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Prokaryotic and eukaryotic translational machineries respond differently to the frameshifting RNA signal from plant or animal virus

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Abstract

Many mutational and structural analyses of the RNA signals propose a hypothesis that programmed frameshifting occurs by a specific interaction between ribosome and frameshifting signals comprised of a shifty site and a downstream RNA structure, in which the exact nature of the interaction has not yet been proven. To address this question, we analyzed the frameshifting sequence elements from animal or plant virus in yeast and *Escherichia coli*. Frameshifting efficiencies varied in yeast, but not in *E. coli*, depending on the specific conformation of mouse mammary tumor virus (MMTV) RNA pseudoknot. Similar changes in frameshifting efficiencies were observed in yeast, but not in *E. coli*, for the mutations in frameshifting sequence elements from cereal yellow dwarf virus serotype RPV (CYDV-RPV). The differential response of MMTV or CYDV-RPV frameshifting signal to prokaryotic and eukaryotic translational machineries implies that ribosome pausing alone is insufficient to mediate frameshifting, and additional events including specific interaction between ribosome and RNA structural element are required for efficient frameshifting. These results supports the hypothesis that frameshifting occurs by a specific interaction between ribosome and frameshifting signal.
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Keywords: CYDV; Frameshifting; MMTV; Pseudoknot; Ribosome–RNA interaction

1. Introduction

Programmed minus-one frameshifting is a strategy utilized by many RNA viruses that regulate protein expression at translational level (Chamorro et al., 1992; Rohde et al., 1994; Brierley, 1995; Miller et al., 1995; Maia et al., 1996). Mutational and sequence analyses of the viral RNA have suggested that two sequence elements on messenger RNA are required for ribosomal frameshifting. One element is a slippery heptanucleotide shift site where frameshifting actually occurs, and the other element is a downstream enhancer structure that forms either a secondary or tertiary structure. The shift site and the downstream structure are separated by a spacer with a confined number of nucleotides. The importance of these sequence elements for an efficient frameshifting in several animal and plant viruses has been verified by mutational analyses. Although the mechanism of minus-one frameshifting is not fully understood, a model suggests that the stable hairpin or pseudoknot structure acts as a passive barrier to the approaching ribosome by slowing or stalling its migration, which, therefore, increases the probability of slippage at the shifty site (Jack et al., 1988; Horsfield et al., 1995). Extensive mutational and structural analyses of mouse mammary tumor virus (MMTV) RNA pseudoknots revealed that efficient frameshifting pseudoknots adopt a specific bent conformation (Chen et al., 1995; Shen and Tinoco, 1995; Chen et al., 1996; Kang et al., 1996; Kang and Tinoco, 1997; Sung and Kang, 1998a), suggesting that a specific conformation of

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downstream RNA is recognized by ribosomes or ribosome-related translational factors during translation of the viral RNA. The structure, stability, and function of RNA pseudoknots involved in programmed ribosomal frameshifting have been investigated in many other animal and plant viruses (Giedroc et al., 2000 and references therein). A more recent study revealed that ribosome pausing alone is insufficient to mediate frameshifting and additional events including specific interaction between ribosome and RNA structural element are required for efficient frameshifting (Kontos et al., 2001). However, the functional role of RNA pseudoknot and the exact nature of the interaction between ribosome and the RNA with specific conformation have not yet been verified. To understand the detailed mechanism of RNA structure-induced frameshifting, it is of critical importance to search for the factors that are involved in the interaction between ribosome and downstream RNA structure from various animal and plant viruses. Analysis of the effect of RNA structure with different conformation on in vivo frameshifting event in prokaryotic and eukaryotic translational systems would provide valuable clues for possible interaction between frameshifting sequence elements and translational machinery. The dependence of frameshifting efficiency on shift site, downstream RNA structure, and the spacing between these two sequence elements has been extensively investigated in many viral sequences. The in vitro analyses were carried out using either a rabbit reticulocyte lysate or a wheat germ extract, and the in vivo analyses were performed in *E. coli*, yeast, or mammalian cells. However, the efforts investigating and comparing directly the same frameshifting signals in both *E. coli* and yeast were relatively scanty.

