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Identification of the +1 Ribosomal Frameshifting Site of LRV1-4 by Mutational Analysis

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Leishmania virus (LRV)1-4 has been reported to produce a fusion of ORF2 and ORF3 *via* a programmed +1 frameshift in the region where ORF2 and ORF3 overlap (Lee *et al.*, 1996). However, the exact frameshift site has not been identified. In this study, we compared the frameshift efficiency of a 259bp (nt. 2565-2823), frameshift region of LRV1-4, and the 71bp (nt. 2605-2678) sub-region where ORF2 and ORF3 overlap. We then predicted the frameshift site using a new computer program (Pseudoviewer), and finally identified the specific region associated with the mechanism of the LRV1-4's +1 frameshift by means of a mutational analysis based on the predicted structure of LRV1-4 RNA. The predicted structure was confirmed by biochemical analysis. In order to measure the frameshift efficiency, constructs that generate luciferase without a frameshift or with a +1 frameshift, were generated and *in vitro* transcription/translation analysis was performed. Measurements of the luciferase activity generated, showed that the frameshift efficiency was about 1% for both the 259bp (LRV1-4 259FS) and 71bp region (LRV1-4 71FS). Luciferase activity was strongly reduced in a mutant (LRV1-4 NH: nt. 2635-2670) with the entire hairpin deleted and in a mutant (LRV1-4 NUS: nt. 2644-2659) with the upper stem of the hairpin deleted. These results indicate that the frameshift site in LRV1-4's is in the 71bp region where ORF2 and ORF3 overlap, and that nt. 2644-2659 (the upward hairpin stem) play a key role in generating the +1 frameshift.

Key words: LRV (Leishmania virus)1-4, Ribosomal +1 frameshift, Luciferase activity

INTRODUCTION

Leishmania virus (LRV), a double-stranded RNA virus, infects the protozoon, *Leishmania,* the cause of leishmaniasis in humans. Leishmaniasis is a vector-borne disease caused by obligate intramacrophage protozoa (Barbara, 1999). *Leishmania* is the parasites which replicate in macrophage, monocytes and naso-oropharyngeal mucosa (Barbara, 1999). About 21 leishmanial species have been known to cause the various clinical syndromes (Desjeux, 1996; Shaw, 1994; Ashford, 1997). LRV is found in *Leishmania braziliensis, L. guyanensis* (New World parasites) and *L. major* (Old World parasites, Tarr *et al.*, 1988; Widmer *et al.*, 1989; Guilbride *et al.*, 1992: Cadd *et al.*, 1993), and is classified as a member of the *Totiviridae* family (Patterson *et al.*, 1995).

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LRVs have been grouped into 12 strains from LRV1-1 to LRV1-12 (in New World parasites, Patterson *et al.*, 1992) or 13 strains including LRV2-1 (in Old World parasites). Recently, the complete sequences of LRV1-1 (Stuart *et al.*, 1992), LRV1-4 (Scheffter *et al.*, 1994), and LRV2-1 (Scheffter *et al.*, 1995) have been reported. The genome of LRV1-4 consists of 5283 nucleotides, and has 4 open reading frames (ORF1, ORFx, ORF2, ORF3, Scheffter *et al.*, 1994). ORF1 and ORFx, small open reading frames located at the 5' terminus of the genome, encode 34- and 60-amino acid polypeptides, respectively, whose functions have yet to be identified. ORF2 and ORF3, with a 71 nucleotide overlap, encode the 82 kDa major capsid protein and the 98 kDa RNA-dependent RNA polymerase, respectively (Scheffter *et al.*, 1994).

It has been reported that ORF3 does not contain a translation initiation sequence (Kozak, 1986, 1987), and that instead a 180 kDa fusion protein, in which ORF 2 and ORF3 are fused together, is formed by a programmed +1 frameshift in the region where ORF2 and ORF3 overlap

(Lee *et al.*, 1996). However, the exact frameshift site and the mechanism by which the frameshift is generated have not been identified.

