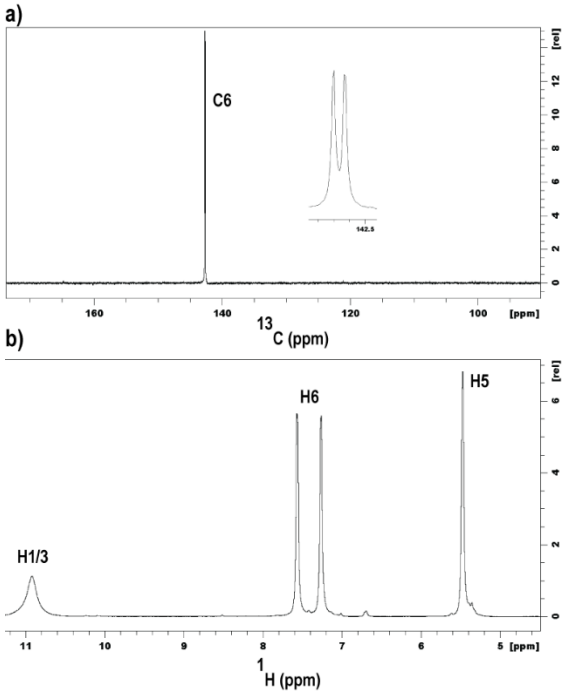
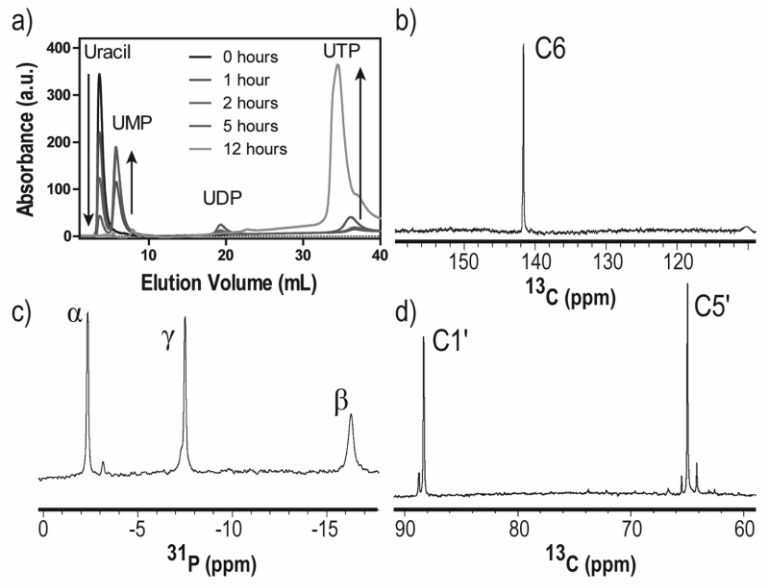
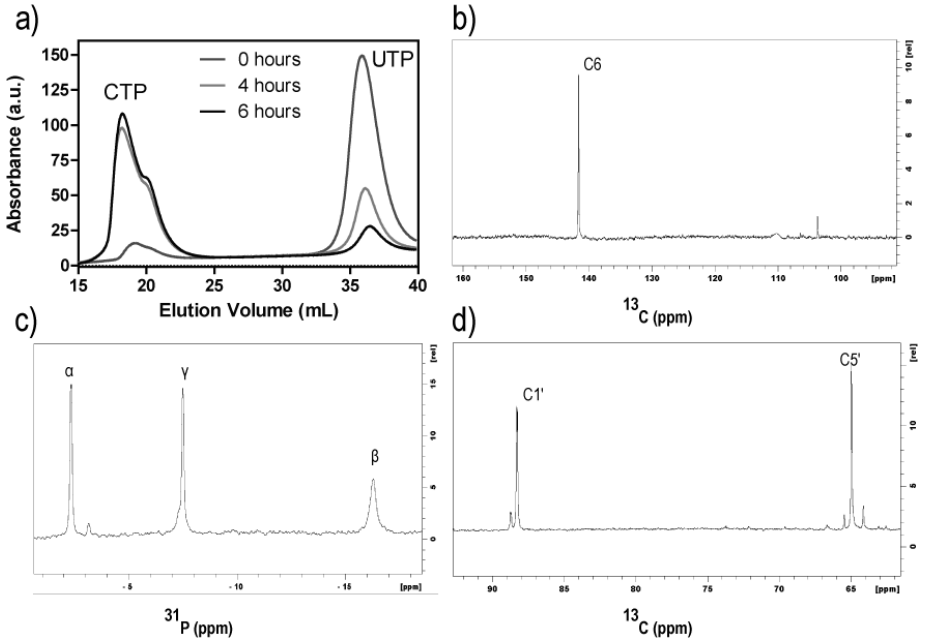
Supplementary Information