In this study, frameshifting abilities of well-known MMTV frameshifting RNA pseudoknots with different conformation, as well as the effect on frameshifting of mutations in frameshifting signals from cereal yellow dwarf virus serotype RPV (CYDV-RPV) (Miller et al., 1995), were analyzed in *E. coli* and yeast translational systems. The structures of MMTV RNA pseudoknots investigated in this study (Fig. 1 and Table 1) have been determined (Shen and Tinoco, 1995; Chen et al., 1996; Kang et al., 1996; Kang and Tinoco, 1997). We specifically aimed to test if the prokaryotic and eukaryotic translational systems respond differently to the sequences and conformation of frameshift signals from either MMTV or CYDV-RPV whose proposed secondary structures were depicted in Fig. 1.

To construct frameshifting assay cassettes, the synthetic oligonucleotides (Table 1) were first subcloned into the B*am*HI and N*ar*I sites of the plasmid pGEM-luc (Promega, Wisconsin, USA) carrying a firefly luciferase gene. A B*am*HI-N*ar*I fragment of 34 bp in length was replaced by a synthetic DNA fragment containing MMTV *gag-pro* and CYDV-RPV ORF2/ORF3 (replicase) frameshifting elements in front of the firefly luciferase. The *Bam*HI-*Sal*I fragment of pGEM-luc carrying the N-terminally modified luciferase gene with MMTV or CYDV-RPV frameshift signal was fused into the downstream region of the GAL4 gene of the yeast expression vector, pAS2-1 (Clontech, Palo Alto, USA) (Fig. 2). For the analysis of the frameshifting in *E. coli*, the S*ma*I-X*ho*I fragment of pAS2-1 carrying the N-terminally modified luciferase gene was fused into the downstream of the GST gene of the *E. coli* expression vector, pGEX-4T-1 (Pharmacia, Uppsala, Sweden). The construct was designed to express the GAL4 DNA-BD-luciferase or GST-luciferase fusion proteins as a consequence of frameshift into minus-one frame (Fig. 2). As a control a zero frame construct was designed to express the active luciferase without frameshift. All constructs were confirmed by the dideoxy chain termination sequencing method using the dye Terminator cycle sequencing kit and ABI prism 310 DNA sequencer (Perkin–Elmer). The plasmids were transformed into a *S. cerevisiae* INVSc1 using lithium acetate transformation procedure, and into an *E. coli* BL21 using standard heat shock procedure as recommended by the manufacturer (Clontech, Palo Alto, USA). The yeast and *E. coli* cells were cultured, harvested and normalized, and same amounts of cells were assayed for the luciferase activity as described (Sung and Kang, 1998b). The luciferase background was measured using the same yeast and *E. coli* cells carrying only the cloning vector without the gene for luciferase. Frameshifting efficiency was calculated as a ratio of the luciferase activity of the minus-one frame construct to that of the zero frame construct. The measurements were repeated nine to ten times with varying amounts of freshly cultured yeast and *E. coli* cells harvested at different growth stage to compensate any possible changes in luciferase activity.

2. Yeast and *E. coli* translational machineries respond differently to the conformation of MMTV frameshifting pseudoknot

To test whether the well-known frameshift signal from MMTV responds differently to prokaryotic and eukaryotic translational systems, the frameshift sequence elements were introduced into the luciferase assay cassette. Especially, we wanted to know whether the *E. coli* and yeast translational machineries respond to the conformational changes of the RNA pseudoknot downstream from the shift site. Two mutant sequence elements (APK and ΔA14U13C in Chen et al., 1996) containing mutated RNA pseudoknot that induced a low level of frameshifting in vitro, as well as two RNA pseudoknots (MMTV and U13C in Chen et al., 1996)