Although programmed +1 frameshifts are less frequent than -1 frameshifts, they have been encountered in bacteria (Craigen *et al.*, 1986), yeast (Belcourt *et al.*, 1990), and mammalian cells (Matsufuji *et al.*, 1995; Ivanov *et al.*, 1998, 2000). Several studies have suggested that +1 frameshifts require a slippage region where ribosomes can switch frame by moving through the 3-dimensional structure of the RNA, though such slippage does not depend on a unique mechanism that is involved in -1 frameshifting (Farabaugh, 1996).

In this study, we inserted the LRV1-4 frameshift region (259bp) proposed by Lee *et al.* and a 71bp sub-region where ORF2 and ORF3 overlap, into a vector (p.cDNA3.1-5'URT-LUC) that can generate luciferase only when +1 frameshift occurs. Our aim was to measure the frequency of +1 frameshifts, and identify the frameshift site. In addition, we attempted to predict the structure of the RNA of LRV1-4 using the computer program (Pseudoviewer). We then tested this structure by biochemical analysis, and identified the specific region responsible for the +1 frameshifts by deleting different segments of the region believed to serve as the frameshift signal.

MATERIALS AND METHODS

Construction of plasmids

The parent plasmid used in this study was pcDNA3.1-5'UTR-LUC (Baril *et al.*, 2003). pcDNA3.1-LUC was initially generated by inserting the luciferase gene downstream from the T7 promoter in pcDNA3.1/hygro(+) (Invitrogen, U.S.A., Dulude *et al.*, 2002), and pcDNA3.1-5'UTR-LUC was created from pcDNA3.1-LUC by inserting a 55nt 5'UTR, an initiation triplet, two codons, and an Eco47 III restriction site between the *Kpn I and BamH I* sites (Baril *et al.*, 2003).

We then inserted the LRV1-4 frameshift region between the *Kpn I and BamH I* sites of pcDNA3.1-5'UTR-LUC. The resulting constructs were designed to generate luciferase only when the ribosome moved +1 nucleotide *via* a +1 frameshift (Fig. 1-A). A control plasmid (0) corresponding to each construct was created that made luciferase without needing a frameshift, by inserting an adenine immediately before the luciferase genes of the (+1) constructs (Baril *et al.*, 2003, Fig. 1-A).

A cDNA of LRV1-4 259FS containing the 71bp overlap region was produced by RT-PCR (Ro *et al.*, 1997), and *Kpn I and BamH I* sites added by PCR (Fig. 1-C). The deletion mutants (H, B, NH, NB, NUS, NDS) created from LRV1-4 71FS and 71FS were made by oligonucleotide synthesis (TaKaRa, Japan, Fig. 1-B). These DNAs were inserted between the *Kpn I and BamH I* sites of pcDNA3.1-5'UTR-LUC, and the inserts were confirmed by DNA sequencing.

In vitro transcription/translation

The plasmids were cut with *Stu I*, extracted with PCI (phenol/chloroform/isoamyl alcohol 25:24:1), and precipitated with ethanol. The DNA pellets were resuspended in TE (pH 8.0), and the DNA measured spectrophotometrically and by agarose electrophoresis. 1 μg aliquots of DNA were used in *in vitro* transcription/translation reactions using a TNT-Quick coupled *in vitro* transcription/translation kit (Promega, U.S.A.). 2.5 μL of the *in vitro* transcription/translation product was added to 50 μL of luciferase reagent (Promega, U.S.A.) and the luciferase activity was measured with a luminometer (Berthold Lumat LB 9507, Australia, Baril *et al.*, 2003).