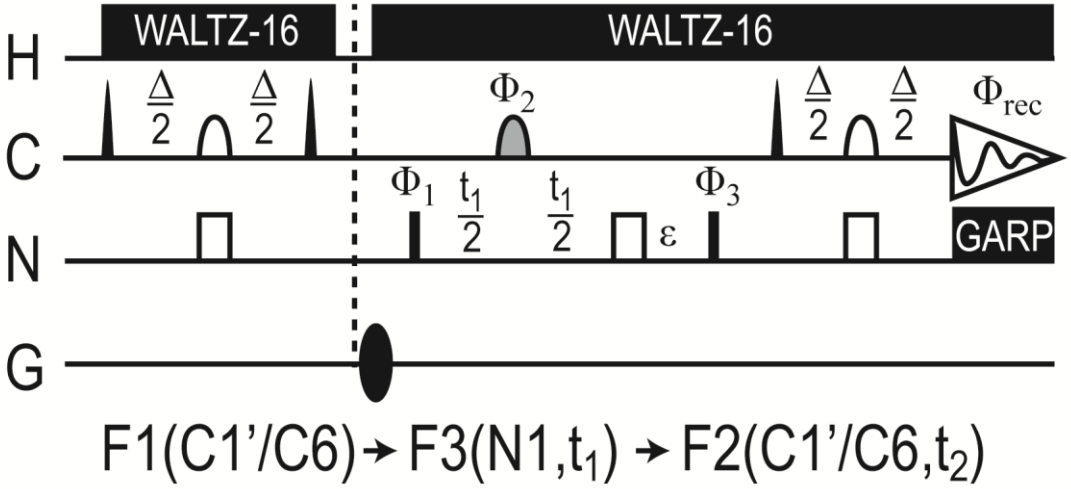
SI-1. 6-$^{13}C_1$-1,3-$^{15}N_2$-Uracil quality control. (a) 1D ^{13}C NMR without ^{1}H decoupling in DMSO-d_6. (b) 1D ^{1}H NMR in DSMO-d_6.



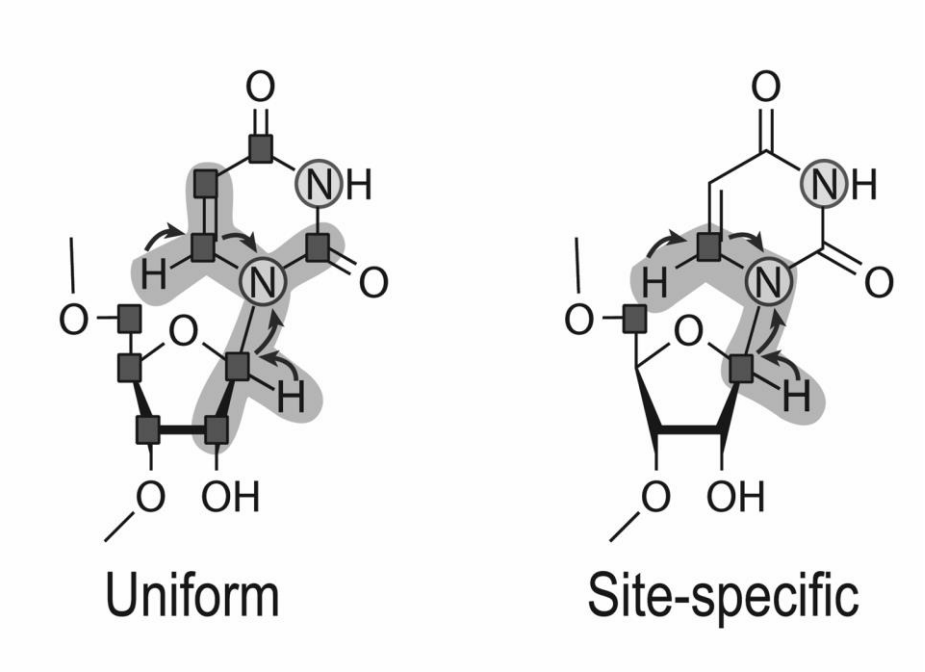
SI-2. Synthesis and quality control of UTP. (a) HPLC chromatogram of the reaction time course of UTP synthesis from ribose and uracil. (b,d) 1D ^{13}C NMR of 1′,5′,6-$^{13}C_3$-1,3-$^{15}N_2$-UTP. (c) 1D ^{31}P NMR of 1′,5′,6-$^{13}C_3$-1,3-$^{15}N_2$-UTP.



