Mutational analysis of the RNA pseudoknot involved in efficient ribosomal frameshifting in simian retrovirus-1

Deukyong Sung and Hunseung Kang*

Kumho Life and Environmental Science Laboratory, Korea Kumho Petrochemical Co. Ltd, 572 Ssangam-dong, Kwangsan-ku, Kwangju, Korea

Received January 22, 1998; Revised and Accepted January 27, 1998

ABSTRACT

Mutational effects on frameshifting efficiency of the RNA pseudoknot involved in ribosomal frameshifting in simian retrovirus-1 (SRV-1) have been investigated. The primary sequence and the proposed secondary structure of the SRV-1 pseudoknot are similar to those of other efficient frameshifting pseudoknots in mouse mammary tumor virus (MMTV) and feline immunodeficiency virus (FIV), where an unpaired adenine nucleotide in between stem 1 and stem 2 has a potential to form an A-U base pair with the last uridine nucleotide in the loop 2, resulting in a continuous A-form helix with coaxially stacked stem 1 and stem 2. To test whether this A-U base pair is absolutely required for efficient frameshifting in SRV-1, a series of mutants changing this potential A-U base pair to either G+C base pair or A+A, A+G, A+C, G+A, G+G mismatch is generated, and their frameshifting efficiencies are investigated in vitro using rabbit reticulocyte lysate translation assay. The frameshifting abilities of these mutant pseudoknots are similar to that of the wild-type pseudoknot, suggesting that the A-U base pair in between stem 1 and stem 2 is not necessary to promote efficient frameshifting in SRV-1. These results reveal that coaxial stacking of stem 1 and stem 2 with a Watson–Crick A-U base pair in between two stems is not a required structural feature of the pseudoknot for promoting efficient frameshifting in SRV-1. Our mutational data suggest that SRV-1 pseudoknot adopts similar structural features common to other efficient frameshifting pseudoknots as observed in MMTV and FIV.

INTRODUCTION

Retroviruses use a programmed minus-one frameshifting to encode essential proteins from the polycistronic gag, gag-pro or gag-pro-pol overlapping genes. Two definite sequences, a heptanucleotide shift site and a downstream RNA secondary or tertiary structure on messenger RNA, serve as a signal for this frameshifting event. Numerous mutational studies have been done to investigate the roles of the downstream RNA structure on ribosomal frameshifting, and it is known that an RNA pseudoknot is required for high level of frameshifting in many retroviruses (1–3).

Three-dimensional structures of the RNA pseudoknots involved in ribosomal frameshifting in mouse mammary tumor virus (MMTV) have been determined by NMR (4–7). Direct comparison of the structures of the RNA pseudoknots that promote efficient frameshifting with those of the mutant pseudoknots that promote a low level of frameshifting, reveals the structural requirement of RNA pseudoknots in MMTV. One characteristic structural feature of the efficient frameshifting pseudoknots, is that two stems are separated by a single unpaired nucleotide (usually adenine), which prevents the pseudoknot from forming a continuous coaxially stacked conformation. This unpaired nucleotide serving as a hinge, and the steric constraint induced by the short loop 1, cause the two stems to bend relative to each other (4,6).

The RNA pseudoknot involved in ribosomal frameshifting at gag-pro overlapping region in SRV-1, contains similar structural features. Figure 1 compares the primary sequences and the proposed secondary structures of the RNA pseudoknots from simian retrovirus-1 (SRV-1), MMTV and feline immunodeficiency virus (FIV). For SRV-1 and FIV pseudoknots, two stems are separated by a single adenine nucleotide. This unpaired A has a potential to form an A-U base pair with a uridine nucleotide at the end of loop 2. In a mutational study, Chen et al. (6) showed that changing this U in loop 2 to A does not alter the frameshifting efficiency in FIV, indicating that this A-U base pair in between stem 1 and stem 2 is not necessary to promote efficient frameshifting. Loop 1 of the SRV-1 and FIV pseudoknots consists of 1 and 2 nt, respectively, which provides the steric hindrance for the two stems to bend relative to each other. Based on these observations, Chen et al. (6) postulated that SRV-1 and FIV pseudoknots may also adopt a characteristic bent conformation similar to that of MMTV pseudoknot.

In contrast to these mutational studies, a recent NMR study of SRV-1 pseudoknot by Du et al. (8) observed a peak from the base paired A-U imino proton, arguing the adenine nucleotide in between stem 1 and stem 2 does base pair with the last uridine nucleotide in loop 2. However, they observed an extremely broad U imino resonance, indicating that this U imino proton exchanges rapidly with the solvent. Since the three-dimensional structure of the SRV-1 pseudoknot is not yet available, the question whether the SRV-1 pseudoknot adopts a characteristic bent conformation, as for the MMTV pseudoknot, remains to be proved.

