Supporting information for:

Protein binding in an mRNA 5'-UTR sterically hinders translation

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Descriptions of modeling MCP-M, plasmid maps, RT-qPCR primers, two figures, and one table.

Modelling of MCP-M with MS2 hairpin

The 3D structure of the RNA hairpin used in this study was created using the trRosettaRNA server (Wang et al. 2023); the resulting model had an estimated RMSD of 0.5 Å. The MCP-M model containing an internal $(His)_6$ linker was created using AlphaFold2 (Jumper et al. 2021) as informed with a non-tethered MCP dimer structure (PDB ID 1MSC). Model is shown in Figure 1B.

Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, et al. 2021. Highly accurate protein structure prediction with AlphaFold. *Nature* **596**: 583–589.

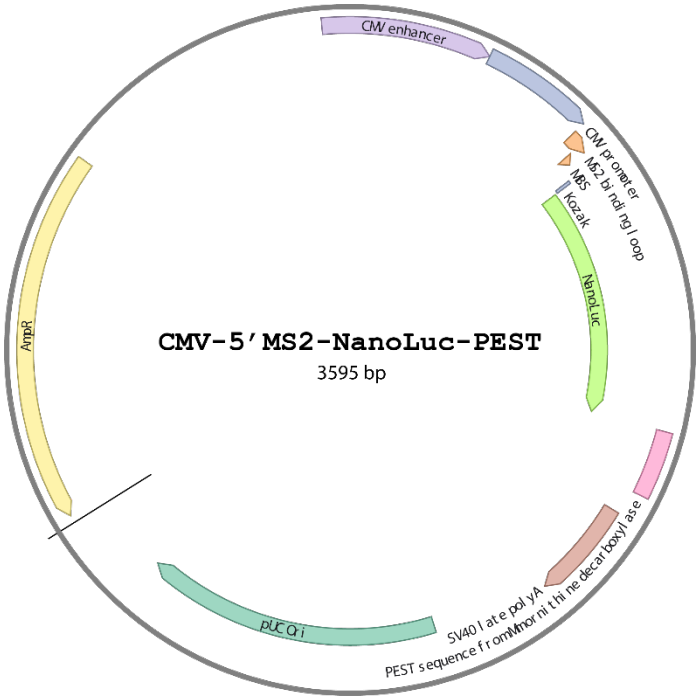
Wang W, Feng C, Han R, Wang Z, Ye L, Du Z, Wei H, Zhang F, Peng Z, Yang J. 2023. trRosettaRNA: automated prediction of RNA 3D structure with transformer network. *Nat Commun* **14**: 7266.

Plasmid Maps

Legend:

CMV enhancer
CMV promoter
MS2 Hairpin
Kozak sequence
NanoLuc coding sequence (***Start site***) – PEST sequence (***Stop site***)
Sv40 late PolyA
Replication origin
Ampicillin resistance gene
Transcribed region
qPCR primers

CMV-5'MS2-NanoLuc-PEST



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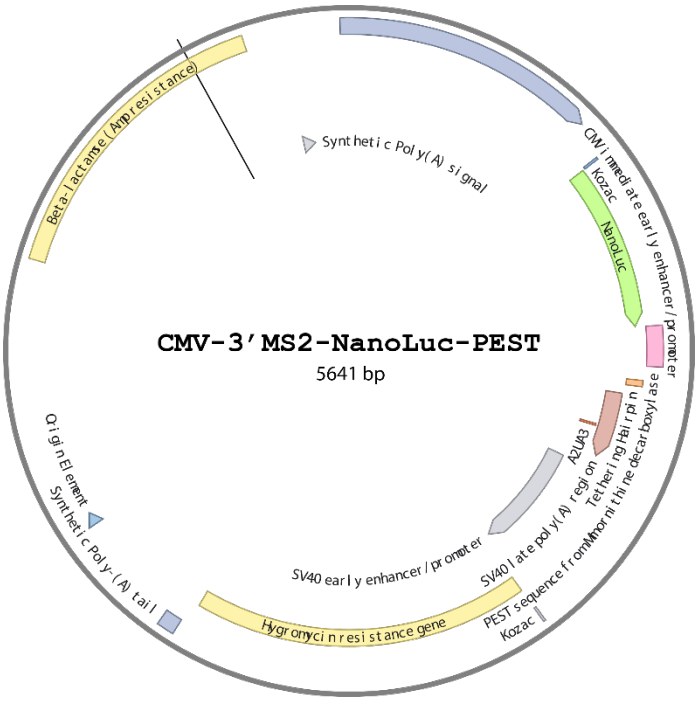
CMV-3'MS2-NanoLuc-PEST



