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Use of *cis*- and *trans*-ribozymes to remove 5′ and 3′ heterogeneities from milligrams of *in vitro* transcribed RNA

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In vitro transcription with phage T7 RNA polymerase is the method of choice for obtaining multi-milligram quantities of RNA for structural studies. However, run-off transcription with this enzyme results in molecules that are heterogeneous at their 3′-, and depending on template sequence, 5′-termini. For transcripts longer than ~50 nucleotides (nt), these impurities cannot be removed by preparative purification techniques. Use of *cis*-delta, or *trans*-VS ribozymes allows preparation of homogeneous RNA with any 3′-terminal sequence. If present, 5′ heterogeneity can be overcome with a *cis*-hammerhead ribozyme.

During the course of a structural investigation of Group II self-splicing introns, we sought to prepare a 70 nt RNA molecule comprising domains V and VI (d56) of the ai5γ intron (Fig. 1). Run-off transcription from a plasmid linearized with the restriction enzyme *Bsa*I to generate a DNA terminus ...TAGCC-3′ on the template strand resulted in six to eight different molecules, the shortest of which (~30% of the full-length transcript) is the desired product (Fig. 2, lane A). Although resolved on an analytical gel, these different molecules could not be separated on a preparative scale either by gel electrophoresis or chromatography, making the resulting RNA inadequate for biophysical studies. Addition of random nucleotides to the 3′-terminus of run-off transcripts by T7 RNA polymerase is well documented (1); we chose to use a ribozyme to cleave the 3′-end of our transcript to make it homogeneous.

The hammerhead (HH) and hairpin ribozymes have been employed previously to cleave transcripts (2, and references therein). However, the well-characterized hammerhead has sequence requirements 5′ to the cleavage site that are incompatible with our desired product. This catalytic RNA needs the sequence UX (X ≠ G) to precede the cleavage site. The hairpin ribozyme, which needs (G/C/U)N instead, is prone to aberrant cleavage (2). Furthermore, when used to remove 3′-termini, both require sequence complementarity with nucleotides internal to the product, necessitating ribozymes of different sequence for cleaving different constructs. In contrast, the hepatitis delta virus ribozyme (δ) and the *Neurospora* Varkud satellite RNA ribozyme (VS) have minimal sequence requirements: δ will cut after any base other than G (3, and references therein), while VS will cleave efficiently after any base other than C (4). Thus, these two ribozymes used in concert should allow cleavage after any desired sequence.

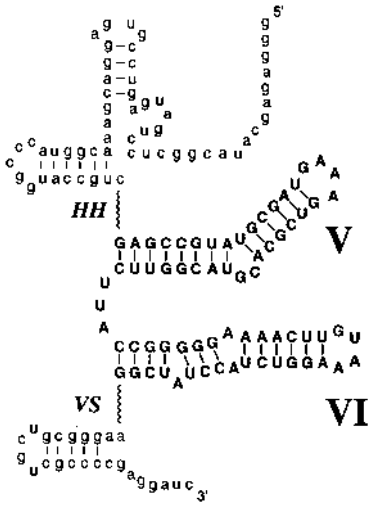

Figure 1. Schematic representation of the hammerhead-d56-VSsl construct. The sequences of domains V and VI are shown in upper case letters, in their conventional base-paired representation. The hammerhead ribozyme (HH) and VS ribozyme substrate stem–loop (VSsl) are in lower case. Bonds that are cleaved by the ribozymes are shown in broken lines. Note that the first 11 nt of domain V are complementary to a portion of HH. VSsl is longer than that of (4) because the template plasmid was linearized with *Bam*HI rather than *Ava*I.

Run-off transcription from a template encoding d56 followed by the 24 nt substrate stem–loop required by VS to cut in *trans* (VSsl) resulted in the expected 94 nt product. In the presence of the *trans*-acting ribozyme RNA, the desired 70 nt d56 was obtained, contaminated, however with ~10% of RNAs that appear to be 1 and 2 nt longer (Fig. 2, lanes C and D). To investigate if these were the result of aberrant cleavage by VS, we transcribed a d56 which is followed by a single 3′-terminal U, in turn followed by the δ ribozyme. This resulted in a similar pattern of contaminants (Fig. 2, lane B). These longer products are not artifacts of partial dephosphorylation, since acid treatment of the full-length RNA to open the 2′–3′ cyclic phosphate followed by phosphatase treatment preserves the pattern, with slightly slower mobilities overall (data not shown).