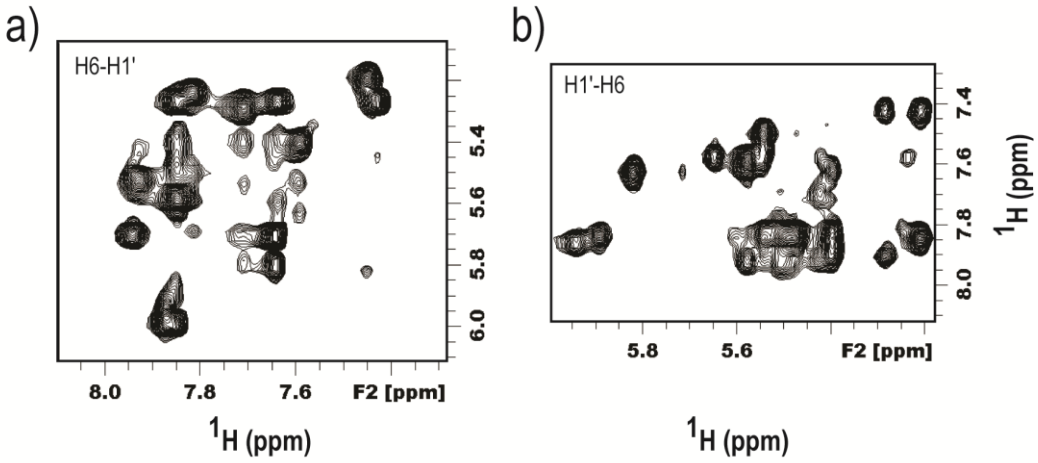
SI-3. Synthesis and quality control of CTP. (a) HPLC chromatogram of the reaction time course of CTP synthesis from UTP. (b,d) 1D ^{13}C NMR of 1′,5′,6-$^{13}C_3$-1,3,4-$^{15}N_3$-CTP. (c) 1D ^{31}P NMR of 1′,5′,6-$^{13}C_3$-1,3,4-$^{15}N_3$-CTP.



SI-4. Direct carbon-nitrogen HSQC pulse scheme based on CON experiment without the need for an IPAP/AP module.[1] Filled symbols stand for 90° and open symbols represent 180° rf pulses respectively, and rectangular and rounded pulses symbolize hard and band selective pulses. For band selective pulses, ^{13}C excitation and inversion/refocusing Q5 (or time reversed Q5) and Q3 shapes[2] are used with durations of 384 μs and 307 μs respectively, and for the pulse with phase Φ_2 a 500 μs Chirp shape[3] for adiabatic inversion is applied. The delays are: Δ = 16 ms; ε=t_1(0). The phase cycle used is Φ_1 = x,-x; Φ_2 = 2x,2(-x); Φ_3= 4x,4(-x); and Φ_{rec} = x, (-x),x, (-x), (-x),x, (-x),x; all other pulses have phase x. Quadrature detection in the F1 dimension is obtained by incrementing Φ_1 in a States-TPPI fashion. Decoupling of ^{1}H and ^{15}N was achieved with 2.5 kHz Waltz-16 and 1.0 kHz Garp-4 schemes, respectively.[4,5]



SI-5. Magnetization transfer in HCN experiments. Black arrows represent the transfer of magnetization from H1′/H6 to C1′/C6 and then to N1. The shaded segments represent all possible pathways that reduce transfer efficiency.