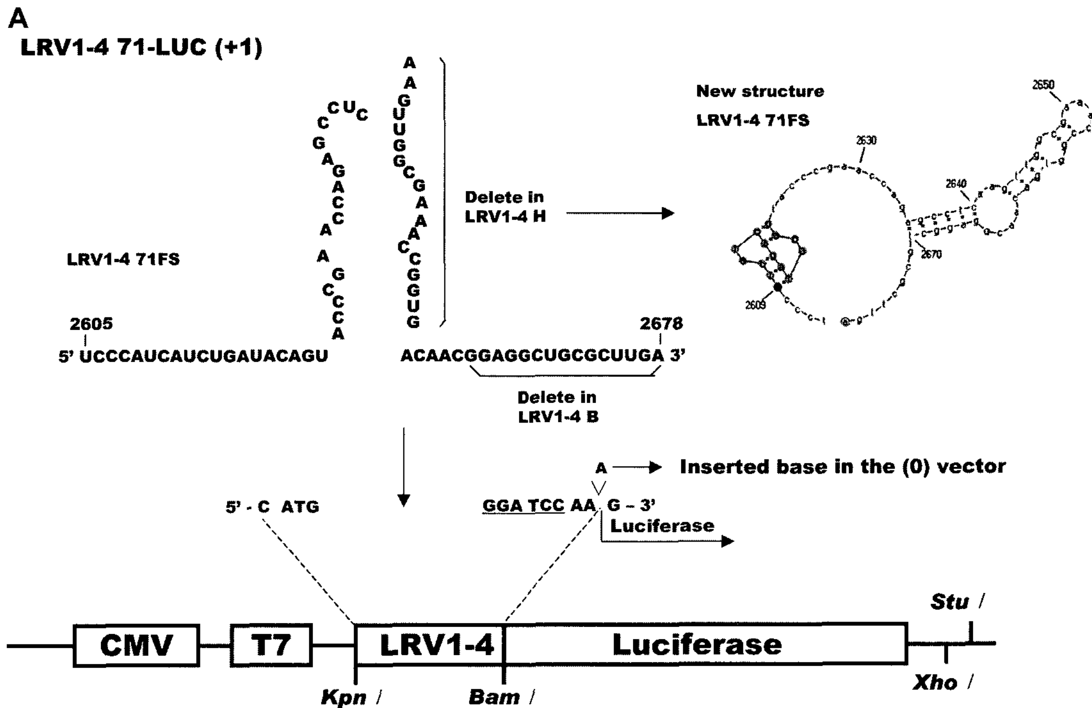
RNase mapping

In vitro transcribed RNA was dephosphorylated with calf intestine phosphatase (New England Biolabs, U.S.A.) and labeled with [γ-32P] ATP (Amersham, U.S.A.). The labeled RNA (56 nM) was denatured and renatured in RNA folding buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM DTT). This RNA was then incubated with RNase T1 (0.2 U) or RNase S1 (5 U and 0.5 U) at 37°C for 10 min, or with RNase V1 (0.005 U and 0.0025 U) at room temperature for 15 min. Nucleotide ladders were generated by alkaline hydrolysis of 0.25 mg/mL tRNA in 50 mM $NaHCO_3$ (pH 9.0), 1 mM EDTA, for 15 min at 90°C. After ethanol precipitation of the samples, the pellets were dissolved in loading dye, denatured for 5 min at 65, and immediately placed on ice. The samples were then loaded on 10% polyacrylamide/7 M urea gels in 1X TBE for 3 h at 1500V, and the gels were dried and analyzed with a PhosphoImager (FUJIX Bio Image Analyzer System, Japan).

RESULTS

Identification of the frameshift region of LRV1-4

In order to measure the efficiency of frameshifting of LRV1-4, the 71bp overlap region (nt. 2605-2678) (Fig. 1-A), of ORF2 and ORF3, and 259bp (nt. 2565-2823) containing the overlap region were inserted in a vector that would express the luciferase gene only when a frameshift was generated, These constructs were used in an *in vitro* transcription/translation system, and luciferase activity was measured (see Materials and Methods for details). Similar levels of luciferase activity were generated from the 71bp and 259bp inserts and comparison with the LRV1-4 71FS(0) and LRV1-4 259FS(0) constructs that can generate luciferase without a frameshift showed that the efficiency of frameshifting was about 1% in each case