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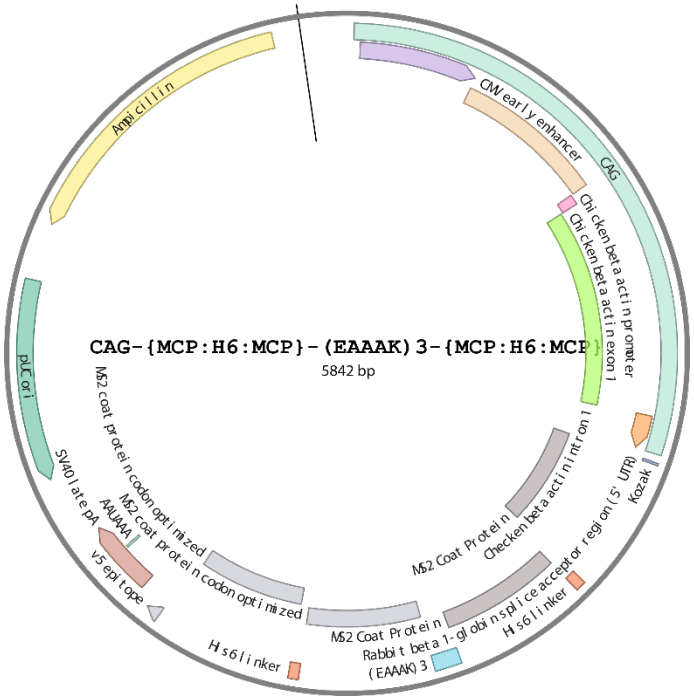
MCP constructs

Native Sequence dimer (MCP-D; K_D of monomer = 10 nM)

Structure: MCP H6 Linker MCP $(EAAAK)_3$ MCP H6 Linker MCP
Restriction sites: XbaI, BamHI



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Fragment sequences for Gibson Assembly

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TAAGCCTAT

MCP-M 1000:
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GCTACCAACTCAGACTGTGAACTCATCGTTAAGGCGATGCAGGGCCTTCTGAAAGACGGAAATCCC
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GCCTAT

qPCR Primers

NanoLuc-Forward: CATCATCCCGTATGAAGGTCTGAGC
NanoLuc-Reverse: AGTGTGCCATAGTGCAGGATCAC
Firefly-Forward: AGACCGACTACC AGGGCTTCC
Firefly-Reverse: TCAGGGCGATGGTTTTGTCCC