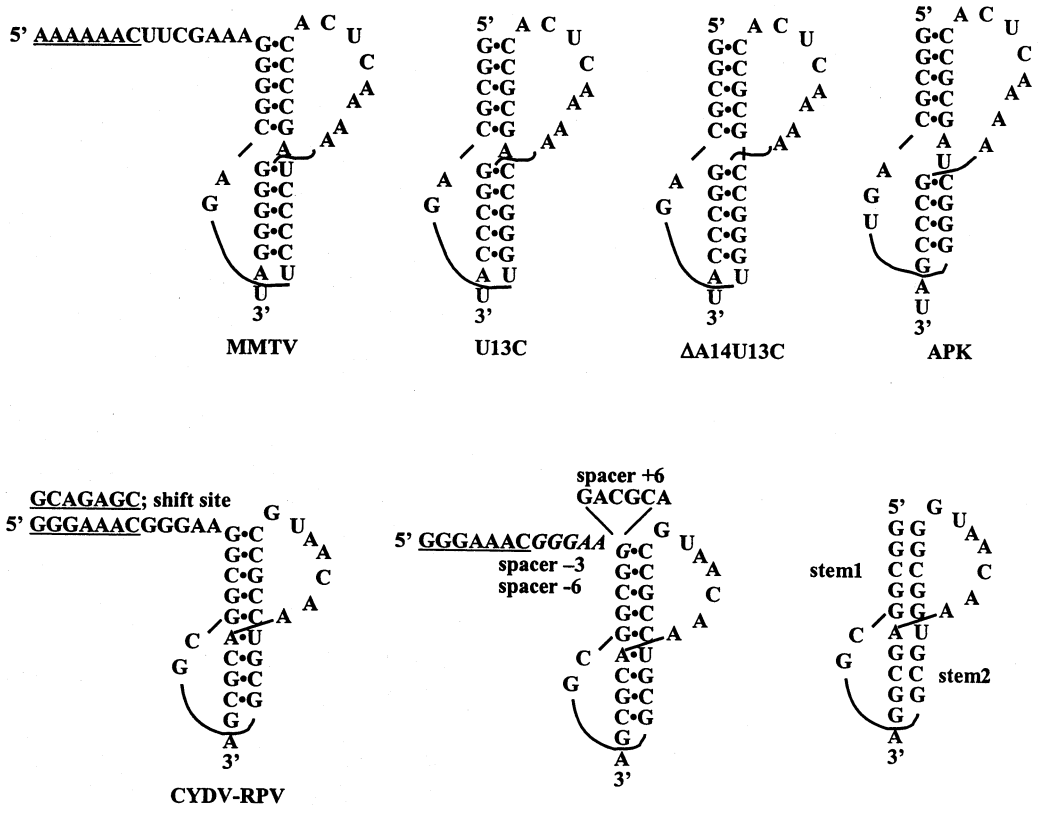

Fig. 1. Sequence elements and proposed structures of wild type and mutant frameshifting RNA signals from MMTV and CYDV-RPV. The mutations at shift site and both stems were indicated. The length of spacer was either shorten by 3–6 nucleotides or lengthen by six nucleotides.

that induced a wild type level of frameshifting in vitro, were introduced into the frameshift assay cassette. The three-dimensional structures of these frameshifting and non-frameshifting RNA pseudoknots have been determined by NMR. The efficient frameshifting pseudoknots adopted a well-defined conformation in which stems 1 and 2 bent relative to each other (Shen and Tinoco, 1995; Chen et al., 1996; Kang and Tinoco, 1997), whereas the non-frameshifting pseudoknots adopted an entirely different conformation (Chen et al., 1996; Kang et al., 1996). As summarized in Table 2, MMTV frameshift sequence element containing an RNA pseudoknot with a specific bent conformation (MMTV or U13C) directed about 7 and 6% frameshifting in *E. coli* and yeast, respectively. The level of frameshifting in yeast containing a non-frameshifting mutant pseudoknot with an entirely different conformation (APK or ΔA14U13C) was decreased to 1–2%. In contrast, in *E. coli* the non-frameshifting mutant pseudoknots showed a similar level of frameshifting comparable with those of frameshifting pseudoknots. This observation indicates that the eukaryotic translational machinery recognizes the conformational change of the downstream RNA pseudoknot, thus directing a different level of frameshifting depending on the structure. In contrast, the downstream RNA structure is not recognized by the prokaryotic translational machinery. These results imply that a particular conformation or certain specific residues may have dynamic interactions with the ribosome or with ribosome-related factors during the slippage into −1 frame.