To understand the base pairing pattern of the two stems and to provide better insight into the structural feature of the RNA pseudoknot involved in ribosomal frameshifting in SRV-1, we

*To whom correspondence should be addressed. Tel: +82 62 970 2648; Fax: +82 62 972 5085; Email: hskang@ksc.kumho.co.kr
carried out a systematic mutational study for the nucleotides at the junction of stems and loop of the RNA pseudoknot. We are particularly interested in determining whether the SRV-1 pseudoknot has similar base pairings to the MMTV pseudoknot where an unpaired adenine nucleotide at the junction of two stems makes the RNA pseudoknot bent. We generated a series of mutants which changes the possible A•U base pair at the junction of stem and loop to several mismatch, A•A, A•G and A•C. We also mutated the A•U base pair to G•C base pair, and to G•U, G•G and G•A mismatch. Our mutational studies, together with other already verified mutational data, clearly demonstrate that the base pairing pattern of the SRV-1 pseudoknot is quite similar to other efficient frameshifting pseudoknots, suggesting that the SRV-1 pseudoknot adopts similar structural features to MMTV and FIV pseudoknots for promoting efficient ribosomal frameshifting during translation of the messenger RNA.

MATERIALS AND METHODS
Site-directed mutagenesis
All pseudoknot mutants were constructed by subcloning synthetic oligonucleotide fragments into the BstBI/SmaBI sites of plasmid
pMGPP containing the MMTV gag, pro, and pol genes fused to the 5′ portion of RSV gag downstream of an SP6 promoter as described. A BstBI–SnaBI fragment of 47 bp in length was replaced by a synthetic DNA fragment containing SRV-1 gag/pro frameshifting sequence to prepare SRV-1 pseudoknot mutants. The inserts of all these plasmids were confirmed by both automated sequencing, using ALFexpress automatic DNA sequencer (Pharmacia), and dideoxy sequencing using sequencing (USB).

**In vitro frameshifting assay**

The mutant SRV plasmids were linearized with BglII and transcribed in vitro by SP6 RNA polymerase for 1.5 h at 37°C as described. The RNAs were translated in vitro in rabbit reticulocyte lysates (Promega) for 50 min at 30°C and the [35S]methionine-labeled proteins were analyzed by 10% SDS–PAGE as described. After electrophoresis, the gels were dried and exposed to X-ray films. The relative amounts of gag, gag-pro, and gag-pro pol translation products were quantified by a PhosphorImager (Fuji), and the frameshifting efficiencies were calculated after correcting for different methionine content of the products.

**RESULTS AND DISCUSSION**

**The potential A·U base pair in between stem 1 and stem 2 is not necessary for efficient frameshifting in SRV-1 pseudoknot**

To investigate whether the A·U base pair in between stem 1 and stem 2 in SRV-1 pseudoknot is necessary to promote efficient frameshifting, we generated a series of mutants which change the possible A·U base pair to the A·A, A·C or A·G mismatch. The frameshifting abilities of these mutant pseudoknots were tested in vitro using a rabbit reticulocyte lysate translation assay. As shown in Figure 2, the frameshifting efficiencies of the SRV-1 mutants were not changed by mutating the U, the last nucleotide in the loop 2, to either A, C or G. These results imply that the A·U base pair is not necessary for promoting efficient frameshifting in SRV-1.

To further test the need for formation of an A·U base pair in the SRV-1 pseudoknot to promote efficient frameshifting, we generated another series of mutants which change the potential A·U base pair in between stem 1 and stem 2 to either the G·C base pair or to the G·A or G·G mismatch. Figure 3 shows the in vitro frameshifting efficiencies of these SRV-1 mutants. Replacing the A·U base pair with G·C base pair does not alter the frameshifting efficiency. In addition, the mutant carrying either G·A or G·G mismatch retains the wild-type level of frameshifting efficiency.

Changing the U, the last nucleotide in the loop 2, to either the A or G introduces a stop codon (UAU or UAG), which terminates the translation 10 amino acids earlier than the normal translation event. It is not clear whether the different location of the stop codon relative to the shift site affects the frameshifting in retroviruses. However, since the A·C mismatch mutant that does not contain a premature stop codon has the same frameshifting efficiency to the A·A or A·G mismatch mutants, it is considered that introducing a stop codon at a position earlier than the normal position does not influence the frameshifting efficiency, unless it is very close to the shift site.

All of these mutational results indicate that in the RNA pseudoknot involved in ribosomal frameshifting in SRV-1, the formation of the A·U base pair in between stem 1 and stem 2 is not necessary to promote efficient frameshifting. In a recent NMR report, Du et al. (8) showed the formation of the A·U base pair in between stem 1 and stem 2. However, the U imino peak is extremely broad, which indicates that the imino proton exchanges
rapidly with the solvent. Du et al. (8) discussed that the relatively rapid exchange rate is perhaps due to the A•U base pair being at the center of a pseudo-continuous, rather than continuous, A-form helix. It is considered that the SRV-1 pseudoknot adopts a structure that the sequence at the junction of the stems and loop is in equilibrium, between A•U base pairing and no A•U base pairing. Our results, of all mutants containing a mismatch instead of the A•U base pair retain the wild-type of frameshifting efficiency, support this speculation. Although there is no direct evidence showing the absence of the formation of A•A, A•C, A•G, G•A or G•G mismatches in the SRV-1 pseudoknot, it is very unlikely to imagine that all of these mismatches discussed above contribute equally to the structure and/or stability of the SRV-1 pseudoknot, giving rise to the same frameshifting efficiency for the different mutants.

