Supplementary Online Material:

Material and Methods:

RNA synthesis and labeling

Hammerheads were generated by *in vitro* transcription of the corresponding recombinant plasmids digested with *BamHI* (see Material and Methods in the main text). After denaturing PAGE in 5% gels, 250 ng of each gel-eluted hammerhead were heat-denatured (95°C for 1.5 min) and snap-cooled on ice, and then dephosphorylated using 20 U of calf intestinal alkaline phosphatase (CIAP, Roche) according to the manufacturer instructions. Following incubation at 50°C for 30 min, RNAs were extracted with buffer-saturated phenol (Tris-HCl 10 mM pH 7.5, EDTA 1 mM and SDS 0.1%), recovered by ethanol precipitation and resuspended in deionized sterile water. Dephosphorylated hammerheads were 5′-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Perkin Elmer) and T4 polynucleotide kinase (Fermentas) (1), gel-filtrated using mini Quick Spin™ columns (Roche) and recovered by ethanol precipitation. The integrity and concentration of 5′-labeled hammerheads were assessed by denaturing PAGE in 15% gels and silver-staining and labeling was evaluated by gel scanning with a Phosphoimager.

RNA ladders

RNA ladders were generated by incubation with $NaHCO_3/Na_2CO_3$ as described previously (2, 3) with some modifications. Briefly, 2-5 ng of 5′-labeled hammerheads were heat-denatured (95°C for 1.5 min) and immediately snap-cooled on ice. After 5 min, the remaining components of the reaction (50 mM $NaHCO_3/Na_2CO_3$ pH 9, 1 mM EDTA pH 8, and 1 μg of a mixture of 16S and 23S ribosomal RNAs from *Escherichia coli*, Roche, in a final volume of 30 μl) were added, mixed and incubated at 95°C. Aliquots were removed at 2, 5, 10 and 15 min, and quenched with 1 volume of gel loading solution (10 M urea, 1.5 mM EDTA pH 8, 0.25% w/v bromophenol blue and xylene cyanol).

T1 RNase protection assay

0.5 ng of 5′-labeled hammerhead RNAs were heat-denatured at 95°C for 2 min in 5 mM Tris-HCl pH 8 containing EDTA 0.5 mM, a 50-fold molar excess of the short oligonucleotides (RF-979 or RF-994) or the long monomeric PSTVd (-) RNA and a mixture of 16S and 23S RNAs to reach a final mass of 240 ng in volume of 12 μl. Additional reactions without substrate, or including a short non-substrate oligonucleotide (RF-494 5′-AGAGUCUGUGC-3′) or a long non-substrate RNA (333 nt) of *Eggplant latent viroid,* were used as controls. After the initial denaturation step, the samples were snap-cooled on ice for 10 min, mixed with 6 μl of 3.3X RNA folding mix (333 mM HEPES pH 8, 20 mM $MgCl_2$ and 333 mM NaCl) (4), and divided into four aliquots to which decreasing amounts (0.5, 0.05, 0.005 and 0 units) of T1 RNase (Ambion´s RNA grade ribonucleases, Ambion) were added. Following incubation at 25°C for 15 min, RNAs were recovered by ethanol precipitation, resuspended in deionized sterile water, mixed with 3 volumes of gel loading buffer B solution and heat-denatured, and fractionated by denaturing PAGE in 15% gels with the appropriate RNA ladders. The resulting bands were scanned with a Phophoroimager analyzer.

References

1. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989). Molecular cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Knapp,G. (1989). Enzymatic approaches to probing of RNA secondary and tertiary structure. *Methods Enzymol*., **180**, 192–212.
3. Soukup,G.A. and Breaker,R.R. (1999). Relationship between internucleotide linkage geometry and the stability of RNA. *RNA*, **5**, 1308-1325.
4. Wilkinson,K.A., Merino,E.J. and Kevin M Weeks,K.M. (2006). Selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution. *Nature Protocols*, **1,** 1610-1616.

