Cotranscriptional 3'-end processing of T7 RNA polymerase transcripts by a smaller HDV ribozyme

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Abstract

In vitro run-off transcription by T7 RNA polymerase generates heterogeneous 3'-ends because the enzyme tends to add untemplated adenylates. To generate homogeneous 3'-termini, HDV ribozymes have been used widely. Their sequences are added to the 3'-terminus such that co-transcriptional self-cleavage generates homogeneous 3'-ends. A shorter HDV sequence that cleaves itself efficiently would be advantageous. Here we show that a recently discovered, small HDV ribozyme is a good alternative to the previously used HDV ribozyme. The new HDV ribozyme is more efficient in some sequence contexts, and less efficient in other sequence contexts than the previously used HDV ribozyme. The smaller size makes the new HDV ribozyme a good alternative for transcript 3'-end processing.

Introduction

T7 RNA polymerase is an important tool in RNA biochemistry, by transcribing long sequences of RNA from DNA templates that contain the 17 base pair long promoter (Milligan et al., 1987). However, this enzyme tends to generate heterogeneous 3'-termini, consisting of untemplated adenylates (Milligan and Uhlenbeck, 1989) or more complicated sequences that result from self-priming of the 3'-terminus (Triana-Alonso et al., 1995; Zaher and Unrau, 2004).

Homogeneous 3'-termini are sometimes required for the pool molecules of in vitro selection and evolution experiments. If the selection procedure requires catalysis to occur at an RNA 3'-terminus then heterogeneity at this 3'-terminus can result in a large fraction of the pool being inactive, and a consequent loss of pool complexity. Ribozymes that react at their 3'-termini have been selected multiple times (Curtis and Bartel, 2005; Illangasekare et al., 1995; Lau et al., 2004; Saito and Suga, 2001; Unrau and Bartel, 1998), and in one case an HDV ribozyme was used to generate homogeneous 3'-termini of the pool RNA (Chumachenko et al., 2009). In addition to generating homogeneous 3'-termini, these ribozymes (or deoxyribozymes) usually generate 2',3'-cyclic phosphates and 5'-hydroxyl groups. The 2',3'-cyclic phosphates can be used as chemical activation group to react with 5'-hydroxyl groups and form linear 3',5'-phosphordiester bonds. This has been used during the in vitro selection of catalytic DNAs ligating 2',3'-cyclic phosphates with 5'-hydroxyl

groups (Hoadley et al., 2005; Kost et al., 2008; Semlow and Silverman, 2005). These examples show that the efficient processing of RNA 3'-termini is an important step for a range of in vitro selection experiments.

To generate homogeneous 3'-termini in transcripts of T7 RNA polymerase, different ribozymes and deoxyribozymes have been used (Avis et al., 2012). The cis-acting HDV ribozyme is widely used due to its convenience: The sequence of the ribozyme is simply added to the 3'-end of the desired transcript. During co-transcriptional self-cleavage, the HDV ribozyme cleaves itself off the desired sequence at one defined position (Ferre-D'Amare and Doudna, 1996; Walker et al., 2003). Other ribozymes that have been used for 3'-end processing are the hammerhead ribozyme (Price et al., 1995), the hairpin ribozyme (Price et al., 1995), and the VS ribozyme (Ferre-D'Amare and Doudna, 1996). However, the hammerhead ribozyme and the hairpin ribozyme have a significant sequence requirement on the desired transcript, and the hairpin ribozyme is prone to aberrant cleavage (Price et al., 1995). The HDV ribozyme is most general because it will cut efficiently after any nucleotide other than G (Tanner et al., 1994), where cleavage kinetics are ~10-fold slower (Kellerman et al., 2015). Different upstream sequences appear to interfere with the ribozyme's formation of its P1.1 helix (Figure 1A), leading to reduced ribozyme efficiency (Chadalavada et al., 2000; Jeong et al., 2003; Mörl and Hartmann, 2014; Nishikawa et al., 1999; Ruminski et al., 2011).

