Structure of an RNA Interacting with the Stem-loop Sequence at the Gag-pol Junction

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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 been used widely for the analysis of RNA structure.[2-8] 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 a 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 (·OH) has also been used for the structural 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 is 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 stem-loop sequence at the gag-pol junction of HIV-1 RNA, were selected from a random-sequence RNA library.[19] In this work, the secondary structure of the RNA aptamer 13-1-15, one of the selected RNA aptamers (Fig. 1) was examined with the MFOLD program accessed on the internet (www.bioinfo.rpi.edu/applications/mfold/old/rna) and 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 hairpin RNA, the determination of the structure of RNA aptamers is important. The secondary structure of RNA aptamer 13-1-15 was also compared with those of RNA aptamer 13-1-3 and 12-1-5, which were studied previously.

GGGCGAAUAGGGAAGCUUGAU

Figure 1. Sequence of the randomized region in RNA aptamer 13-1-15.

The structure of RNA aptamer 13-1-15 was probed in binding buffer with RNase T1, RNase V1, and nuclease S1 (Fig. 2). G12, G23, G24 G29 and G33 were cleaved by RNase T1, especially strong cleavages at G5, G10 and G11 so these guanines are thought to be in single-strand region of the secondary structure model of RNA aptamer 13-1-15. But G19 and G22 in double-strand region were also digested by RNase T1, suggesting that these two Gs are unstable because G19 and G22 are near a bulge G33 and single strand region, respectively.

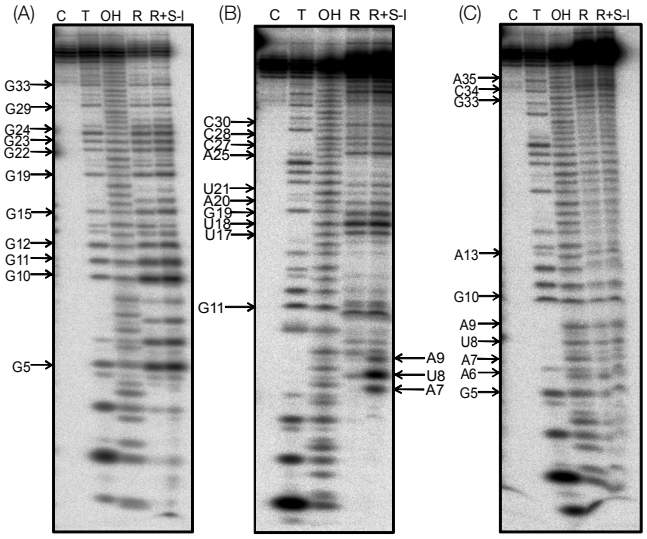

Figure 2. Enzymatic probing of RNA aptamer 13-1-15 labeled at the 5'-end. The RNA was partially digested with RNase T1 (A), RNase V1 (B) and nuclease S1 (C). The cleaved nucleotides are indicated by arrows. Region A7UA9 was protected from RNase V1 digestion by the hairpin RNA. Lane C, control; lane T, the denatured RNA treated with RNase T1; lane OH, partial alkaline ladder; lanes R and R+S-l, enzymatic cleavages in the absence of and the presence of stem-loop RNA, respectively.

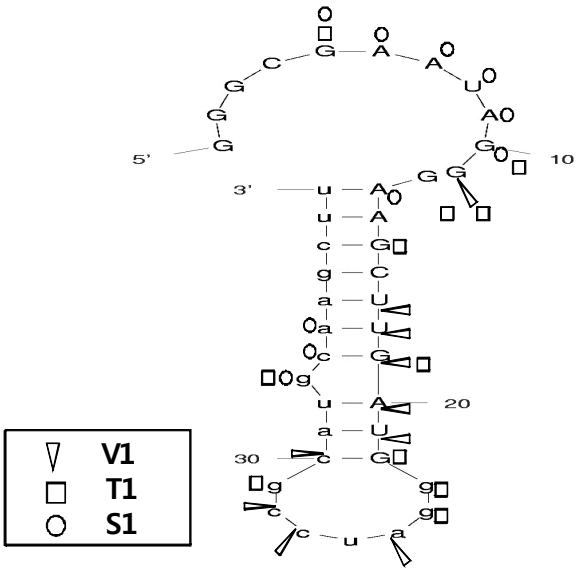

Figure 3. Schematic representation of possible secondary structure of RNA aptamer 13-1-15. Triangles indicate the sites cleaved by RNase V1, squares indicate the sites cleaved by RNase T1 and circles indicate the sites cleaved by nuclease S1.

A20, U21 and C30 were cleaved by RNase V1, especially strong cleavages at region U17UG19 so these regions are thought to be located in double strand. But G11, A25 and C27C28 which were thought to be in single strand, were susceptible to RNase V1, suggesting that these regions were stacked from intramolecular interaction and became accessible to RNase V1 in solution. Region A7UA9 was protected from RNase V1 digestion by the hairpin RNA, a ligand used for selection of this RNA aptamer. This region of RNA aptamer 12-1-5 is thought to be important for the interaction between the RNA aptamer and the hairpin RNA and this will be discussed later in footprinting assay.

Residues G5AAUA9 and G33 were cleaved by nuclease S1, especially strong cleavages at G10, so these regions are thought to be in single-strand region of the secondary structure model of RNA aptamer 13-1-15. The residue C34 was susceptible to nuclease S1 and residue G19 was attacked by RNase T1, and so C34:G19 base pair is thought to be unstable because of the adjacent presence of a bulge G33. This instability of C34:G19 base pair was also detected in C11:G25 base pair of RNA aptamer 13-1-3 and C11:G24 base pair of RNA aptamer 12-1-5.[6,7]

In conclusion, the structure of RNA aptamer 13-1-15 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 result suggested that 1) this molecule has two single strand regions and one double strand region which has a bulge G33, and 2) C34:G19 base pair is unstable because of the adjacent presence of a bulge G33.

Experimental Section

Preparation of RNA. RNA aptamer 13-1-15 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-^{32}P]ATP and T4 polynucleotide kinase.

Enzymatic cleavage reaction. 5'-terminal radiolabeled RNA aptamer 13-1-15 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 37 ^{o}C for 20 min and allowed to cool to RT (~21 ^{o}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 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 7M urea.

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