Figure Legends

Supplementary Figure S1. RNAse T1 digestion profile of the discontinuous hammerhead HHd-ELVd-UA/UACG and its potential complexes with different RNA substrates. (A) Schematic representation of the hammerhead secondary structure of minimal free energy inferred by the Mfold program. Predicted RNAse T1 cleavage sites are indicated with arrowheads, with strong and weak (or absent) sites denoted with black and grey arrowheads, respectively. (B) Schematic representation of the complex formed by the hammerhead and the short substrate (SS) RF-979. Hammerhead and substrate nucleotides are shown with black and red fonts, respectively. RNAse T1 cleavage sites are marked as above, and bands indicating formation of the hammerhead-substrate complex are denoted with blue arrowheads: filled for new bands and empty for bands with decreased intensity. Plasmid nucleotides flanking the 3' end are in lower case. (C) RNAse T1 digestion products analyzed by denaturing PAGE in 15% gels. Decreasing RNAse T1 amounts (0.05 and 0.005 U, respectively) are represented with a right-angled triangle on the top. Symbol (Ø) refers to reactions without substrate and abbreviations SS, SNS, LS and LNS refer to the short-substrate RNA (RF-979), to a short non-substrate RNA (RF-494), to the long substrate PSTVd (-) monomeric RNA, and to the long non-substrate ELVd (+) monomeric RNA, respectively. RNAse T1 cleavage sites are indicated as before with their size (in nucleotides) at the sides of the gel. L refers to the ladder generated by alkaline partial cleavage of the hammerhead RNA. Other details as in Figures 1 and 2.

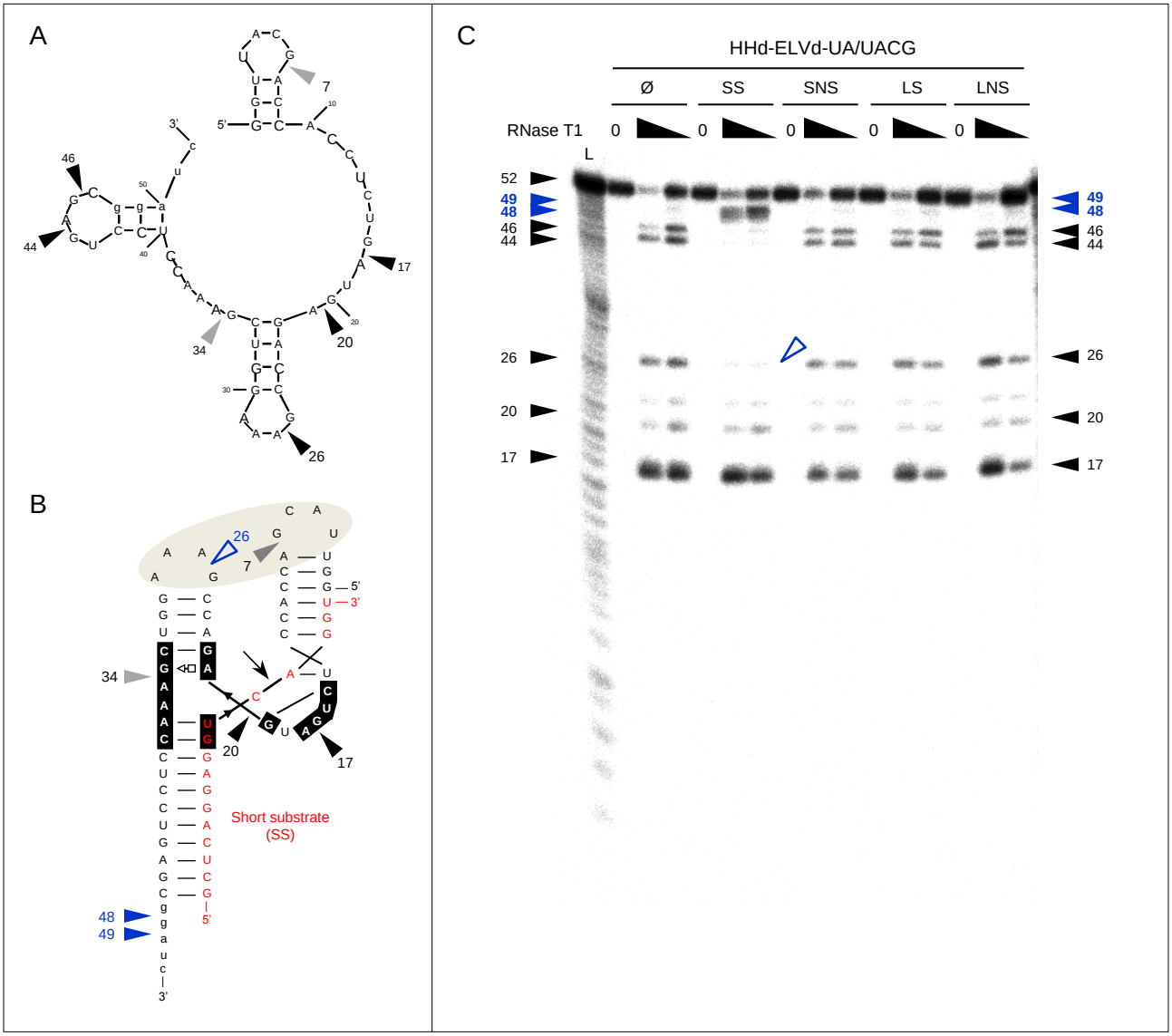
Supplementary Figure S2.