B

LRV1-4 71FS(+1) TCCCATCATCTGATACAGTACCCGAACCAGAGCCTCAAGTTGGCGAAACCGGTGACAACGGAGGCTGCGCTTGA

LRV1-4 71FS(0) TCCCATCATCTGATACAGTACCCGAACCAGAGCCTCAAGTTGGCGAAACCGGTGACAACGGAGGCTGCGCTTGAAA

LRV1-4 H TCCCATCATCTGATACAGTACCCGAACCAGAGCCTC..ACAACGGAGGCTGCGCTTGA

LRV1-4 B TCCCATCATCTGATACAGTACCCGAACCAGAGCCTCAAGTTGGCGAAACCGGTGACAAC......................................

LRV1-4 NH TCCCATCATCTGATACAGTACCCGAACCAG...GCGCTTGA

LRV1-4 NB TCCCATCATCTGATACAGTACCCGAACCAGAGCCTC......GTTGGCGAAACCGGTGAC..........GAGGCTGCGCTTGA

LRV1-4 NDS TCCCATCATCTGATACAGTACCCGAACCAGAG..........AAGTTGGCGAAACCGGTGACAACGGAGGCTGCGCTTGA

LRV1-4 NUS TCCCATCATCTGATACAGTACCCGAACCAGAGCCTCAAG..CAACGGAGGCTGCGCTTGA

C

LRV1-4 259FS cDNA

2565 GTTGAGTTACCACTAGCCCCACGTTGTACATCTTCTTTAATCCCATCATCTGATACAGTACCCGAACCAGAGCCTCAAGTTGGCGA

AACCGGTGACAACGGAGGCTGCGCTTGATTGTTTTGAAAATGTGCGACGTATGATTAGTGGTTTCTTGAATCTGTCGTGGCTGACAGTAAC

ATCTAAAGAAAGGCAACATAAAAGTGACAGTAATTATTTCTATGATTATAATCTCTTCTTTTCCCAGATGCCAGCGATAGTT 2823

Forward primer 5'- GGGGTACCATGGTTGAGTTACCACTAGCCCCA -3'
Kpn I

Reverse primer 5'- CGGGATCCAACTATCGCTGGCATCTGGGA -3'
BamH I

Fig. 1. The luciferase-expressing plasmid used in analyzing the LRV1-4 +1 frameshift. (A) The LRV1-4 frameshift region (LRV1-4 259FS) proposed by Lee *et al.* and deletion mutants (LRV1-4 H, B, NH, NB, NDS, NUS) of the overlap region (LRV1-4 71FS) of ORF2 and ORF3, were inserted between the *Kpn I and BamH I* site, located before the coding sequence of the luciferase reporter gene. The LRV1-4 71FS RNA structure (left) is the structure predicted by Lee *et al.* while the structure (right) was predicted by Pseudoviewer. See Materials and Methods for further details. (B) The synthetic oligo sequence corresponding to the frameshift region (71bp) used in analysis of the +1 frameshift and deletion mutants. (C) The 259bp (nt. 2565-2823) sequence containing the overlap region (underline) of ORF2 and ORF3, and the oligonucleotide primers used with added *Kpn I and BamH I* sites (underline).

Table I. Luciferase assays with various constructs

Construct	Description	Luciferase activity (RUL)[b] *in vitro*
pcDNA3.1/zeo(+)	Negative control	13.5±2
T7 luciferase control DNA	Positive control	1018747±115613
LRV1-4 259FS(0)	frameshift region of LRV1-4[a]	279460±10836
LRV1-4 259FS(+1)	frameshift region of LRV1-4	2346±156
LRV1-4 71FS(0)	Overlap region (71bp) of LRV1-4	2732215±14618
LRV1-4 71FS(+1)	Overlap region (71bp) of LRV1-4	2172±56
H	Hairpin deleted	1240±20
B	Hairpin binding site deleted	990±77
NH	Hairpin region deleted	216±20
NB	lower stem deleted	1076±74
NUS	upper stem deleted	104±8
NDS	bulge deleted	558±59