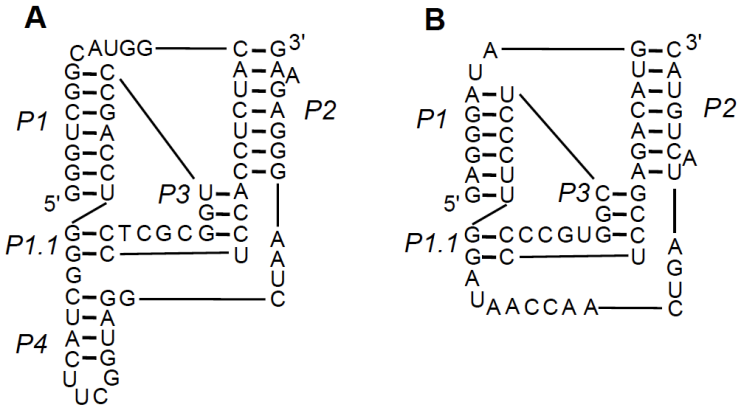

Figure 1. Secondary structure representations of the two HDV ribozyme variants compared in this study. **(A)** The previously shortest HDV ribozyme (Schurer et al., 2002) with a length of 67 nt (here termed HDV67). The secondary structure was based on the sequence / secondary structure consensus model given in Riccitelli et al. (Riccitelli et al., 2014). **(B)** The small HDVdm3 ribozyme with a length of 56 nt (here termed HDV56), with the secondary structure given in Riccitelli et al. (Riccitelli et al., 2014).

The ideal HDV ribozyme for homogeneous 3'-termini would be small so that it does not significantly increase the size of the transcript, and its co-transcriptional self-cleavage would be efficient and sequence general. Previously used HDV ribozymes have lengths of 67 nucleotides (Schurer et al., 2002), 84 nucleotides (Avis et al., 2012), and 86 nucleotides (Ferre-D'Amare and Doudna, 1996). In several cases the self-cleavage efficiency is low. This can be addressed partially by temperature cycling of the transcription mixture between 25°C and 60°C for ~10 times, with or without increase of the total magnesium ion concentration to 30-40 mM $MgCl_2$ (Mörl and Hartmann, 2014). In our hands such incubation conditions led to significant degradation of the desired transcript (data not shown).

Previously, a shorter variant of the HDV ribozyme was identified in a metagenomic data set of human sewage by a systematic, computational search for truncated HDV ribozyme variants (Riccitelli et al., 2014). This HDV ribozyme (drz-Mtgn-3) shows cleavage kinetics on par with many larger HDV ribozymes (Webb et al., 2009) and has a size of only 56 nucleotides. This ribozyme is the focus of this study, and was termed HDV56 for simplicity.

The current study examined the HDV56 ribozyme for its ability to process 3'-termini of transcripts by T7 RNA polymerase, and compared it to the performance of the shortest previously used, 67-nucleotide long HDV ribozyme variant (Mörl and Hartmann, 2014; Schurer et al., 2002), which we termed HDV67. When compared on three different upstream sequences, HDV56 showed the highest and the lowest efficiency between the two ribozymes. To obtain a more general statement regarding the sequence generality, a random sequence with 30 nucleotides was inserted upstream of the HDV ribozyme variants. From this unbiased library, HDV56 generated the same amount of product with the correct length as the HDV67 ribozyme, within error. Together, these results show that HDV56 is a shorter alternative for HDV ribozymes that generate homogeneous 3'-end by co-transcriptional processing.