RNAse T1 digestion profiles of three discontinuous hammerheads and its potential complexes with different RNA substrates analyzed by denaturing PAGE in 15% gels. (A) HHd-ELVd-UA/GUGU, (B) HHd-ELVd-UA/UACG and (C) HHd-ELVd-CG/AAAA. Other details as in Supplementary Figure S1, with the exception that the

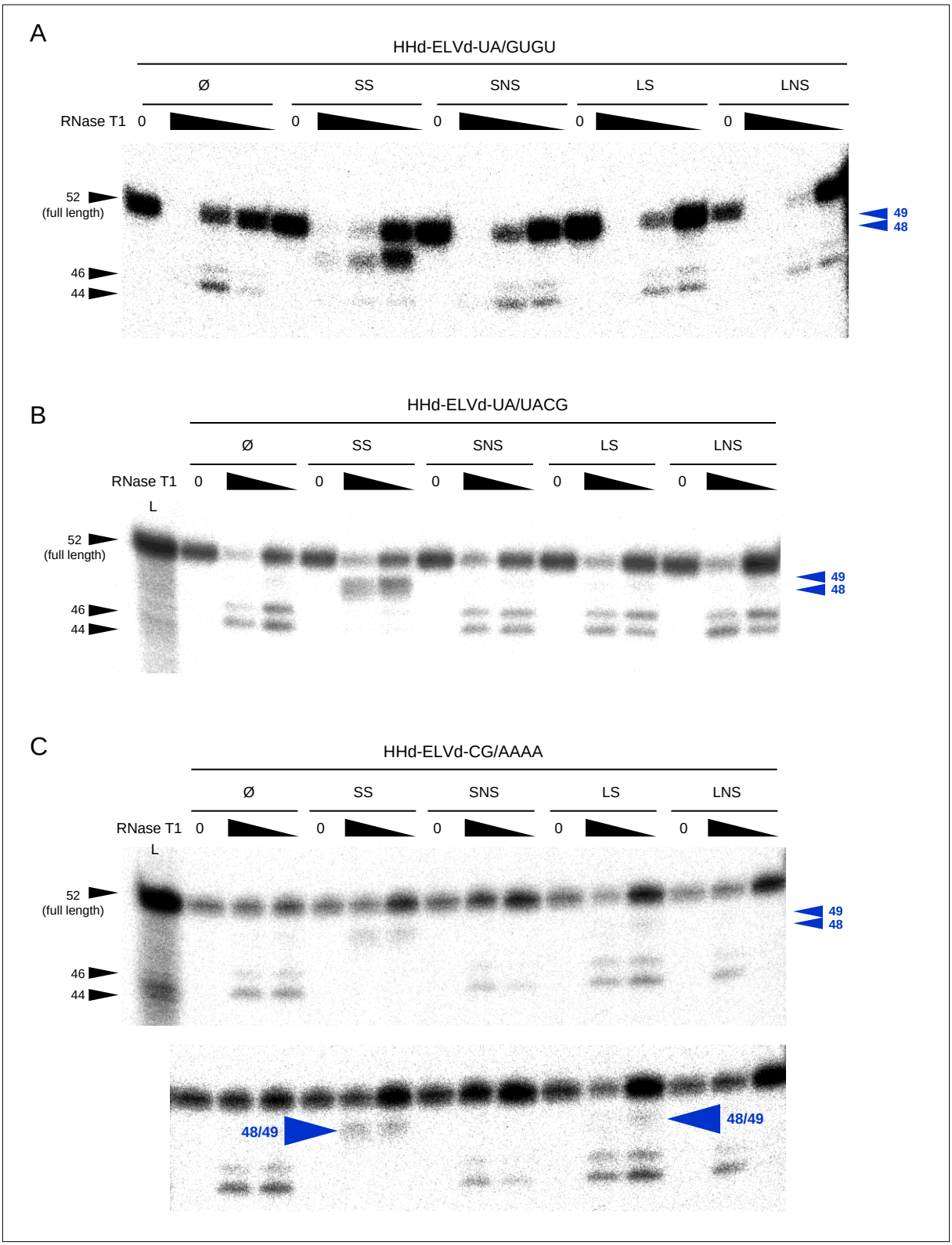
triangle in panel (A) refers to 0.5, 0.05 and 0.005 U of RNase T1 concentrations. Lower panel in (C) corresponds to an exposition longer than that in the upper panel.