SI-6. Zoomed in view of the off-diagonal crosspeaks of the NOESY spectrum of the IRE RNA. The spectrum shown in Figure 2b appears asymmetrical, however, the symmetrical windows showing the contacts between (a) H6-H1′ and (b) H1′-H6 have similar signal-to-noise ratios. The 3D NOESY-HSQC was ran with a spectral width of 6394 Hz in both ^{1}H dimensions, and 4024 Hz in the ^{13}C dimension. 1024, 128, and 512 complex points were acquired in t_3, t_2, and t_1, respectively, with 16 scans per slice. The recycling delay used was 2.0 s. The mixing time was 250 ms and $^{1}J_{CH}$ was 145 Hz. The 90° ^{1}H pulse was 9.1 μs on the 800 MHz Bruker Avance III spectrometer equipped with a cryoprobe.

Materials and Methods:

UTP synthesis:

The site-specifically labeled 1′,5′,6-$^{13}C_3$-1,3-$^{15}N_2$-Uridine triphosphate was enzymatically synthesized *in vitro*. First, uridine monophosphate (UMP) was synthesized from uracil and ribose. The reaction was carried out in 50 mM Na_3PO_4 pH 7.5, 10 mM $MgCl_2$, 2 mg/mL ampicillin, 10 mM DTT, 0.5 mM dATP, 100 mM creatine phosphate, 8 mM uracil, 10 mM ribose, 50 U/mL creatine kinase, 50 U/mL myokinase, 0.4 U/mL thermostable inorganic pyrophosphatase (New England Biolabs), 5 U/mL ribokinase, 3 U/mL phosphoribosyl pyrophosphate synthetase, and 5 U/mL uridine phosphoribosyl transferase. The reaction was incubated at 37 °C for 5 hours. UMP was then purified by boronate affinity chromatography and lyophilized to a powder. The powder was then resuspended, and assayed for UMP concentration by its UV trace at 262 nm wavelength (ε = 10,000 $M^{-1}cm^{-1}$). Our typical yield was 90 % based on starting uracil. The phosphorylation of UMP was achieved *in situ* immediately after its synthesis. Addition of 20 mM KCl and 50 µg/mL nucleoside monophosphate kinase from bovine liver (Roche), followed by incubation at 37 °C for 6 hours achieved complete phosphorylation of UMP to UTP. The pH of the reaction was adjusted with either HCl or NaOH to be between 7.5 and 8.0. In most cases, the reaction mixtures were also spiked with 0.2 mM dATP and 20 mM creatine phosphate. Our typical overall yield with this method was 90 % with respect to input uracil**.**

CTP synthesis:

The site-specifically labeled 1′,5′,6-$^{13}C_3$-1,3-$^{15}N_2$-Cytidine triphosphate was enzymatically synthesized *in vitro* in a single-step reaction.[16] The previously synthesized uridine triphosphate (UTP) was converted in a reaction with dATP and ammonium chloride, catalyzed by CTP synthetase. The reaction was carried out in 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 2 mg/mL ampicillin, 4 mM dATP, 2 mM UTP, 20 mM ^{15}N-NH_4Cl, and 0.1 mg/mL CTP synthetase.[16] The reaction was incubated at 37 °C for 6 hours. The reaction was then filtered through a 3,000 molecular weight cut-off filtering device. The flow-through was collected and assayed for CTP concentration by its UV trace at 273 nm wavelength (ε = 9,000 $M^{-1}cm^{-1}$). The recovered CTP at nearly quantitative yields was used directly in *in vitro* transcriptions. Alternately, newly synthesized CTP was purified by boronate affinity chromatography in an identical manner to the purification of UTP. Our typical yield was 95 %.

RNA Preparation:

IRE RNA:

(5′GGAGUGCUUCAACAGUGCUUGGACGCUCC) was synthesized *in vitro* from synthetic DNA templates (Integrated DNA Technologies Inc.). The transcriptions were carried out in transcription buffer [40 mM Tris-HCl pH 8.0, 0.1 mM spermidine, 0.01 % Triton X-100, 10 mM DTT, 80 mg/mL PEG (8000MW)], supplemented with 2.0 U/mL thermostable inorganic pyrophosphatase (New England Biolabs, Inc.), 0.3 µM each DNA template, 10 mM $MgCl_2$, 5 mM NTPs (1.25 mM each NTP), and 0.25 mg/mL T7 RNA polymerase. The T7 RNA polymerase was expressed in *E. coli* BL21(DE3) and purified on pre-packed Ni-NTA beads in column mode as previously described.[6,7] The T7 promoter sequence used was 5′ CTA ATA CGA CTC ACT ATA G. The template strands of wild-type IRE RNA was 5′GGAGCGTCCAAGCACTGTTGAAGCACTC CTATAGTGAGTCGTATTAG. Two terminal 2′-O-methyl modifications in the template strands were introduced to substantially reduce transcript heterogeneity.[8,9] All purchased DNA oligonucleotides were PAGE-purified. The transcription conditions were optimized by a sparse-matrix approach. The reactions were incubated at 37 °C for 3h. The reactions were then phenol-chloroform extracted and then ethanol-precipitated overnight with three volumes of absolute ethanol and 0.3 M sodium acetate pH 5.2. The precipitates were then re-suspended in a minimum amount of water and PAGE-purified in 8 M Urea/13 % acrylamide-bis (19:1) gels. The gel was UV-shadowed, the corresponding band was then excised and the RNA was then electro-eluted on an Elutrap electro-

separation system (Schleicher and Schuell) at 200 V for 6-7 h. The elution fractions were pooled, concentrated and extensively dialyzed against a high-EDTA buffer (10 mM Na_3PO_4 or Tris-HCl pH 6.5, 10 mM NaCl, 10 mM EDTA) and a low-EDTA buffer (same as above, 0.2 mM EDTA). Prior to use, the RNA was heated to 90 °C for two minutes and immediately cooled on ice for 10 minutes.

ESS 3 RNA:

ESS3 was prepared using recombinant T7 RNA polymerase that was overexpressed and purified from BL21 (DE3) cell. Synthetic DNA templates used for transcription were purchased from Eurofins MWG Operon. The transcriptions were carried out in transcription buffer [30 mM Tris, 2 mM spermidine, 0.01% Triton X-100, 25 mM $MgCl_2$, 10 mM DTT] and consisted of 10 mL reaction volumes containing ~0.3 µM each DNA template, ^{13}C uniformly labeled rNTPs (Isotech) or newly site-selectively made NTPs from this work. The reaction was carried at 37^0 C for 2.5 hrs. Following synthesis, ESS3 was purified to homogeneity by 20% denaturing PAGE, excised from the gel and electroeluted. After electroelution desalting were carried out by exhaustively washing the sample with RNase free water using a Millipore Amicon® Ultra-4 centrifugal filter device. Purified ESS3 was dried under vacuum and stored at -20° C until further use.

Pro-tRNA:

Three complementary DNA strands corresponding to the template DNA were purchased from Dharmacon Research Inc. Each strand was combined with its complementary strand in the anneaing buffer [10 mM Tris-HCl pH 7.0, 10 mM NaCl], boiled for two minutes and slowly cooled down to room temperature. Each of the three annealed double-stranded DNA pieces were combined together in the presence of T4 DNA ligase and incubated at room temperature for ligation overnight. The reaction was quenched at 65 °C for 10 min. This DNA was then directly used for in vitro transcription of $tRNA^{Pro}$.The DNA template was transcribed *in vitro* by T7 RNA polymerase in 30 mL reactions containing the optimized amount of template, 20 mM $MgCl_2$, 2 mM spermidine, 80 mM Tris-HCl (pH 8.1), 2 mM DTT, 10 % DMSO, 0.3 mg T7 RNA polymerase and the optimized amount of each NTP. Reactions were incubated at 37 °C for three hours and quenched with 25 mM EDTA. The RNA was ethanol precipitated, air-dried and re-suspended in water containing 4 M urea. After denaturation at 96 °C for five minutes, the RNA was purified by denaturing polyacrylamide gel electrophoresis. The concentration of each sample was determined by measuring the optical absorbance at 260 nm.