SI Figures

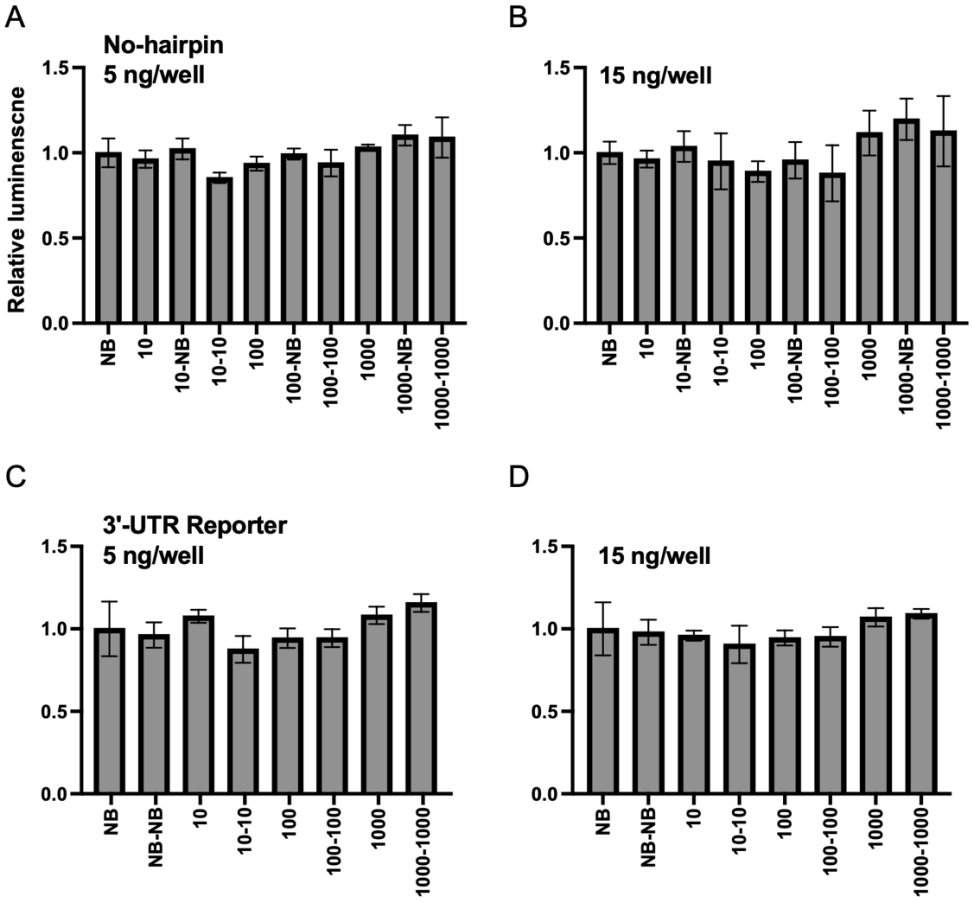

Figure S1. Luciferase signals for the no-hairpin and 3'-UTR target hairpin NanoLuc reporters, co-transfected with MCP-M and MCP-D proteins, and their variants. Experiments performed with no-hairpin reporter at (A) 5 or (B) 15 ng of plasmid per well. Experiments performed with 3'-UTR reporter at (C) 5 or (D) 15 ng of plasmid per well. NanoLuc luminescence was normalized to firefly luciferase signal and plotted relative to signal in the presence of the non-binding variant (NB). Mean ± SEM of biological triplicates are shown.

