

Secondary Structure Analysis of a G-rich Sequence Recognizing RNA Aptamer with Structure Specific Enzymes

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RNA structure has been analyzed by biophysical method such as NMR (nuclear magnetic resonance), which has not been popular because the RNA with small size only is available for the structural analysis with NMR.¹ So biochemical methods using structure specific enzymes and chemicals have widely been used for the analysis of RNA structure.^{2-8,19} Enzymes and chemical which have mainly been used for probing RNA structure in solution, are double-strand-specific RNase V1, single-strand-specific nuclease S1, RNase T1 which has the specificity for a guanine in single strand region, and kethoxal (3-ethoxy-1,1-dihydroxy-2-butanone), which modify the N1 and N2 of guanine in the single strand. Hydroxyl radical ($\bullet\text{OH}$) has also been used for the high-order structure analysis of RNA. Exposed nucleotides are damaged by hydroxyl radical while nucleotides involved in tertiary contacts are protected from damage, making it a favorable approach for establishing exterior/interior relations for RNA.⁹⁻¹⁵ Radicals are generated from Fe(II)-EDTA with hydrogen peroxide (H_2O_2). Ascorbate (or DTT) is added to reduce Fe(III) to Fe(II). Hydrogen abstraction from the ribose 4' carbon leads to strand scission. In-line probing is also an RNA-structure probing method recently developed by Breaker group.¹⁶⁻¹⁸ This method has been used to examine secondary structure of RNAs and whether RNAs undergo structural rearrangements under the different incubation conditions. In-line probing takes advantage of the fact that the spontaneous cleavage of RNA is dependent on the local structure at each internucleotide linkage. RNA degrades through a nucleophilic attack by the 2' oxygen on the adjacent phosphorus. Cleavage occurs efficiently when the attacking 2' oxygen, the phosphorus and the departing 5' oxygen of the phosphodiester linkage are in a linear configuration. Linkages in double strand region of a folded RNA show resistance to cleavage because it is difficult for the atoms to be held in an in-line configuration. However, if folding does not restrict its structure, linkages occasionally take on in-line geometry through random motion and therefore are subject to a spontaneous cleavage.

RNA aptamers which are capable of interacting with the guanine-rich sequence, were selected from a random-sequence

GGGAUCCGCAUGCAAGCUUACAGCCGGUGU

Figure 1. Sequence of the randomized region in RNA aptamer 11-48-2. The consensus sequence is underlined in bold letters.

RNA library. In this work, the secondary structure of the RNA aptamer 11-48-2, one of the selected RNA aptamers (Fig. 1) was predicted with the CLC RNA workbench ver. 4.2 program accessed on the internet (www.clcbio.com/index.php?id=1042) and examined with RNA structural probes such as RNase T1, RNase V1 and nuclease S1. Because prior to getting the information for the interaction between an RNA aptamer and a ligand RNA, the determination of the structure of selected RNA aptamers is important.

The structure of RNA aptamer 11-48-2 including primer sequence was probed in binding buffer with RNase T1, RNase V1, and nuclease S1 (Fig. 2). G16, G23 and G45

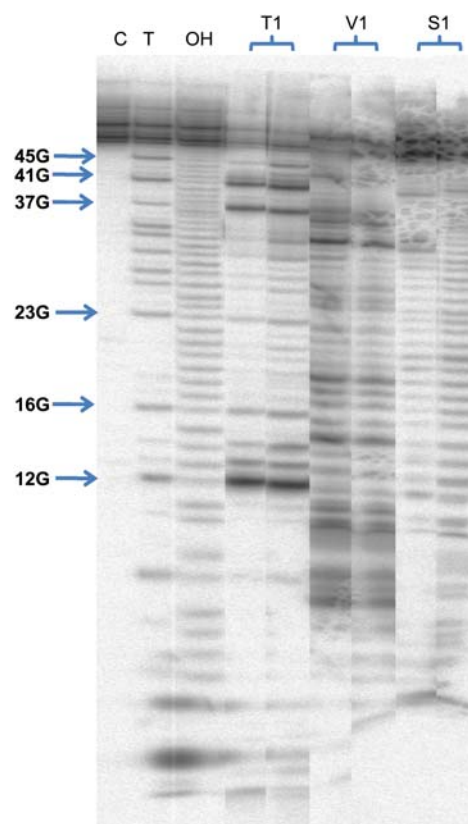


Figure 2. Enzymatic probing of RNA aptamer 11-48-2 labeled at the 5'-end. The RNA was partially digested with RNase T1, RNase V1 and nuclease S1. The cleaved nucleotides by RNase T1 are indicated by arrows. Lane C, control; lane T, the denatured RNA treated with RNase T1; lane OH, partial alkaline ladder.

