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Secondary Structure Analysis of an RNA Interacting with Guanine-rich Sequence

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RNA structure has sometimes 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 and a large amount of RNA is required 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.[2-11] Enzymes which have mainly been used for probing RNA structure in solution, are double-strand-specific RNase V1, single-strand-specific nuclease S1 and RNase T1 which has the specificity for a guanine in single strand region. Chemicals used for probing RNA structure, are DMS (dimethyl sulfate) which methylates position N-1 of adenines and, to a lower extent, N-3 of cytosines, CMCT (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate) which modifies position N-3 of uridine and, to a weaker degree, N-1 of guanines and kethoxal (3-ethoxy-1,1-dihydroxy-2-butanone) which modifies position N-1 and N-2 of guanine in the single strand.

Hydroxyl radical (·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.[12-18] Radicals are generated from hydrogen peroxide (H_2O_2) with Fe(II)-EDTA. 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 developed by Breaker group.[19-21] 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 inter-nucleotide 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 binding to the guanine-rich sequence RNA (5'-GGGAGGGGCGGGUCUGGG-3') in the 5'-UTR (untranslated region) of N-ras oncogene, were selected from a random-sequence RNA library with RNA-RNA interaction.[22] The selected RNA aptamers can recognize the specific domain of RNA structure like a monoclonal antibody and be candidates of the anticancer agent at the genetic level. The determination of the structure of selected RNA aptamers is very important prior to getting the information for the interaction between an RNA aptamer and a ligand RNA used for selection. So in this work, the secondary structure of an RNA aptamer 11-30-36, one of the selected RNA aptamers (Fig. 1) was predicted with the theoretical method, the CLC RNA workbench ver. 4.2 program accessed on the internet (www.clcbio.com) and biochemically examined with RNA structural probes such as RNase T1, RNase V1 and nuclease S1.

5'-**GGGAUCCGCAUGCAAGCUUA**CUGCCACGUUG-3'

Figure 1. Sequence of the randomized region in RNA aptamer 11-30-36. The consensus sequence is underlined in bold letters.

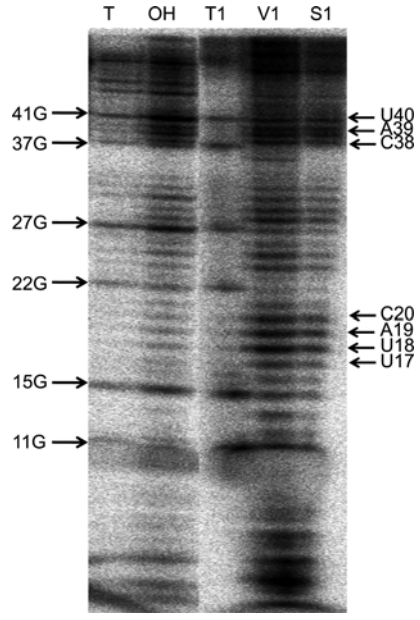

Figure 2. Enzymatic probing of RNA aptamer 11-30-36 labeled at the 5'-end. The RNA was partially digested with RNase T1, RNase V1 and nuclease S1. The nucleotides cleaved by RNase T1 and nuclease S1 are indicated by arrows. Lane T, the denatured RNA treated with RNase T1; lane OH, partial alkaline ladder.

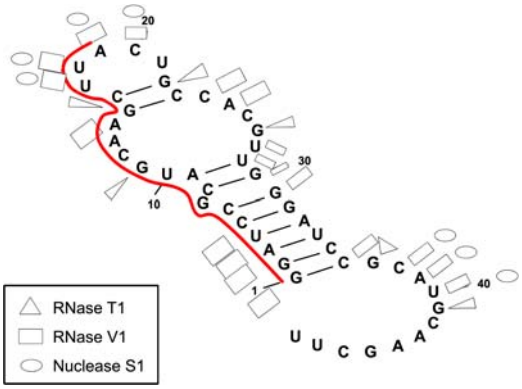

Figure 3. Schematic representation of possible secondary structure of RNA aptamer 11-30-36. Triangles indicate the sites cleaved by RNase T1, squares indicate the sites cleaved by RNase V1 and circles indicate the sites cleaved by nuclease S1.

The structure of RNA aptamer 11-30-36 including primer sequence was probed in binding buffer with RNase T1, RNase V1, and nuclease S1 (Fig. 2). Nucleotides G11, G15, G22, G27, G37 and G41 were strongly cleaved by RNase T1, suggesting that these guanines are thought to be in single-strand region of the secondary structure model of RNA aptamer 11-30-36. And G1G2, G7 and G30GG32 were not or weakly cleaved by RNase T1 so these guanines are thought to be in double-strand region. Nucleotides G15 and G22 in double-strand region of the secondary structure model were strikingly digested by RNase T1, suggesting that base pairs G15:C23 and C16:G22 are not stable by the presence of two adjacent single-strand loops and exist in single stranded and double stranded region at the equilibrium state. The accurate identity for these two base pairs can be got after getting the information for the tertiary structure of RNA aptamer 11-30-36.

G1GAU4, A14, U17UAC20, C24A25, U28UGG31, C36 and C38AU40 were strongly cleaved by RNase V1 so these nucleotides are thought to be located in double strand. But sequences U17UAC20 and C38AU40 which were thought to be in single strand, were strongly susceptible to RNase V1, suggesting that these nucleotides were stacked from intramolecular interaction and became accessible to RNase V1 in solution.

Sequences U17UAC20 and C38AU40 were strongly cleaved by nuclease S1 so these nucleotides are thought to be in single-strand region of the secondary structure model of RNA aptamer 11-30-36. These two sequences were also digested by RNase V1 and so it seems likely that RNA aptamer 11-30-36 has a dynamic structure with multiple conformers.

In conclusion, the structure of RNA aptamer 11-30-36 in solution was analyzed 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 three single strand regions and two double strand regions with a bulge G7, 2) sequences U17UAC20 and C38AU40 are stacked from intramolecular interaction and 3) base pairs of G15:C23 and C16:G22 are not stable because of the adjacent presence of two single stranded regions.

Experimental Section

Preparation of RNA. RNA aptamer 11-30-36 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 PAGE (polyacrylamide gel electrophoresis) and gel elution of the crush and soak method.[23] The purified RNA was treated with CIP (calf intestinal alkaline phosphatase) to remove 5' terminal phosphate and then labeled at the 5' end using [γ-^{32}P]ATP and T4 polynucleotide kinase. The 5' end radiolabeled RNA was purified by PAGE.

Enzymatic Cleavage Reaction. The 5'-terminal radiolabeled RNA aptamer 11-30-36 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 Manheim 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 $ZnCl_2$ for only nuclease S1 cleavage. The cleavage products were recovered by ethanol precipitation and separated on a 15% polyacylamide gel in 90 mM Tris-borate (pH 8.3) and 2.5 mM EDTA containing 7 M urea.

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