Results

To test whether the small HDV ribozyme HDV56 (Riccitelli et al., 2014) is not only smaller than previously studied HDV ribozymes but also similarly efficient for in vitro co-transcriptional 3'-end processing, the HDV56 sequence was cloned to the 3'-terminus of three unrelated sequences. These three sequences were the highly structured RNA 5'-triphosphorylation ribozyme TPR1e (96 nt), which was developed by in vitro selection (Dolan et al., 2015), and two less structured RNAs: A 115-nucleotide long portion of the mRNA of *E. coli* EF-Tu2, and a 191-nucleotide long portion of the mRNA for *E. coli* DNA polymerase III subunit alpha. Upstream of each sequence, the promoter for T7 RNA polymerase was added to facilitate in vitro transcription. The HDV67 ribozyme (Mörl and Hartmann, 2014; Schurer et al., 2002) was used as comparison to the HDV56 sequence. The sequence of each construct was confirmed by cloning into the vector pUC19 and sequencing. PCR amplification of these constructs resulted in PCR product lengths of 173 - 279 base pairs. These PCR products were used as templates for the transcription reactions.

The transcription products were internally labeled through the presence of $\alpha[^{32}P]$-ATP in the transcription reaction. During transcription, samples were taken at different times, and separated by denaturing polyacrylamide gel electrophoresis (figure 2A). This procedure allowed following the buildup of full-length transcripts, as well as the processed transcripts. The parameter of most importance to an application of this ribozyme is the molar amount of processed RNA produced with the correct length. This amount was highest after one hour for all three upstream sequences, therefore the one-hour time point was used to compare the constructs.

