preparative denaturing polyacrylamide gel electrophoresis (PAGE) (3) and characterized by nicking with S1 nuclease; the product has the mobility of the linear full-length precursor for cyclization (13). DNA circle solutions of 15 µM were prepared, and 10-µL aliquots (enough for 10 transcription reactions) were stored at -20°C. These storage conditions maintained activity of the templates for at least several months.

Transcription of each of the four DNA circles is carried out in standard fashion with E. coli RNA polymerase. Alternatively, T7 RNA polymerase can be used; however, of these four circles, it only successfully transcribes 67TRSV and 83ASBV. Although previous work has shown that certain synthetic circular oligodeoxynucleotides can be transcribed by both of these RNA polymerases (3,5), there have been a number of cases of efficient transcription by only one or neither enzyme, which is apparently due to as yet undefined secondary-structure preferences for initiation. The transcription buffer contains 3 mM Mg²⁺ to support both polymerase and the ribozyme activities; 1.5 h of incubation was found to be sufficient to give bands of reasonably good size distribution. The RNAs are readily radiolabeled by uptake of [α-³²P]UTP, and the products are visualized by autoradiography or phosphor-imaging.

Simultaneous with transcription, the resulting concatemeric RNAs undergo self-processing, which generates a regular ladder of bands when the reaction is stopped at 1.5 h. The self-processing produces band distributions that shorten over time; we found that a relatively low level of magnesium (3 mM) helps to minimize this problem, and it is recommended that transcription reactions be used within 72 h to keep product distribution even. Figure 2A shows the results for the four DNA circles described above; a 10% polyacrylamide gel was electrophoresed a short distance (xylene cyanol to 22 cm) to resolve lower molecular weight (mol wt) products for low-range RNA size markers. Figure 2B shows results from a gel run further (xylene cyanol to 34 cm) to allow better resolution of higher mol wt bands. The autoradiograms show monomer-length RNAs as the fastest major bands (63–83 nt), with regularly spaced multimers up to at least 9-mers (567–747 nt) clearly visible. Additionally, with use of a lower percentage acrylamide, the slower mobility bands can be resolved more readily (data not shown).
Rolling circle transcription of ssDNA circles encoding self-processing ribozymes is a novel, simple and rapid means to obtain small, regularly spaced, RNA size marker ladders. To our knowledge, the combination of small-size RNA production represented as a regularly spaced ladder found in our system is currently not available from any single in vitro transcription system. The DNA circle synthesis is easily carried out, and the stability of these DNAs allows for a single synthesis to create ample template for long-term usage.

REFERENCES


Address correspondence to Dr. Eric Kool, University of Rochester, Department of Chemistry, College of Arts & Sciences, Rochester, NY 14627-9000, USA. Internet: etk@etk.chem.rochester.edu

Received 8 June 1998; accepted 13 August 1998.

Amy M. Diegelman, Sarah L. Daubendiek and Eric T. Kool
University of Rochester
Rochester, NY, USA

Combined PCR/Gapped-Duplex Method for Site-Directed Mutagenesis


Here we present a new method for oligonucleotide-mediated, site-directed mutagenesis (SDM) of cloned DNA sequences that combines the high mutagenic efficiency of polymerase chain reaction (PCR)-based methods (2,4,9,10,13) with the cloning flexibility provided by the gapped-duplex method (5).

Mutagenesis techniques involving one mutagenic oligonucleotide and a single-stranded (ss) (8,14) or double-stranded (ds) (5) template have variable efficiencies of mutagenesis. These protocols, however, have the advantage of not requiring specifically located restriction sites. In the gapped-duplex method (5), any pair of restriction sites that flank the region to be mutated is sufficient.

PCR-based mutagenesis (4) has the advantage of using either ss- or dsDNA as template and of producing large quantities of amplified ds products containing the desired sequence. For further manipulation, it is often desirable or necessary to clone these mutant PCR products into plasmid or phage vectors. This cloning step requires appropriate unique restriction enzyme sites that are either present naturally in the cloning vector and in the amplified sequence or that were specifically designed into the primers used for amplification. On occasion, this seemingly trivial cloning step presents unforeseen difficulties for any one of a number of reasons, including: (i) absence of appropriate naturally occurring restriction sites in the amplified sequence, (ii) the use of amplification oligonucleotides with new restriction sites that may introduce unwanted mutations (however, see Reference 13) and (iii) inefficiency of ligation of the amplified PCR product into the parental vector after restriction enzyme digestion.

To overcome such difficulties, we have successfully used a variation of the gapped-duplex method for oligonucleotide-mediated mutagenesis (5) in which ds PCR product containing the desired mutation is used instead of a ss mutagenic oligonucleotide. Figure 1 shows the general scheme of the protocol.