3. Differential response of CYDV-RPV frameshifting signals in *E. coli* and *S. cerevisiae*

To test the effect of a plant viral RNA signal on frameshifting in vivo, the wild type and modified versions of the putative CYDV-RPV frameshift signal (Fig. 1 and Table 1) were placed in front of the luciferase gene (Fig. 2). Frameshifting efficiencies caused by the wild type CYDV-RPV frameshift sequence in *E. coli* and yeast were 2 and 1.5%, respectively (Table 2). This level of frameshifting in *E. coli* caused by the CYDV-RPV sequence is comparable with that directed by beet western yellow virus (BWYV) sequence that contains an identical shift site G GGA AAC (Garcia et al., 1993). Similar level of frameshifting in *E. coli* was also observed in BYDV-PAV that contains different shift site G GGU UUU (Di et al., 1993). A marked reduction in frameshifting abilities (to 0.1–0.4%) in yeast was observed for mutations that disrupted either stem 1 or stem 2, that deleted the entire downstream structural element, or that modified the length of the spacer

Table 1
Oligonucleotide sequences of the constructs used to test the role of ribosomal frameshifting sequence elements from CYDV-RPV or MMTV

Construct	Sequence
CYDV: 0	5′ *GGGAAAC***GGGAA**GGCGGCGGCGTCCGCCGTAACAAACGCG[A]AA 3′
Wild type	5′ *GGGAAAC***GGGAA**GGCGGCGGCGTCCGCCGTAACAAACGCGAA 3′
Stem 1	5′ *GGGAAAC***GGGAA**GGCGGCGGCGTGGCGGGTAACAAACGCGAA 3′
Stem 2	5′ *GGGAAAC***GGGAA**GGCGGCGGCGTCCGCCGTAACAAAGCGGAA 3′
Del enhancer	5′ *GGGAAAC***GGGAAG**——————————————GCGAA 3′
Shift site	5′ *GCAGAGC***GGGAA**GGCGGCGGCGTCCGCCGTAACAAACGCGAA 3′
Spacer (−3)	5′ *GGGAAAC***G**——**A**GGCGGCGGCGTCCGCCGTAACAAACGCGAA 3′
Spacer (−6)	5′ *GGGAAAC*——————GCGGCGGCGTCCGCCGTAACAAACGCGAA 3′
Spacer (+6)	5′ *GGGAAAC***GGGAAGACGCA**GGCGGCGGCGTCCGCCGTAACAAACGCGAA 3′
MMTV: 0	5′ *AAAAAAC***TTCGAAA**GGGGCAGTCCCCTAGCCCCACTCAAAAGGGGGAT[A]G 3′
MMTV: −1	5′ *AAAAAAC***TTCGAAA**GGGGCAGTCCCCTAGCCCCACTCAAAAGGGGGATG 3′
APK	5′ *AAAAAAC***TTCGAAA**GGCGCAGTGGGCTAGCGCCACTCAAAAGCCCGATG 3′
U13C	5′ *AAAAAAC***TTCGAAA**GGCGCAGTGGGCCAGCGCCACTCAAAAGGCCCATG 3′
ΔA14U13C	5′ *AAAAAAC***TTCGAAA**GGCGCAGTGGGCC-GCGCCACTCAAAAGGCCCAT[A]G 3′

These sequences correspond to the frameshift (FS) signal located in front of the luciferase in Fig. 2. The shift sequences are italics, and the stems 1 and 2 of the proposed pseudoknots are underlined. The spacers between shift sites and downstream pseudoknots are bold. The zero frame constructs (CYDV: 0 and MMTV: 0) contains an extra A (boxed) and produce a full-length luciferase without frameshift. The structures of the APK, U13C and ΔA14U13C pseudoknots have been determined (Chen et al., 1996; Kang et al., 1996; Kang and Tinoco, 1997).

between shifty site and downstream structural element. In contrast, the same set of mutations did not change the frameshifting efficiency in *E. coli* (Table 2). These results further support the suggestion that prokaryotic and eukaryotic translational machineries recognize differently the downstream structural element, implying that frameshifting occurs by a specific interaction between frameshift signal and ribosome.