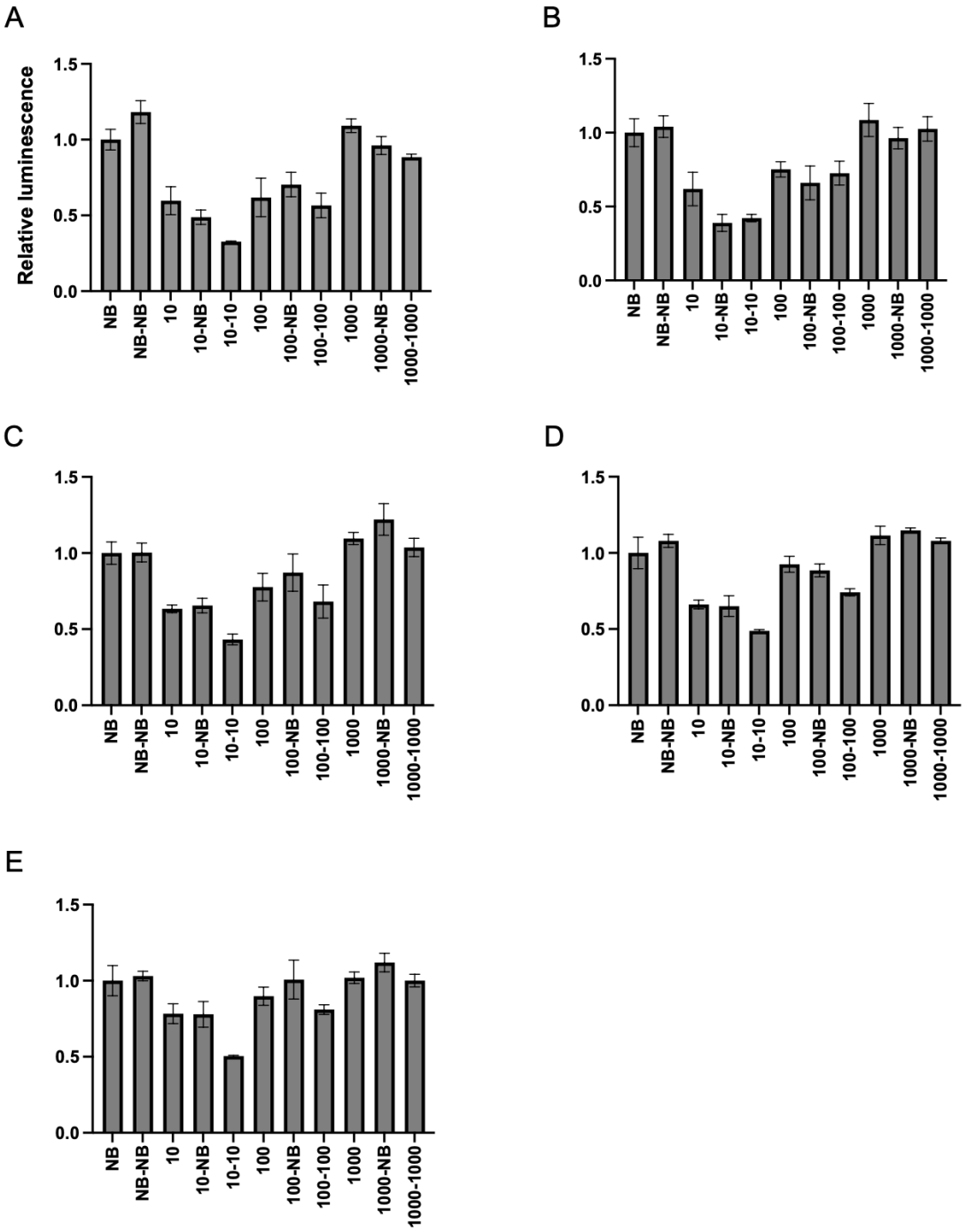

Figure S2. Relative luciferase signal for the NanoLuc reporter containing the 5'-UTR MS2 target hairpin co-transfected with MCP-M and MCP-D proteins, and their variants at (A) 15, (B) 10, (C) 5, (D) 2.5 or (E) 1.25 ng of plasmid per well. Mean ± SEM of biological triplicates are shown.

MCP version	Relative inhibition [MCP-D]/[MCP-M]		
	1.25/2.5	2.5/5	5/10
10	1.5	1.5	1.4
100	2.4	1.2	1.2

Table S1. Comparison of relative inhibition ratios between MCP-D and MCP-M across different binding affinities. Ratio of relative inhibition (1-relative luminescence) for MCP-D and MCP-M at different binding affinities (10 nM, 100 nM, and 500 nM), calculated as a function of transfection amount (1.25/2.5, 2.5/5.0, and 5.0/10. ng/well). Ratios greater than 1 indicate stronger translational repression by the MCP-D dimer compared to an equivalent concentration of MCP-M monomers.