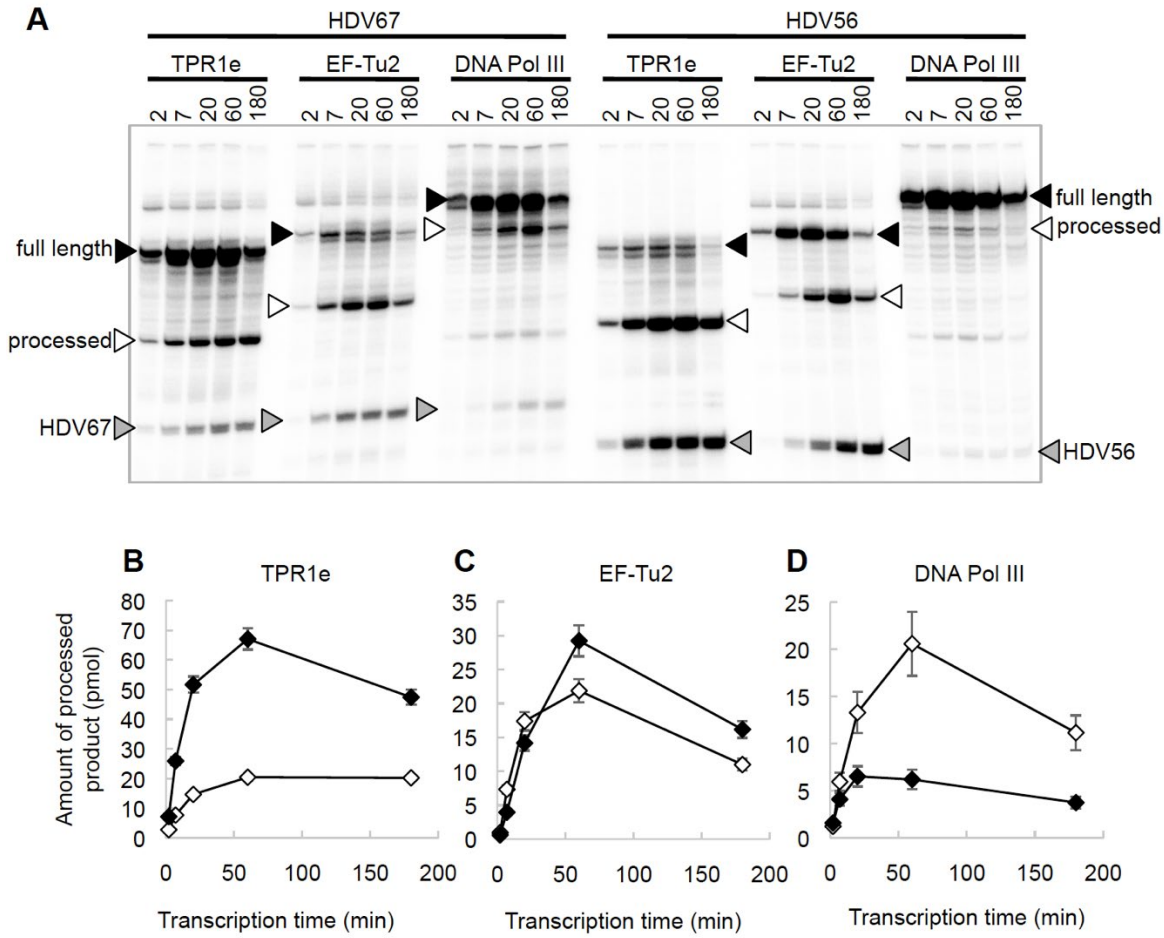

Figure 2. Co-transcriptional 3'-processing by HDV67 and HDV56, using three different sequences upstream of the HDV ribozyme. **(A)** Phosphorimage of reaction products separated by 15% polyacrylamide gel electrophoresis. Samples were taken from the transcription reactions at the indicated time points. The signals stem from internal labels caused by traces of α-[^{32}P] ATP included in the transcription mixture. The three time courses on the left stem from the HDV67 ribozyme, the three time courses on the right from the (new) HDV56 ribozyme. For each ribozyme, the upstream sequence is indicated with (from left to right) the triphosphorylation ribozyme TPR1e, a 115-nucleotide fragment of the mRNA for *E. coli* eF-Tu2, and a 191-nucleotide fragment of *E. coli* DNA polymerase III. **(B-D)** Quantitation of processed transcript, as a function of transcription time. The previously used HDV67 ribozyme (white symbols) and the new HDV56 ribozyme (black symbols) are shown for each of the three upstream sequences. Error bars denote standard deviations from three replicate experiments. In cases where the error bars are not visible they are smaller than the symbols.

The processed sequence TPR1e was produced 3.3-fold more efficiently with HDV56 than with HDV67 (Figure 2B). The processed sequence EFTu2 was produced 1.3-fold more efficiently with HDV56 than with HDV67 (figure 2C). The processed sequence DNA pol III was produced 3.3-fold more efficiently with HDV67 than with HDV56 (figure 2D). These results show that HDV56 and HDV67 have a different preference for upstream sequences. However, the number of three different upstream sequences was not sufficient to obtain a general picture about the processing efficiency of the two tested HDV ribozyme variants.

To arrive at a more general statement on the 3'-processing efficiency, a random sequence with 30 nucleotides was inserted upstream of the HDV ribozyme variants. Co-transcriptional processing resulted in the same cleavage efficiency by HDV56 when compared to HDV67, within error (figure 3).

These results show that the average cleavage efficiency of HDV56 is similar to that of HDV67. Due to its shorter size, HDV56 may be the first choice to test for 3'-end processing of a new transcript.