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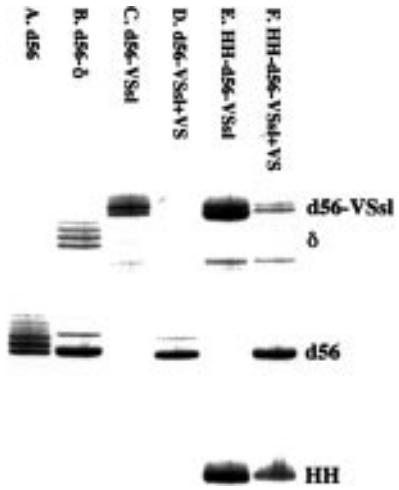

Figure 2. Production of homogeneous d56 RNA with use of *cis*- and *trans*-ribozymes. Lane A, d56; lane B, d56-δ; lane C, d56-VSsl in the absence of VS ribozyme; lane D, as C with VS ribozyme added; lane E, HH-d56-VSsl without VS ribozyme; lane F, same as E, with VS added. Transcription reactions (10 µl each) contained 50 µg/ml linearized plasmid DNA, 25 mM $MgCl_2$, 2 mM spermidine, 30 mM Tris–HCl pH 8.1, 10 mM DTT, 0.01% Triton X-100, 2.5 mM of each nucleotide triphosphate, enzyme, and trace amounts of [α-^{32}P]ATP, and were incubated for 2 h at 37°C. Reactions on lanes C and F also contained 20 µg VS ribozyme. After addition of 90% formamide–1× TBE and heating to 90°C for 2 min, reactions were analyzed directly on a 10% polyacrylamide–8 M urea gel, and visualized by autoradiography on imaging plates. The δ ribozyme used has the sequence of Rz89cc described by (3). The positions of HH (the self-cut hammerhead ribozyme), d56, δ (the self-cut δ ribozyme), and d56-VSsl (d56 followed by the VS ribozyme substrate stem–loop) are indicated.

In order to determine the nature of this residual heterogeneity, the dephosphorylated RNAs were 5′-end-labeled, separated on a denaturing gel, excised individually, and subjected to partial RNase T1 digestion. This revealed that they differ exclusively at their 5′-terminus (data not shown). 5′-terminal heterogeneity in T7 RNA polymerase transcripts is thought to result from incorporation of abortive dinucleotides and trinucleotides during initiation of further rounds of transcription (5). We examined the effect of varying the nucleotide triphosphate concentrations during transcription on the ratio of product to contaminants, without succeeding in obtaining a more pure product.

The problem was solved by incorporating a hammerhead ribozyme on the 5′-end of d56 together with the VSsl at the 3′-terminus (Fig. 1). The hammerhead ribozyme cleaved itself off the product almost completely during the course of a 2 h transcription (Fig. 2, lane E). Addition of VS ribozyme in *trans* resulted in the generation of a homogeneous d56 RNA of the expected mobility (Fig. 2, lane F). We note that the hammerhead ribozyme itself is uniform in length, implying it has a homogeneous 5′-terminus.

The analytical transcription of the homogeneous d56 presented in Figure 2 was carried out in the presence of an approximately equimolar amount of separately transcribed and purified VS ribozyme. Titration experiments showed that after incubation for 4 h under transcription conditions, one-tenth of this amount of ribozyme achieved complete cleavage of substrate (data not shown). For large scale (20 ml) transcription reactions, we found that simultaneous transcription, with a 1:10 ratio of plasmids encoding the *trans*-VS ribozyme and HH-d56-VSsl, resulted in almost quantitative conversion of precursor into processed, homogeneous d56, which was easily separated from the ribozymes and cleaved VSsl by preparative denaturing polyacrylamide gel electrophoresis. The yield of purified d56 was ~0.5 mg RNA/ml transcription reaction.

The use of the *trans*-acting VS ribozyme for the preparation of large quantities of homogeneous RNA transcripts that we introduce here has several advantages over the previously well-documented use of *cis*-hammerhead ribozymes. First, the VS ribozyme has minimal sequence requirements 5′ to the cleavage site. Secondly, introduction of the VS substrate stem–loop into the template is accomplished readily, using PCR, with a 3′ primer of modest length. Thirdly, one *trans*-acting VS ribozyme can be used to cleave multiple RNA constructs, and even recycled for further reactions. Fourthly, in many cases, it is sufficient simply to transcribe in the same vessel the RNA of interest and the VS ribozyme. Finally, when valuable nucleotides (for instance, uniformly isotopically labeled nucleotides for NMR spectroscopy) are employed in transcription, use of a *trans*-acting ribozyme prepared separately with conventional nucleotides would allow considerable savings of precursors, which would otherwise be incorporated into a *cis*-ribozyme.

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