a: The proposed LRV1-4 frameshift region (Lee *et al.*, 1996)
LRV1-4 259FS-LRV1-4 nt. 2565-2823 (259bp); LRV1-4 71FS- nt. 2605-2678 (71bp); H- nt. 2641-2658 deleted; B- nt. 2664-2678 deleted; NH- nt. 2635-2670 deleted; NB- nt. 2641-2642, 2661-2664 deleted; NUS- nt. 2644-2659 deleted; NDS- nt. 2635-2640, 2665-2670 deleted.
b: RLU= Relative Light Units

(Table I). This result confirmed that the 71bp region of overlap between ORF2 and ORF3 plays a significant role in generating the frameshift.

Analysis of the frameshift region (71bp) of LRV1-4 using deletion mutants

Based on the structure (Fig. 1-A) of the overlap region of ORF2 and ORF3 predicted by Lee *et al.* (1996), we deleted the hairpin part (H; nt. 2641-2658), and the region (B; nt. 2664-2678) that is thought to bind to the hairpin, and these constructs were in turn put through *in vitro* transcription/translation. Luciferase activity was reduced in both deletion mutants, but the effect was surprisingly small (Table I). It therefore appeared desirable to reconsider the structure of LRV1-4 RNA.

Analysis of the LRV1-4 frameshift region using a Computer program

The structure of the LRV1-4 frameshift region was analyzed with a web-based program (http://pseudoviewer.inha.ac.kr), referred to as Pseudoviewer (Fig. 3-A). In the newly generated structure the hairpin region was formed by nt. 2635-2670, whereas it was generated by nt. 2624-2657 in the previously predicted structure.