Supplementary Figure S3. RNAse T1 digestion profile of an extended hammerhead (HHe-ELVd) and its potential complexes with different RNA substrates. (A) Schematic representation of the first suboptimal hammerhead secondary structure of minimal free energy predicted by the Mfold program. (B) Schematic representation of the complex formed by the hammerhead and the short substrate (SS) RF-994. (C) RNase T1 digestion products analyzed by denaturing PAGE in 15% gels. Other details as in Supplementary Figures 1 and 2.

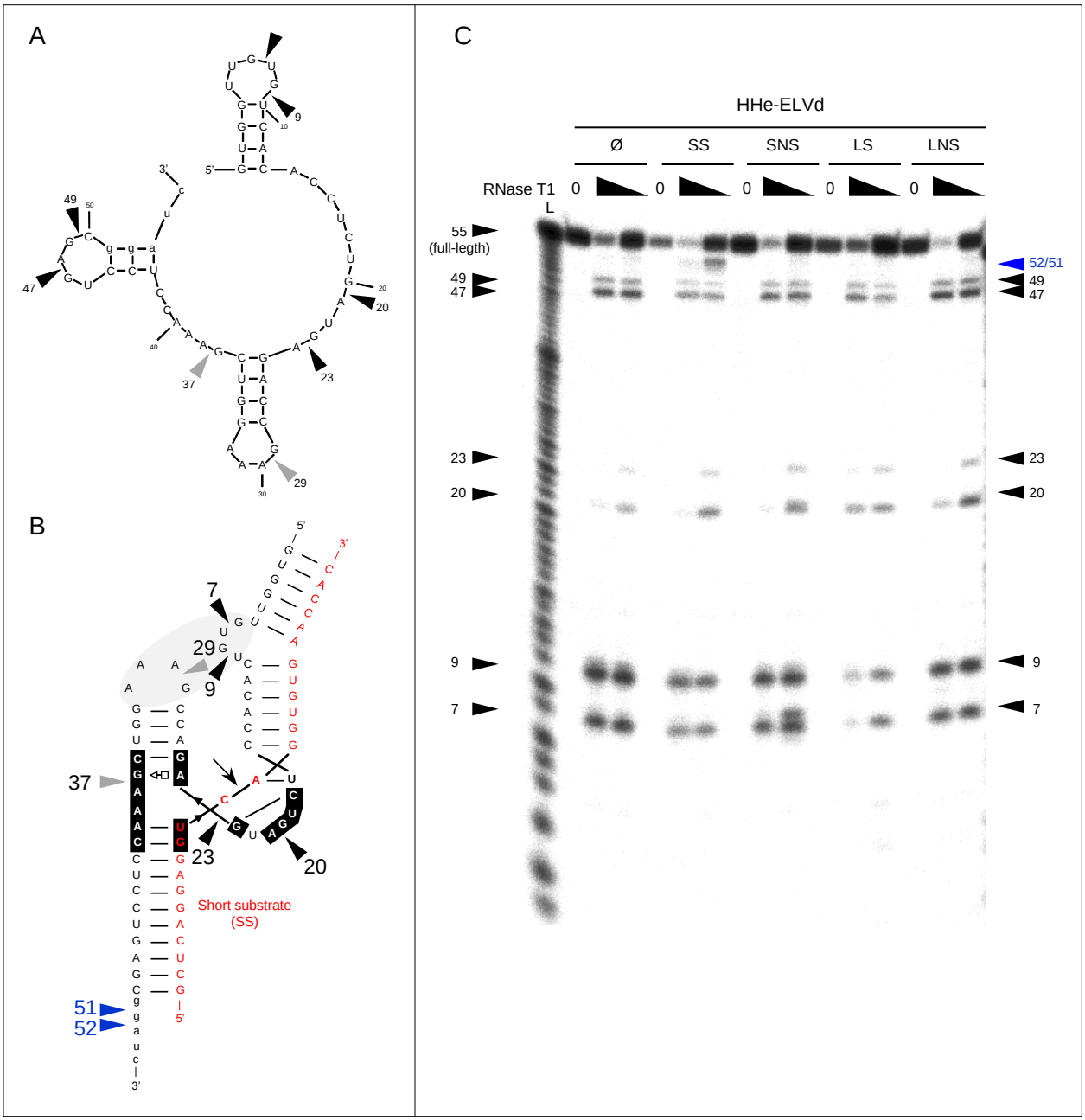
