Supplementary Experimental Section

Construction of the bicistronic reporter constructs: Cloning of all bicistronic reporter constructs (Figure 2A) followed identical procedures with the exception of ΔTS1. Oligonucleotides for the cloning are outlined in Supplementary Table 1. All TS1 containing constructs had oligonucleotides (sense/antisense) ligated into the pSP-luc+NF plasmid (Promega, Madison, WI). The resulting TS site 1 hairpin+firefly luciferase sequence was then digested in its entirety and ligated together with equimolar amounts of HCV IRES sequence (see below), and pRL-null vector. This three-component ligation mixture was transformed into Neb5α cells, plated on LB-Amp medium and incubated at 37º C overnight. Individual clones were sequenced to confirm correct insertion.

Oligos encoding TS1 and TS-mt 1-4 were phosphorylated, annealed at 65°C, then ligated into BstEII and HinDIII digested pSP-luc+NF plasmid. Once sequenced, the constructs were digested with NdeI and EcoRI to extract the TS1+Firefly Luciferase, gel purified, then ligated together with the EcoIR-BstBI digested HCV IRES for 30 minutes. Then NdeI-BstBI (250ng) digested pRL-null was added to the ligation mixture which was incubated for another 30 minutes. The resulting product is (5'-3') TS Hairpin+Firefly Luciferase→44bp→HCV IRES→Renilla luciferase.

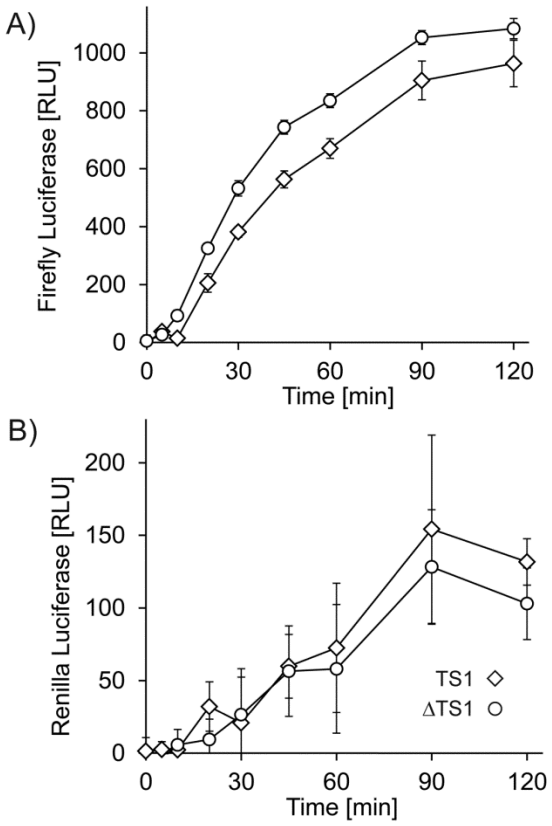
The ΔTS1 construct had the NdeI-EcoRI restriction product (firefly luciferase) ligated with the IRES/pRL-null mixture without undergoing any insertions in the 5' end. The resulting product is (5'-3') Firefly luciferase→44bp→HCV IRES→ Renilla luciferase.

Construction of the HCV IRES component: Oligonucleotides for the cloning of the HCV IRES are outlined in the Supplementary Table 2. Synthetic oligonucleotides were phosphorylated, annealed pairwise (IR-nT with IR-nB), and then ligated together. To increase the yield of full length IRES construct, a polymerase chain reaction (PCR) was conducted on the resulting ligation mix. Primers used for the PCR reaction were designed to amplify the IRES region, while retaining the original EcoRI and

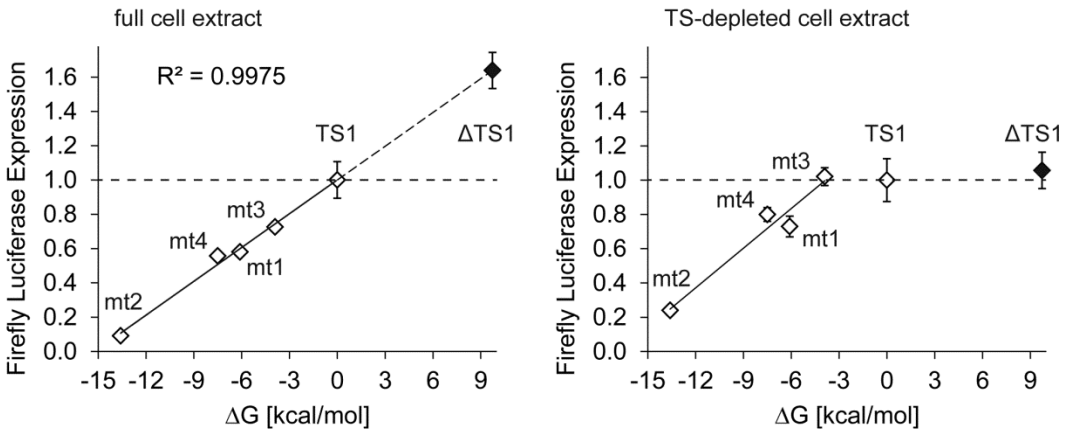
BstBI restriction sites. The PCR product was purified with a QIAquick PCR Purification Kit, digested with EcoRI and BstBI. After further purification the IRES was saved for ligation into the TS constructs.