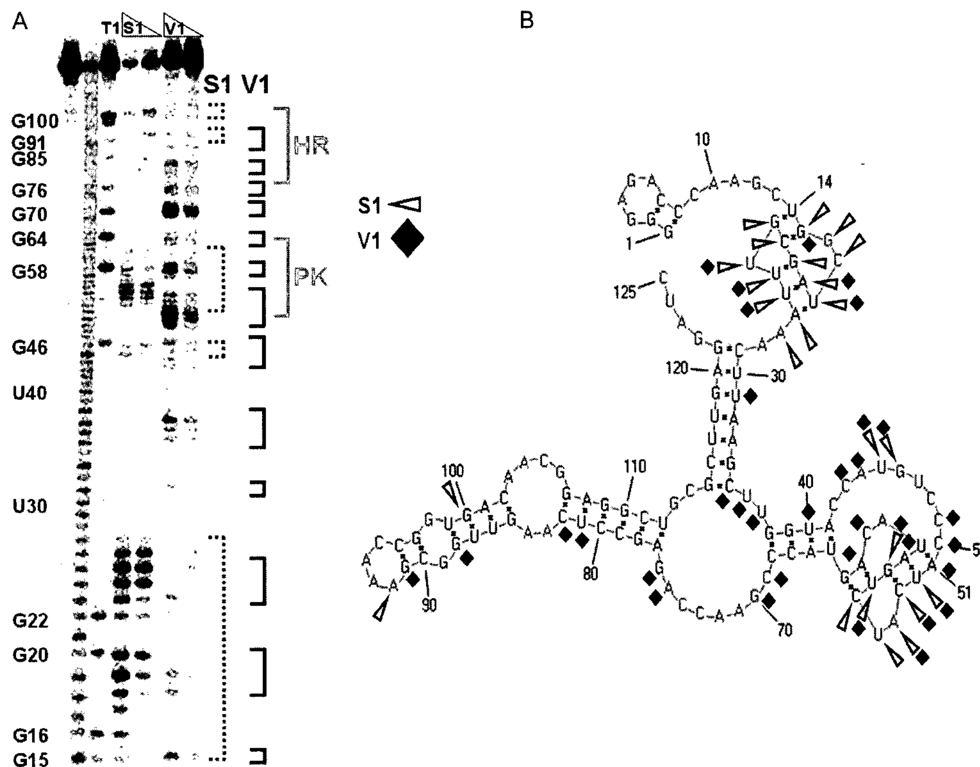


Fig. 2. Determination of the structure of pLRV RNA. (A) pLRV RNA was end-labeled and digested with RNase T1 (T1), RNase S1 (S1) or RNase V1 (V1). Lane 1, no treatment; lane 2, alkaline hydrolysis; lane 3, RNase T1 (0.2U); lane 4-5, RNase S1 (5U and 0.5U); lane 6-7, RNase V1 (0.005U and 0.0025U). Nucleotide positions are indicated on the left side of the gel. RNase-digested nucleotides are marked by dashed lines (S1) and solid lines (V1). HR: Hairpin region. PK: Pseudoknot region. (B) Diagram of the secondary structure of pLRV RNA. The RNase sensitive nucleotides are indicated by triangles (S1) and squares (V1). Viral RNA sequences (nucleotides 38-125) are indicated by the gray shading.

Determination of the RNA structure

To test whether LRV1-4 RNA had the newly predicted pseudoknot structure, we examined it (Fig. 2) by RNase mapping with G nucleotide-specific RNase T1, single strand-specific RNase S1, and double-strand-specific RNase V1. The *in vitro* transcribed RNA has extra vector flanking sequences as well as the viral RNA sequences (nucleotides 38-125). Based on the results from the RNase mapping and the computer program, the predicted frameshifting pseudoknot and the hairpin region structure existed in the viral RNA (Fig. 2-B). Therefore, the results showed that the predicted frameshifting pseudoknot structure was correct (nucleotides 51-64).

Analysis of the frameshift region (71bp) using further deletion mutants

In order to pinpoint the specific region controlling the LRV1-4 +1 frameshift, we measured the luciferase activities resulting from transcription/translation of additional deletion mutants of the hairpin region as specified in the new structure (Fig. 3-B). Luciferase activity was greatly reduced in mutant (NH) lacking the entire hairpin region, and in mutant (NUS) with the upper stem of the hairpin deleted, whereas it was not greatly affected when the lower stem of the hairpin (NDS) or the loop region (NB) was deleted (Table I). These findings confirm that the upper stem of the hairpin (nt. 2644-2659) plays a major role in frameshifting.

DISCUSSION

The results of this study indicate that the signal stimulating the LRV1-4 frameshift is located in the upper stem of the RNA hairpin. The similar luciferase activity