Since the frameshifting efficiencies are calculated by measuring the enzymatic activity of luciferase synthesized as a consequence of frameshift event in cells, it is important to investigate whether the luciferase expression vectors carrying the frameshifting assay cassette are transcribed at a same level in all yeast or *E. coli* cells. To test the expression level of the luciferase transcript in yeast or *E. coli*, RNAs were extracted from the same batch of the cell cultures used to measure the luciferase activity, and the abundance of luciferase frameshift reporter mRNA was determined by an RNA gel blot analysis. Similar amounts of RNA transcript were detected in all *E. coli* or yeast constructs, indicating that the expression levels of the vectors carrying GAL4 BD-luciferase or GST-luciferase fusion construct are similar in mutants and wild type constructs (data not shown). These results indicate that different frameshifting efficiencies measured for each mutant discussed above arose from the different frameshifting event, but not from different levels of transcripts.

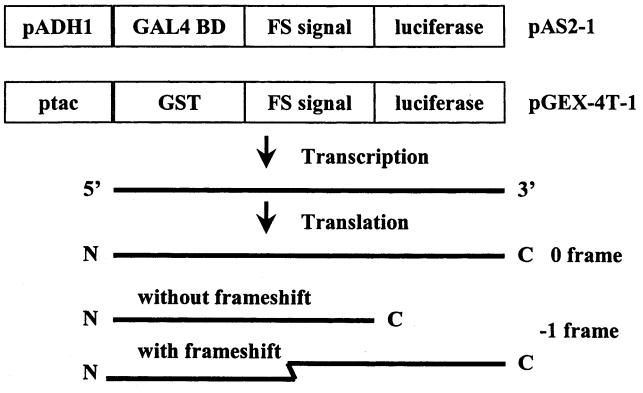

Fig. 2. Frameshifting assay constructs used to test the effect of RNA signal from CYDV-RPV or MMTV on frameshifting. The assay cassettes in *S. cerevisiae* and *E. coli* use pAS2-1 and pGEX-4T-1 vector, respectively, and contain frameshift signal in N-terminal region of luciferase. The −1 frame was designed to synthesize an active GAL4 BD-luciferase or GST-luciferase fusion protein as a consequence of frameshift into −1 frame, and the 0 frame was designed to synthesize active GAL4 BD-luciferase or GST-luciferase fusion protein without frameshift.

We observed that different levels of frameshifting were directed by the MMTV frameshifting signals depending on the conformation of the downstream RNA pseudoknot in the yeast translational system. In contrast, no change in frameshifting efficiency was detected for the signals with different conformation of downstream structure in *E. coli* translational system. This different response could stem from the different overall geometry of eukaryotic and prokaryotic ribosomes. Eukaryotic and prokaryotic translational systems also responded differently to the CYDV-RPV frameshift signals, which is in agreement with the previous report for the BWYV frameshift signal (Garcia et al., 1993). Although the dependence of downstream RNA structure on frameshifting in vivo was extensively investigated for human immunodeficiency virus (HIV), controversy still exist on the role of downstream sequence elements. In an early report by Wilson et al.

Table 2
Frameshifting efficiencies of wild type and mutant RNA signals from CYDV-RPV or MMTV in *E. coli* and yeast *S. cerevisiae*