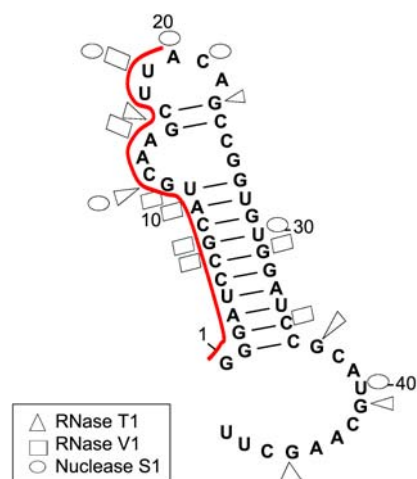


Figure 3. Schematic representation of possible secondary structure of RNA aptamer 11-48-2. Triangles indicate the sites cleaved by RNase T1, squares indicate the sites cleaved by RNase V1 and circles indicate the sites cleaved by nuclease S1.

were cleaved by RNase T1, especially strong cleavages at G12, G37 and G41 so these guanines are thought to be in single-strand region of the secondary structure model of RNA aptamer 11-48-2. And G8, G26, G27, G29, G31 and G32 were not or weakly cleaved by RNase T1 so these guanines are thought to be in double-strand region. But G16 and G23 in double-strand region were strikingly digested by RNase T1, suggesting that these two base pairs of G16:C24 and C17:G23 are not stable and exist in single stranded or double stranded region at the equilibrium state..

C7, G8, A10, U11, G31 and C35 were strongly cleaved by RNase V1 so these nucleotides are thought to be located in double strand. But A15 and U19 which were thought to be in single strand, were susceptible to RNase V1, suggesting that these nucleotides were stacked from intramolecular interaction and became accessible to RNase V1 in solution.

Nucleotides G12, U19, A20, C21 and U40 were cleaved by nuclease S1 so these nucleotides are thought to be in single-strand region of the secondary structure model of RNA aptamer 11-48-2. But U30 in double-strand region were strikingly digested by RNase S1, which further study such as NMR is needed for.

In conclusion, the structure of RNA aptamer 11-48-2 was analyzed in solution with probes such as RNase T1, RNase V1, and nuclease S1 and its possible secondary structure was represented (Fig. 3). The results suggested that 1) this molecule has two single strand regions and one double strand region without any bulge, and 2) two base pairs of G16:C24 and C17:G23 are not stable because of the adjacent presence of single stranded region.

Experimental Section

Preparation of RNA. RNA aptamer 11-48-2 was synthesized by run-off *in vitro* transcription with T7 RNA polymerase from the DNA template to which the T7 promoter was annealed and purified by gel elution of the crush and soak method.²⁰ The resulting RNA was treated with CIP (calf intestinal alkaline phosphatase) to remove 5' terminal phosphate and then labeled at the 5' end using [γ -³²P]ATP and T4 polynucleotide kinase.

Enzymatic Cleavage Reaction. 5'-Terminal radiolabeled RNA aptamer 11-48-2 was heated in binding buffer (30 mM Tris-acetate, pH 7.5, 60 mM magnesium acetate, 120 mM potassium acetate, and 120 mM ammonium acetate) at 90 °C for 2 min and allowed to cool to RT (~21 °C). Then 0.1-1 unit of nuclease S1 (Boehringer Mannheim GmbH, W.-Germany) or 0.001-0.01 unit of RNase V1 (Pierce Molecular Biology, Perbio) or 0.1-1 unit of RNase T1 (Industrial Research Limited) was added to the above mixture and then the reaction mixture was incubated for 25 min at RT. The reaction volume included an additional 1 mM ZnCl₂ for nuclease S1 cleavage. The cleavage products were recovered by ethanol precipitation and separated on a 15% polyacrylamide gel in 90 mM Tris-borate (pH 8.3) and 2.5 mM EDTA containing 7 M urea.

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