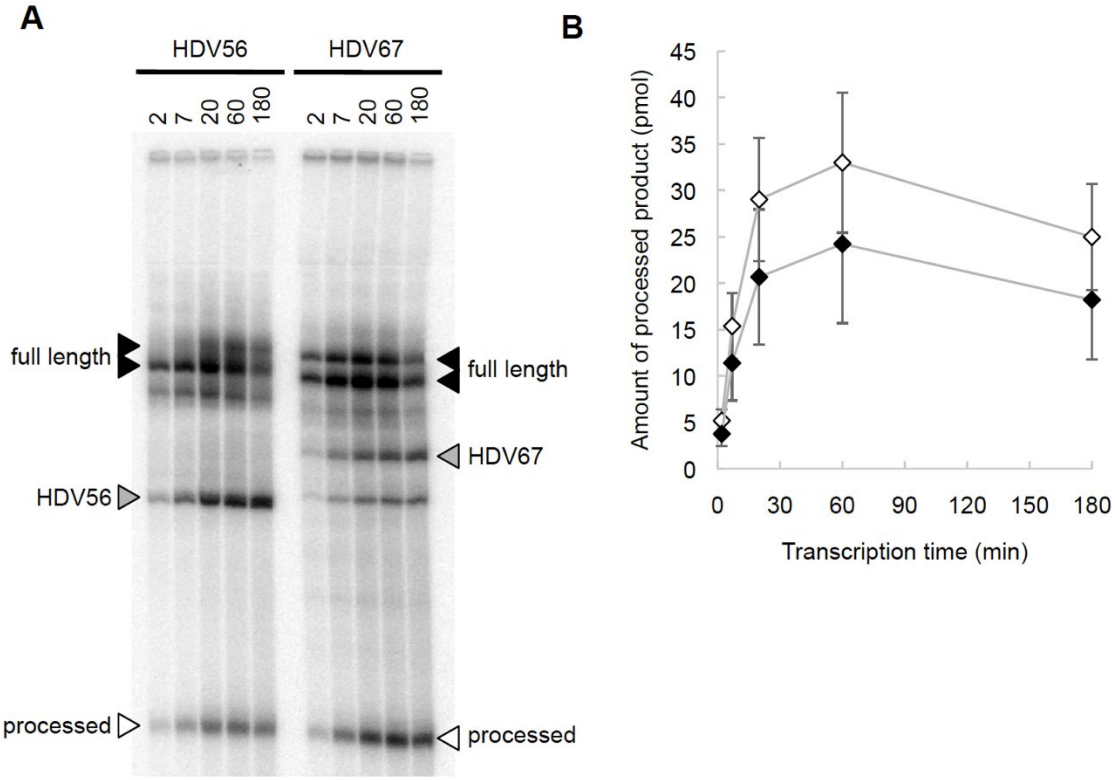

Figure 3. Co-transcriptional 3'-processing by HDV67 and HDV56, using a randomized N30 sequence upstream of the HDV ribozyme. **(A)** Phosphorimage of reaction products separated by 15% polyacrylamide gel electrophoresis. Samples were taken from the transcription reactions at the indicated time points. The signals stem from internal labels caused by traces of α-[^{32}P] ATP included in the transcription mixture. The identity of each ribozyme is indicated on the top. **(B)** Quantitation of processed transcript, as a function of transcription time. The molar amount of processed product was calculated from the signal strengths using a separate calibration (see materials and methods). The previously used HDV67 ribozyme is shown in white symbols and the new HDV56 ribozyme is shown in black symbols. Error bars denote standard deviations from three replicate experiments. In cases where the error bars are not visible they are smaller than the symbols.

Discussion

Our study found that HDV56 is a good alternative to the HDV67 ribozyme for co-transcription processing. The availability of multiple alternative HDV ribozymes for a given upstream sequence is useful because different upstream sequences appear to lead to differences in inefficient folding of the ribozyme (Mörl and Hartmann, 2014); the different effect of upstream sequences on HDV ribozymes was confirmed in our study (see figure 2). Importantly, our study showed that HDV56 and HDV67 showed a different preference of three upstream sequences. This characteristic may be useful because if one upstrem sequence does not work well with one ribozyme then the other ribozyme may be efficient.