HIV-1 RNA:

A plasmid containing the DNA coding for the HIV-1 core encapsidation signal was amplified using *E. coli* DH5α and purified (Mega Kit, Qiagen). The plasmid was linearized with Sma1 (New England Biolabs) and the linear template was then extracted with phenol-chloroform and precipitated with ethanol. The pellet was dissolved in sterile water and desalted. The HIV-1 core encapsidation signal RNA was synthesized by *in* vitro transcription using T7 RNA polymerase in reactions that contained 40 mM Tris-base, 5 mM DTT, 1 mM Spermidine, 0.01% Triton X-100, pH 8.5 with 15 mM $MgCl_2$, 6 mM NTPs, 1.25 mg DNA template, and 0.3 mg T7 RNA polymerase. The transcription reaction was incubated for 3h at 37 °C. The reaction was then quenched by addition of a 10% v/v mixture of 250 mM EDTA and 7 M urea. The transcription reaction was purified on urea-containing polyacrylamide gels (National Diagnostics) run on a FisherBiotech DNA sequencing system at 20W overnight. The RNA was visualized by UV-shadowing, excised, and eluted using elutrap electroelution systems (Whatman) at 120 V overnight. The eluted RNA was concentrated using a 10K Amicon Ultra-4 Centrifugal Filter Device, washed with high-purity 2M NaCl, and then extensively washed with water. The sample was then lyophilized, and dissolved in D_2O containing 10 mM deuterated Tris (CIL) pH 7.0.

Nuclear magnetic resonance spectroscopy:

Table S1. The HSQC and TROSY parameters for TROSY enhancement comparison of selectively labeled UTP samples.

NMR Sample	Region	NS	TD2	TD1	^{1}H SW	^{13}C SW	^{1}H Carrier	^{13}C Carrier
IRE RNA	C5'	8	1024	256	6	6	4.7	63.5
Human Pro-tRNA	C1'	32	1024	128	10	4.6	4.7	89.4
	C5'	32	1024	128	10	5.5	4.7	64.15
	C6	32	1204	128	10	4	4.7	139.9
HIV-1 core encapsidation signal RNA	C1'	128	1024	96	8	6	4.7	89
	C5'	128	1024	96	8	5.5	4.7	64.25
	C6	128	512	80	6	5	7.5	140.5

The column headings are: NS, number of scans; TD, complex points; ^{1}H SW, proton sweep width (p.p.m.); ^{13}C SW, carbon sweep width (p.p.m.); ^{1}H Carrier, proton carrier in p.p.m.; and ^{13}C Carrier, carbon carrier in p.p.m. Concentrations of the IRE, human pro-tRNA, HIV-1 core encapsidation signal RNAs used were respectively 1 mM, 0.3 mM, and 0.2 mM.

Longitudinal (R_1) and transverse (R_2) relaxation rates were measured for ribose C1′ and pyrimidine nucleobase C6 carbons using previously published pulse sequences optimized for RNA.[10] The T_1 mixing times were 40 (2x), 100, 150, 270, 450, 750, and 1000 ms. The $T_{1\rho}$ mixing times were 4 (2x), 10, 16, 24, 32, 40, 50, and 60 ms with a spin-lock field strength of 3 kHz. For uniformly labeled samples to maintain the increased resolution and sensitivity comparable to the site-selectively labeled samples, the C1'/C6 carbons were selectively decoupled from the C2'/C5 carbons using the WUSRT-2 adiabatic band-selective ^{13}C-^{13}C decoupling scheme.[11–13] Parameters used for each sample are listed in Table S2.

Table S2. NMR relaxation experimental parameters for uniform and site-specific U-labeled ESS3 and uniform and site-specific UC-labeled IRE RNA.

NMR Sample	Experiment	Region	NS	TD2	TD1	^{1}H SW	^{13}C SW	^{1}H Carrier	^{13}C Carrier
ESS3: site-specific	T_1	C1'	64	1024	32	6	4.5	4.7	89.25
	T_1	C6	128	1024	32	6	3.3	7.1	139.8
	$T_{1\rho}$	C1'	64	512	32	4	4.5	4.7	89.25
	$T_{1\rho}$	C6	64	1024	64	4	3.3	7.1	139.8
ESS3: uniform	T_1	C1'	16	1024	64	6	4.5	4.7	89.25
	T_1	C6	16	1024	64	6	3.3	4.7	1398
	$T_{1\rho}$	C1'	16	512	64	4	4.5	4.7	89.25
	$T_{1\rho}$	C6	128	512	32	4	4	7.7	139.8
IRE: site-specific	T_1	C1'	8	1024	64	4	5.2	4.7	89.4
	T_1	C6	8	1024	64	4	5.5	7.1	138.75
	$T_{1\rho}$	C1'	16	1024	64	4	5.2	4.7	89.4
	$T_{1\rho}$	C6	16	1024	64	4	5.5	7.1	138.75
IRE: uniform	T_1	C1'	64	1024	64	4	8	4.7	90
	T_1	C6	128	1024	64	4	8	7.1	138.5