Supplementary Figure S4. Schematic representation of the secondary structure predicted for the helix I-loop 1 domain of the complexes formed HHe-ELVd and the PSTVd (-) RNA. Most stable conformation for the complex formed between HHe-ELVd and the substrate (right), in which intermolecular interactions (represented with red discontinuous lines) disrupt the ribozyme catalytically-active folding (left). Other details as in Figures 1 and 2.



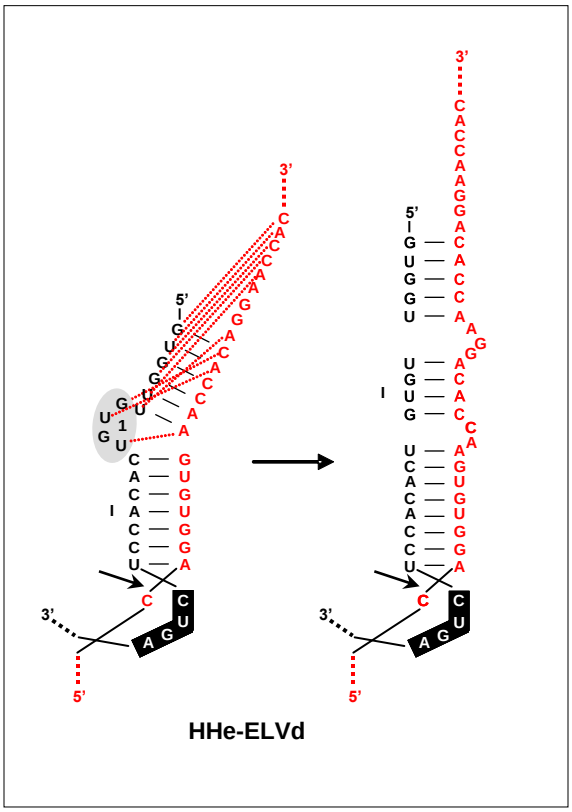
SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2



SUPPLEMENTARY FIGURE 3



SUPPLEMENTARY FIGURE 4