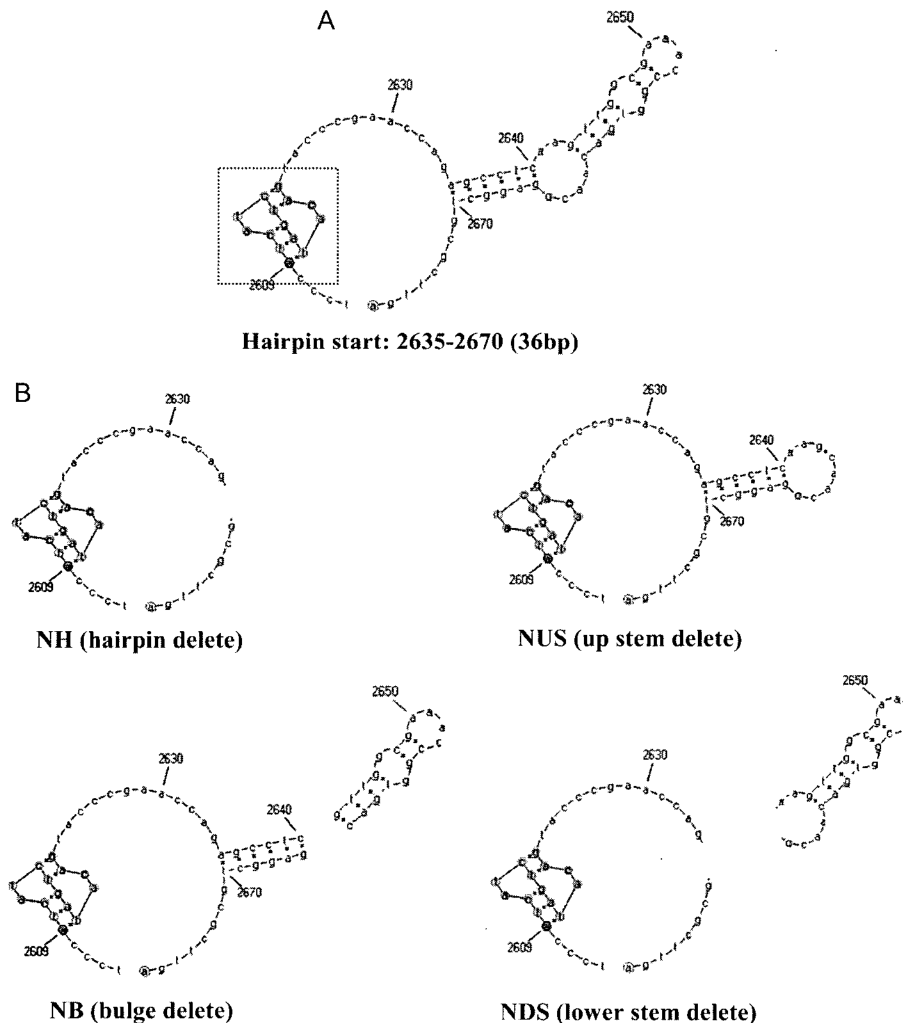


Fig. 3. Predicted RNA structure of the frameshift region of LRV1-4. The new structure of LRV1-4 predicted by the computer program, Pseudoviewer. Dotted square indicate the potential pseudoknot structure. NH; hairpin deleted nt. [2635-2670 deleted (36bp)], NUS; hairpin upper stem deleted [nt. 2644-2659 deleted], NB; bulge deleted [nt. 2641-2642, 2661-2664 deleted], NDS; lower stem deleted [nt. 2635-2640, 2665-2670 deleted].

seen with the complete LRV1-4 frameshift region (LRV1-4 259FS) and the limited LRV1-4's overlap region (LRV1-4 71FS) proposed by Lee *et al.* indicates that the overlap region is critical for generating the frameshift. Given that luciferase activity is similarly reduced in LRV1-4 H, that should not form the hairpin structure because one strand of the hairpin is deleted, and in LRV1-4 B, which is deleted for the region predicted to bind to the hairpin, (based on the structure of the overlap region predicted by Lee *et al.*), it seems clear that the hairpin structure controls the LRV1-4's frameshift. However, since contrary to prediction, luciferase activity was not greatly reduced in LRV1-4 H and LRV1-4 B (Table I), we derived a new structure for the LRV1-4 overlap region using the web-based program known as Pseudoviewer. This predicted RNA structure was verified by RNase mapping, and we showed that luciferase activity was greatly reduced in a mutant (LRV1-4 NH) lacking the whole hairpin region of the new structure (Table I). This result shows that the frameshift stimulating signal is in the hairpin region.

We also showed that luciferase activity was reduced to the same extent by deleting only the upper stem of the hairpin (NUS) as by deleting the whole hairpin (NH). Therefore, we suggest that the upper stem of the hairpin forms a more complex RNA structure after it binds to the hairpin binding site located downstream in the hairpin, thus stimulating the ribosome to move +1 nucleotide.

We are currently investigating further the +1 ribosomal frameshifting mechanism by adding cellular proteins to the LRV1-4 frameshift assay system, to determine whether frameshifting is influenced by some protein.

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