	$T_{1\rho}$	C1'	64	512	64	4	8	4.7	90
	$T_{1\rho}$	C6	64	1024	80	6	7	7.7	138.5

The column headings are: NS, number of scans; TD, complex points; ^{1}H SW, proton sweep width (p.p.m.); ^{13}C SW, carbon sweep width (p.p.m.); ^{1}H Carrier, proton carrier in p.p.m.; and ^{13}C Carrier, carbon carrier in p.p.m. Concentrations of the IRE and ESS3 RNAs used were respectively 1 mM and 0.1 mM.

NMR assignment experiments were performed on site-specifically UC-labeled IRE RNA in order to determine the usefulness of selectively labeled nucleotides for structural assignments. Direct-detect carbon CN experiment (see SI-3) to correlate C1'-N1 and C6-N1 chemical shifts were performed with 256 scans and 512 complex points in t_2 and 96 complex points in t_1. The sweep widths were 15 and 10.6 ppm in the t_2 and t_1 dimensions, respectively. The ^{13}C carrier was optimized for ribose C1′ (89.4 ppm) or pyrimidine nucleobase C6 (138.75 ppm) and the ^{15}N carrier was set at 147.8 ppm for each experiment. A two bond CN coupling constant of 15 Hz was used for both experiments. A ^{13}C-edited 3D NOESY experiment with sensitivity enhancements[14–16] was performed using 16 scans with 1024 complex points in t_3, 64 complex points in t_2, and 128 complex points in t_1. The carriers were 4.7 ppm (t_3), 89.4 ppm, (t_2), and 4.7 ppm (t_1) with sweep widths of 8, 5.2, and 5 ppm, respectively.

References cited in the Supplementary Information:

[1] W. Bermel, I. Bertini, I. C. Felli, R. Kümmerle, R. Pierattelli, *J. Magn. Reson.* **2006**, *178*, 56–64.
[2] L. Emsley, G. Bodenhausen, *Chem. Phys. Lett.* **1990**, *165*, 469–476.
[3] J. M. Bohlen, G. Bodenhausen, *J. Magn. Reson. Ser. A* **1993**, *102*, 293–301.
[4] A. . Shaka, T. Frenkiel, R. Freeman, *J. Magn. Reson.* **1983**, *52*, 159–163.
[5] A. . Shaka, P. . Barker, R. Freeman, *J. Magn. Reson.* **1985**, *64*, 547–552.
[6] P. J. Lopez, J. Guillerez, R. Sousa, M. Dreyfus, *J. Mol. Biol.* **1997**, *269*, 41–51.
[7] J. Guillerez, P. J. Lopez, F. Proux, H. Launay, M. Dreyfus, *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 5958–5963.
[8] J. F. Milligan, D. R. Groebe, G. W. Witherell, O. C. Uhlenbeck, *Nucleic Acids Res.* **1987**, *15*, 8783–98.
[9] C. Kao, M. Zheng, S. Rudisser, *RNA* **1999**, *5*, 1268–1272.
[10] K. T. Dayie, G. Wagner, *J. Magn. Reson. Ser. A* **1994**, *111*, 121–126.
[11] Ē. Kupče, G. Wagner, *J. Magn. Reson. Ser. B* **1995**, *109*, 329–333.
[12] K. T. Dayie, *J. Biomol. NMR* **2005**, *32*, 129–39.
[13] B. Brutscher, J. Boisbouvier, E. Kupče, C. Tisné, F. Dardel, D. Marion, J.-P. Simorre, *J. Biomol. NMR* **2001**, *19*, 141–151.
[14] A. G. Palmer, J. Cavanagh, P. E. Wright, M. Rance, *J. Magn. Reson.* **1991**, *93*, 151–170.
[15] L. Kay, P. Keifer, T. Saarinen, *J. Am. Chem. Soc.* **1992**, *114*, 10663–10665.
[16] J. Schleucher, M. Schwendinger, M. Sattler, P. Schmidt, O. Schedletzky, S. J. Glaser, O. W. Sørensen, C. Griesinger, *J. Biomol. NMR* **1994**, *4*, 301–6.