	E. coli		*S. cerevisiae*	
Construct	Luciferase activity (RLU $\times 10^{-5}$)	FS efficiency (%)	Luciferase activity (RLU $\times 10^{-3}$)	FS efficiency (%)
CYDV: 0	7530±200	(100)	8030±250	(100)
Wild type	150±30	2.0±0.5	120±20	1.5±0.3
Stem 1	140±20	1.9±0.4	30±3	0.4±0.1
Stem 2	155±20	2.1±0.3	29±3	0.4±0.1
Del enhancer	170±30	2.3±0.4	15±1	0.2±0.05
Shift site	6±3	0.08±0.02	4±0.5	0.05±0.02
Spacer (−3)	120±5	1.6±0.1	10±2	0.1±0.03
Spacer (−6)	110±4	1.5±0.1	8±2	0.1±0.03
Spacer (+6)	110±5	1.5±0.1	9±3	0.1±0.04
MMTV: 0	7600±300	(100)	8100±300	(100)
MMTV: −1	530±70	7±1	490±50	6±1
U13C	520±60	7±1	480±50	6±1
APK	520±60	7±1	160±30	2±0.6
ΔA14U13C	450±50	6±1	80±3	1±0.5

Cells were normalized and frameshifting (FS) efficiencies were calculated as the ratio of luciferase activity between the zero frame construct and the −1 frame construct. Data shown here are averages of eight independent measurements.

(1988), it was shown that HIV frameshifting measured in mammalian and yeast systems was not dependent on stem-loop structures downstream from the slippage site. In other studies, removal of a downstream stem-loop region of HIV frameshifting sequence element resulted in a decrease of frameshifting efficiency in yeast and cultured vertebrate cells (Parkin et al., 1992; Cassan et al., 1994; Stahl et al., 1995). Eukaryotic ribosomal frameshifting signals have also been investigated in an *E. coli* system, and the results have been interpreted differently. In an analysis of the MMTV *gag-pro* frameshift site (A-AAA-AAC) and the HIV *gag-pol* frameshift site (U-UUU-UUA) in *E. coli*, only a small decrease in frameshift efficiency (from 1.8 to 1.5% and from 2.1 to 0.9% for HIV and MMTV, respectively) was observed by deleting or disrupting the downstream structure (Weiss et al., 1989). The HIV-1 frameshift has also been reproduced in a bacterial cell-free system (Brunelle et al., 1999), and the study showed that the downstream stem-loop structure is dispensable for frameshifting in a bacterial cell-free system. Brierley et al. (1997) tested the slippery sequence variants of the coronavirus IBV frameshift signal in *E. coli*, and found that at the eukaryotic slippery sequence U-UUA-AAC, deletion of downstream pseudoknot region decreased frameshifting efficiency from 2 to 1%, arguing that frameshifting of prokaryotic ribosome was pseudoknot-independent. These previous observations and our current results indicate that although the degree of alteration in frameshift efficiency varies depending on the different constructs and assay systems, deletion of stem-loop or pseudoknot region downstream of the eukaryotic slippery sequence resulted in a marked decrease in frameshifting efficiency in yeast system, whereas in *E. coli* the viral frameshift signal is poorly recognized.

Many questions still remain regarding the details of the mechanism by which RNA stem-loop or pseudoknots stimulate ribosomal frameshift event. Although the structures and stabilities of wild type and mutant CYDV-RPV RNA pseudoknots are not accessed, our present results clearly show that prokaryotic and eukaryotic ribosomes respond differently to the various conformation of MMTV RNA pseudoknot, and that mutations disrupting the proposed RNA pseudoknot of CYDV-RPV do affect frameshifting only in yeast but not in *E. coli*. These observations support the previous suggestion that ribosome pausing alone is insufficient to mediate frameshifting, and specific interaction between ribosome and RNA structural element are required for efficient frameshifting. In a systematic mutational analysis of BWYV pseudoknot whose crystallographic structure has been determined (Su et al., 1999), specific nucleotide tertiary interactions at the junction between two stems and maintaining the specific conformation at the junction region were found to be crucial for an efficient frameshifting in human embryonic kidney cells (Kim et al., 1999). This suggestion is also in line with the report that the stem-loop in HIV-1 frameshift signal could influence the frameshift through a functional interaction with the ribosome (Brunelle et al., 1999). More structural and mutational analyses of the frameshift sequence elements, and efforts to find any virus or host encoded *trans*-acting factors are required to

elucidate the role of the *cis*-and/or *trans*-acting RNA signals, and completely understand the ribosomal frameshifting mechanism in animal and plant viruses.

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