The preference of upstream sequences is not explained well by a previously identified rule. This rule states that the sequence immediately upstream of the cleavage site can extend the P1 helix, and

thereby interferes with the formation of the correct P1.1 helix (Chadalavada et al., 2000; Jeong et al., 2003; Nishikawa et al., 1999; Ruminski et al., 2011) (see figure 1). In our study, this rule explained the slight advantage of HDV56 over HDV67 on the substrate EF-Tu2 but not the stronger differences between the ribozymes on the substrates TPR1e, and DNA Pol III: For both ribozymes HDV56 and HDV67, the two nucleotides immediately upstream of the cleavage site would have to pair to a GG sequence in the ribozymes, and therefore should not cause a difference in extending the P1 helix. Only the third upstream position the cleavage site should be able to cause a difference, which could pair with a G in HDV56 and with an A in HDV67 (see figure 1). For clarity, the third upstream nucleotide is underlined in the following three tested upstream sequences. First, the upstream sequence 5'-CCU-3' of EF-Tu2 could allow an extension of the P1 helix in HDV67 by three canonical base pairs, and only two canonical base pairs in HDV56. Therefore, HDV67 could be inhibited more by the upstream sequence, explaining the slight advantage of HDV56 over HDV67 on EF-Tu2. In contrast, the upstream sequence TPR1e (5'-TAA-3') cannot explain the much higher cleavage efficiency of HDV56 than HDV67 because both ribozymes would not be able to extend the P1 duplex by a single base pair. Similarly, the upstream sequence of DNA Pol III (5'-UGC-3') cannot explain the lower efficiency of HDV56 than HDV67 because both ribozymes could be extended by only one G:C base pair, with the second position (a G:G pair) destabilizing an elongation of the P1 duplex. These three cases show that additional rules still need to be identified to describe the influence of upstream sequences for the cleavage efficiency of HDV ribozymes. Therefore, some differences in processing efficiencies may not be due to secondary structure formation but due to the sequence itself at the processing site. While classical HDV ribozymes show no strong sequence preference except against G (Kellerman et al., 2015; Tanner et al., 1994) it is possible that the loss of the P4 helix renders the HDV56 ribozyme more sensitive to the upstream sequence.

The efficiency of HDV67 appears to be rather constant across the three individual, tested sequences (always around 20 pmol after 1 hour transcription) while the efficiency of HDV56 is more efficient on substrate TPR1e and less efficient on substrate DNA polymerase III. This suggests that HDV67 is more robust against different upstream sequences than HDV56, and that HDV56 is able to achieve higher cleavage efficiency than HDV67 for sub-groups of substrates (see figure 2A). The identification of molecular causes for these behaviors - or the evolutionary optimization of these ribozymes - may be able to identify HDV variants that are as small as HDV56, display the sequence generality of HDV67, and the high efficiency shown by HDV56 on the substrate TPR1e.

To further improve the efficiency of 3’-end processing by HDV ribozymes it is promising to titrate the Mg^{2+} concentration in the transcription reaction. The assays in this study used the relatively high free Mg^{2+} concentration of 10 mM, resulting from 26 mM total Mg^{2+} and 4 mM of each NTP. This is higher than the standard procedure recommended earlier (Milligan and Uhlenbeck, 1989) with 6 mM free Mg^{2+} (4 mM each NTP and total 22 mM Mg^{2+}). While the HDV ribozyme can be more efficient at Mg^{2+} concentration below 1 mM (Rosenstein and Been, 1990), the T7 RNA polymerase benefits from higher Mg^{2+} concentrations (Lykke-Andersen and Christiansen, 1998). Therefore we recommend testing different Mg^{2+} concentrations for the co-transcriptional processing by HDV ribozymes.

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Materials and Methods:

Preparation of DNA constructs:

The constructs for co-transcriptional 3'-processing were generated by cloning the sequence of TPR1 (96 nt), a 115-nucleotide long fragment of EF-Tu2, and a 191-nucleotide long fragment of DNA Pol III into the vector pUC19. The products were confirmed by sequencing. These plasmids were used as templates to attach the sequence for the T7 promoter at the 5'-terminus, and the sequence for HDV56 or HDV67 at the 3'-terminus. The PCR product lengths were confirmed to be of the right length by agarose gel electrophoresis, and the PCR products were purified on silica-adsorption columns (Machery-Nagel, #740609), to be used as template for the transcription assays.