Base pairing pattern of the SRV-1 pseudoknot common to other efficient frameshifting RNA pseudoknots

In this mutational study, we show indirectly that the formation of an A•U base pair in between stem 1 and stem 2 is not necessary for efficient frameshifting in SRV-1. This leaves the unpaired A stacked in between stem 1 and stem 2. This is one major base pairing pattern for efficient frameshifting RNA pseudoknots in many other retroviruses. In MMTV and FIV, two stems of the pseudoknot are separated by an unpaired A (Fig. 1). It was revealed by NMR studies that this single unpaired nucleotide serving as a hinge, together with the short loop 1, make the two stems of the pseudoknot bent relative to each other. This bending conformation is a unique structural feature among efficient frameshifting pseudoknots in MMTV (4–7).

In the mutational studies for FIV frameshifting pseudoknot, Chen et al. (9) showed that mutating the potential A•U base pair in between stem 1 and stem 2 to the A•A mismatch does not change the frameshifting efficiency (Fig. 1g). This result also indicates that the unpaired A intercalated in between stem 1 and stem 2 is a common structural feature for efficient frameshifting pseudoknot in retroviruses.

In a series of mutational studies for the SRV-1 pseudoknot (Fig. 1), ten Dam et al. (10,11) showed that extending the loop 1 from 1 to 4 nt decreases the frameshifting efficiency from 23 to 11%, indicating that a short loop 1 is necessary for efficient frameshifting. They also showed that decreasing the loop 2 by 2–3 nt increases the frameshifting efficiency from 23 to 33–34%. Chen et al. (9) showed that deleting the intervening A in between stem 1 and stem 2 in SRV-1 pseudoknot dramatically reduces the frameshifting efficiency to 4%. All of these mutational data indicate that the efficient frameshifting pseudoknot adopts a conformation in which a single unpaired nucleotide in between stem 1 and stem 2 serving as a hinge, and the short loop 1, make the two stems of the pseudoknot bent relative to each other. However, it is necessary to determine the three-dimensional structure of the FIV and SRV-1 pseudoknots to conclude that the bending conformation is a common structural feature of the efficient frameshifting pseudoknots conserved in many retroviruses.

Implication for the roles of the RNA pseudoknot in retroviral frameshifting

The mutational data presented here, and all other mutational and structural data published to date, give important clues for understanding the roles of the RNA pseudoknot in ribosomal frameshifting in retroviruses. Based on the mutational and structural analysis of an MMTV pseudoknot, Chen et al. (6) proposed a hypothesis that an RNA pseudoknot may serve actively as a signal for frameshifting, in contrast to the early reports arguing that the RNA pseudoknot acts passively as a simple energetic barrier to the approaching ribosome (12,13). This active role of an RNA pseudoknot implies that there may be a specific interaction between the RNA pseudoknot and the ribosome-related factors during translation of the retroviral messenger RNA. Since the frameshifting occurs in response to two frameshifting signals, the frameshift site and the RNA pseudoknot, these two signals should be located precisely next to each other if there is a specific interaction between ribosome and the RNA pseudoknot. It is true that these two signals are separated by a confined number of nucleotides, a so-called spacer with 6–8 nt. ten Dam et al. (10) showed that shortening or lengthening the spacer by 1 nt dramatically decreases the frameshifting efficiency from 23 to 11–12% in SRV-1 as summarized in Figure 1. The importance of the length of the spacer is also observed in FIV (14). These results imply that the RNA pseudoknot should be located at a precise place relative to the shift site for the ribosome to recognize the two signals. Although no direct evidence has been reported to date for supporting the specific interaction between the ribosome and the pseudoknot, all of these mutational studies indicate that the overall geometry of the RNA, comprising the shift site and the pseudoknot, should be that such the approaching ribosome could recognize the two signals. Since the primary sequence of the stems and the loops of the pseudoknot is not important for the frameshifting, it is feasible that the ribosome interacts with the characteristic bent RNA pseudoknot by geometric fashion, similar to the key and lock model for enzyme–substrate complex formation. More structural and mutational data are needed to answer these questions, and to fully understand the mechanism of frameshifting in retroviruses.

ACKNOWLEDGEMENTS

We thank Professor IGNacio Tinoco, Jr at University of California, Berkeley, in whose laboratory this work was initiated, for valuable comments and advice. We also thank Mr David Koh for synthesizing the DNA templates. This research was supported in part by National Institute of Health grant GM 10840 and by Department of Energy grant DE-FG03-86ER04046 to I. Tinoco. Kumho Life and Environmental Science Laboratory Publication No. 10.