IVT time course: Time course experiments were conducted as per the protocol outlined in the Experimental Section, with the variation of having 9 triplicate samples. Samples were removed from 30º C and placed immediately on ice at each time point (0, 5, 10, 20, 30, 45, 60, 90, 120 minutes) for luciferase quantification. Results of the time course experiment are shown in Supplementary Figure 1. A time point of 45 minutes was chosen for the following experiments to ensure that translation activity was recorded before reaching saturation through substrate depletion.

RNA free energy calculations: Free energies of secondary structures for the site 1 motif RNA and the stabilized mutants mt1-mt4 were calculated using RNAfold in the ViennaRNA package.[S1] Since the model constructs used for energy calculations (G75 – C115, Figure 1) did not contain the mRNA sequences flanking the site 1 motif, base pairing in the closing stem region (G75–C115 & U76○G114) was forced by constraint. See Supplementary Figure 2 for a correlation of estimated stabilization energies introduced by the mutations to reporter expression in full cell extract.



Supplementary Figure 1. Time courses of reporter expression under the control of the TS site 1 regulatory element in a coupled *in vitro* transcription-translation assay (IVT). **A)** Time course of cap-driven firefly luciferase reporter expression from the TS1 and ΔTS1 constructs. **B)** Time course of IRES-driven *Renilla* luciferase reporter expression from the same two constructs. Data are averages of three replicates with error bars showing ±1σ.



Supplementary Figure 2. Correlation of estimated free energy stabilization introduced into the TS site 1 structure by the mutations mt1-mt4 and reporter expression in full reticulocyte extract (left) and cell extract that was depleted of residual TS protein (right). The estimated free energy difference for the ΔTS1 construct was calculated by extrapolating from the experiments with full cell extract. Expression data are averages of three replicates with error bars showing ±1σ.

Supplementary Table 1. Oligonucleotide sequences for the cloning of the TS site 1 motif and mutants mt1-mt4 in the bicistronic reporter constructs (Figure 2A).

Oligo Name	Sequence (5' → 3')
TS1 sense	AGCTTCGTCCCCCGCCCGCCGCGCCATGCCTGTGGCCGGCTCGGAGCTG
TS1 antisense	GTGACCAGCTCCGAGCCGGCCACAGGCATGGCGCGGCGGGCGGGGGACGA
TS1 mt1 sense	AGCTTCGTCCCCCGCCGGCCGCGCATGCCTGTGGCCGGCTCGGAGCTG
TS1 mt1 antisense	GTGACCAGCTCCGAGCCGGCCACAGGCATGCGCGGCCGGCGGGGGACGA
TS1 mt2 sense	AGCTTCGTCCCGAGCCGGCCGCGCCATGCCTGTGGCCGGCTCGGAGCTG
TS1 mt2 antisense	GTGACCAGCTCCGAGCCGGCCACAGGCATGGCGCGGCCGGCTCGGGACGA
TS1 mt3 sense	AGCTTCGTCCCGCGCCGGCCGCGCATGCCTGTGGCCGGCTCGGAGCTG
TS1 mt3 antisense	GTGACCAGCTCCGAGCCGGCCACAGGCATGCGCGGCCGGCGCGGGACGA
TS1 mt4 sense	AGCTTCGTCCCGAGCCGGCCGCGCATGCCTGTGGCCGGCTCGGAGCTG
TS1 mt4 antisense	GTGACCAGCTCCGAGCCGGCCACAGGCATGCGCGGCCGGCACGGGACGA

Supplementary Table 2. Oligonucleotide sequences for the cloning of the HCV IRES in the bicistronic reporter constructs (Figure 2A).

Oligo Name	Sequence (5' → 3')
IR-1T	AATTCCCAGCCCCCGA
IR-1B	CAATCGGGGGCTGGG
IR-2T	TTGGGGGCGACACTCCACCATAGATC
IR-2B	GAGTGATCTATGGTGGAGTGTCGCCCC
IR-3T	ACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGT
IR-3B	GCTAGACGCTTTCTGCGTGAAGACAGTAGTTCCTCACAGGG
IR-4T	CTAGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCC
IR-4B	AGGGGGGGTCCTGGAGGCTGCACGACACTCATACTAACGCCATG
IR-5T	CCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGT
IR-5B	ACTCACCGGTTCCGCAGACCACTATGGCTCTCCCGGG
IR-6T	GAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGG
IR-6B	TGATCCAAGAAAGGACCCGGTCGTCCTGGCAATTCCGGTGT
IR-7T	ATCAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCC
IR-7B	TCGCGGGGGCACGCCCAAATCTCCAGGCATTGAGCGGGT
IR-8T	GCGAGACTGCTAGCCGAGTAGTGTTGGGTCGCGAAA
IR-8B	AGGCCTTTCGCGACCCAACACTACTCGGCTAGCAGTC
IR-9T	GGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCC
IR-9B	CCCGGGGCACTCGCAAGCACCCTATCAGGCAGTACCACA
IR-10T	CGGGAGGTCTCGTAGACCGTGCACCATGAGCACGAATCCTT
IR-10B	CGAAGGATTCGTGCTCATGGTGCACGGTCTACGAGACCT
IRES Forward	AGAGAATTCCCAGCCCCCGATTGG
IRES Reverse	ACTTTCGAAGGATTCGTGCTCATGG

Supplementary Reference

[S1] R. Lorenz, S. H. Bernhart, C. Honer Zu Siederdissen, H. Tafer, C. Flamm, P. F. Stadler, I. L. Hofacker, *Algorithms Mol. Biol. AMB* **2011**, *6*, 26.