To generate the DNA template for transcription of partially randomized sequences, PAGE-purified 69-nucleotide long DNA oligonucleotides (IDT) were used as template for PCR amplication of the sequences [T7 promoter]-[GGG]-[N_{30}]-[HDV56] or [T7 promoter]-[GGG]-[N30]-[HDV67]. The sequence GGG was added upstream of the randomized sequence to allow for efficient transcription. The sequence of the HDV ribozymes was completed through the sequence of the 56-nucleotide long or 67-nucleotide long 3'-PCR primer for HDV56 and HDV67, respectively. The PCR products were purified on silica-adsorption columns (Machery-Nagel, #740609), to be used as template for the transcription assays.

Co-transcriptional 3'-processing assay:

For co-transcriptional 3'-processing, 100 nM DNA template was incubated at 37°C with 26 mM $MgCl_2$, 2.5 mM Spermidine, 0.01% (w/v) Triton X-100, 1 mM of each NTP, 5 mM DTT, and 40 mM Tris/HCl pH 7.9, with trace amounts of α-[^{32}P]-ATP for quantitation, in a total volume of 20 μL. Aliquots of 3.5 μL were removed after indicated time points and quenched with 3.5 μL of formamide PAGE gel loading buffer (90% (v/v) formamide, 45 mM Tris(hydroxymethyl) aminomethane, 45 mM boric acid, and 30 mM Na_2EDTA). Five μL of each timepoint were then loaded onto a 15% denaturing polyacrylamide gel. After separation by electrophoresis, the gel was exposed to a phosphorimaging screen (Kodak screen-K) and scanned on a Personal Molecular Imager (Bio-Rad).

Data processing:

The signals from scanning the phosphorimaging screens were quantitated with the Quantity One software using the 'rectangle' method and background subtraction. To convert the signals (cpm) to molar amounts, transcription experiments were performed for each construct in triplicate, with a 1-hour incubation time and without radiolabel. The products were separated by 15% PAGE and detected by UV shadowing. The band corresponding to processed RNA was excised, the RNA eluted, and quantified by its absorption at 260 nm. The resulting molar amount, and its ratio with the cpm of the same band after one hour transcription was used to used to calculate the molar amount of each band.

Sequences:

The following sequences were used in this study: TPR1e: 5'-
GAGACCGAGATGTTTTTCCCCCGATTACAAGTGTGCCTAAAGGGCTACGGACTCTATTAGAAATG
AGGAGTTCGTTGGGTTTATAAGCACACATAA. EF-Tu2: 5'-
GGAAGTTCGTGAACTTCTGTCTCAGTACGACTTCCCGGGCGACGACACTCCGATCGTTCGTGGT
TCTGCCTGAAAGCGCTGGAAGGCGACGCAGAGTGGGAAGCGAAAATCCT. DNA polymerase III subunit a: 5'-
GGGATGGTGGATAACTTTATCGACCGTAAACATGGTCGTGAAGAGATCTCCTATCCGGACGTAC
AGTGGCAGCATGAAAGCCTGAAACCGGTACTGGAGCCAACCTACGGCATTATCCTGTATCAGGA
ACAGGTCATGCAGATTGCGCAGGTGCTTTCTGGTTATACCCTCGGTGGCGCGGATATGCTGC.
Randomized sequence: 5'-GGG-N_{30}. HDV67: 5'-
GGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCTACTTCGGTAGGCTAAGGGA

GAAG, HDV56: 5'-
GAGGGATAGTACAGAGCCTCCCCGTGGCTCCCTTGGATAACCAACTGATACTGTAC. A plasmid with the sequence of TPR1e was present in the Muller lab from a previous study (Dolan et al., 2015). A plasmid with the sequence for EF-Tu2 was a generous gift from the lab of Simpson Joseph (UCSD). The sequence for DNA polymerase III subunit alpha was amplified by PCR from *E. coli* genomic DNA. All other sequences were assembled by PCR using DNA oligonucleotide primers.

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