

NMR study of 100kDa HCV IRES RNA using segmental isotope labeling

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Supporting Information

1. preparation of ¹⁵N-labeled 5' fragment (domain II) that has hydroxyl groups at both 5' and 3' termini

¹⁵N-labeled 5'-fragment was prepared by *in vitro* transcription with T7 RNA polymerase. A template DNA fragment was designed containing T7 promoter, hammerhead ribozyme, a 64 nucleotide sequence (40-104), and the BsaI restriction site and cloned into pUC119. The BsaI enzyme cleaves 5' to the sequence 5'GAGACC3'. Therefore, the BsaI restriction site is placed downstream of the cleavage site and it allows production of the desired RNA terminating in a 3'-hydroxyl group by runoff transcription. Template DNA was prepared by BsaI digestion of the plasmid in 2 ml containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM MgCl₂, 100 ug of plasmid DNA, and 150 units of BsaI. After overnight incubation at 50°C, the reaction mixture was phenol-extracted and the DNA was ethanol-precipitated. A typical 10 ml transcription reaction mixture contained 40 mM Tris-HCl (pH 8.1), 6 mM MgCl₂, 1 mM spermidine, 5 mM dithiothreitol, 8 mM each ¹⁵N-NTP, 0.5 mg of BsaI-digested DNA, and 12,000 units of T7 RNA polymerase. After incubation for 2 hr at 37°C, EDTA was added to a final concentration of 50 mM, and the reaction mixture was phenol extracted. RNA was purified using preparative polyacrylamide gel electrophoresis, electroeluted from the gel slices with an Elutrap, and centricon filtered 10X with dd H₂O.

2. preparation of unlabeled 3' fragment (domain III and IV) that has phosphate groups at both 5' and 3' termini

A DNA fragment was designed containing T7 promoter, a 150 nucleotide sequence (105-354) of IRES, and a hammerhead ribozyme. Template DNA was prepared by BstZ171 digestion of the plasmid in 0.5 ml containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM MgCl₂, 100 ug of plasmid DNA, and 150 units of BstZ171. After incubation at 37°C for overnight, the reaction mixture was phenol-extracted and the DNA was ethanol-precipitated. A typical 20 ml transcription reaction mixture contained 40 mM Tris-HCl (pH 8.1), 6 mM MgCl₂, 1 mM spermidine, 5 mM dithiothreitol, 16 mM each NTP, 10 mM GMP, 1 mg of BstZ171-digested DNA, and 12,000 units of T7 RNA polymerase. After incubation for 2 hr at 37°C, EDTA was added to a final concentration of 50 mM, and the reaction mixture was phenol extracted. RNA was purified as above.

3. ligation between ¹⁵N-labeled 5' fragment and unlabeled 3' fragment

The purified RNAs were washed several times with pure water by centrifugation with a Centricon-3 cartridge. Optimal conditions of ligation were screened in a series of small reaction. A 10x ligation buffer is added for a final condition of 50 mM Tris-HCl (pH7.5), 3.3mM dithiothreitol, 2.5 % PEG and 60 uM ATP. The concentrations of RNA fragments, MgCl₂, PEG6000, and T4 RNA ligase were optimized. For the HCV IRES, the optimized conditions were: 70.7 nmol of 3' fragment and 66.8 nmol of 5' fragment were mixed in a 40 ml reaction mixture containing 50 mM Tris-HCl (pH7.5), 5 mM MgCl₂, 5.8 % PEG6000, 3.3 mM dithiothreitol, 60 uM ATP, 0.006% BSA, and 2000U of T4 RNA ligase (from New England Biolabs, Beverly, MA). After incubation at 15° for 4 hr, the T4 RNA ligase was heat-inactivated at 50° for 10 min. The final ligated product was purified as above. RNA was centricon-filtered in the final NMR buffer.

4. NMR experiments

NMR data were acquired at either 25°C or 30°C on Varian Inova 800 MHz NMR spectrometers equipped with triple resonance x,y,z-axis gradient probes. NMR samples were in 10 mM phosphate buffer (pH 6.4), 100 mM NaCl. ¹H-¹⁵N HMQC spectrum acquired with 4096 (*t*₂) x 1024 (*t*₁) points, with sweep widths of 17567 Hz in ¹H and 2431 Hz in ¹⁵N and 44 scans per *t*₁ point at 800 MHz.

Longitudinal relaxation (T_1) and transverse relaxation (T_2) experiments were performed using sensitivity-enhanced inversion-recovery pulse sequences with pulsed field gradients. T_1 and T_2 values were obtained by the overall envelop heights of a series of spectra recorded with different delay times, T . T_1 values were measured using spectra recorded with six different delay times; $T=0.1, 0.3, 0.5, 0.7, 0.9, 1.2$ s. T_2 values were measured using spectra recorded with seven different delay times; $T=0.03, 0.05, 0.07, 0.09, 0.11, 0.15$ s. The T_1 and T_2 values were obtained by fitting the intensities of the height of overall envelop in each spectrum to single exponential curve using a non-linear least-squares fitting program, CURVEFIT, available from the web site of A. Palmer, Columbia University (<http://cpmcnet.columbia.edu/depts/gsas/biochem/labs/palmer/software/curvfit.html>).

