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Stem-loop structure of *Cocksfoot mottle virus* RNA is indispensable for programmed −1 ribosomal frameshifting

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ABSTRACT

The −1 programmed ribosomal frameshifting (−1 PRF) mechanism utilized by many viruses is dependent on a heptanucleotide slippery sequence and a downstream secondary structure element. In the current study, the RNA structure downstream from the slippery site of cocksfoot mottle sobemovirus (CfMV) was proven to be a 12 bp stem-loop with a single bulge and a tetranucleotide loop. Several deletion and insertion mutants with altered stem-loop structures were tested in wheat germ extract (WGE) for frameshifting efficiency. The impact of the same mutations on virus infectivity was tested in oat plants. Mutations shortening or destabilizing the stem region reduced significantly but did not abolish −1 PRF in WGE. The same mutations proved to be deleterious for virus infection. However, extending the loop region to seven nucleotides had no significant effect on frameshifting efficiency in WGE and did not hamper virus replication in infected leaves. This is the first report about the experimentally proven RNA secondary structure directing −1 PRF of sobemoviruses.

1. Introduction

Many positive strand RNA viruses use −1 programmed ribosomal frameshifting (−1 PRF) for regulating the expression of viral polymerases. Two *cis*-acting elements are required for an efficient −1 PRF. First, a seven nucleotide slippery sequence is the place where the ribosome will slip back by one nucleotide. The sequence motif for the slip-site is X XXY YYZ (preframeshift codons are indicated), where X can be any nucleotide, Y either A or U, and Z can be any nucleotide except G (Brierley et al., 1992). Secondly, the shifty heptanucleotide is followed by the sequence that is forming a downstream secondary structure. The investigated downstream stimulatory elements can be divided into three groups. The best studied group includes hairpin-type RNA pseudoknot structures formed when the nucleotides of hairpin loop base pair with single-stranded downstream complementary nucleotides (reviewed by Giedroc and Cornish, 2008). The second group of structures consists of pseudoknots having an unusual structure. For example, a three-stemmed pseudoknot structure has been reported for *Severe acute respiratory syndrome coronavirus* (Baranov et al., 2005; Brierley and Dos Ramos, 2006; Plant et al., 2005; Su et al., 2005). This structure is composed of two double-stranded stems connected by a single-stranded loop and a second loop which itself folds into a stem-loop. The stimulatory RNA of the *Visna-Maedi virus* −1 ribosomal frameshifting signal is another unusual pseudoknot with a seven nucleotide interstem element between two stems (Pennell et al., 2008). The third group includes stable stem-loop structures sufficient to promote efficient −1 PRF. Examples include the frameshift-promoting elements at the *gag-pol* junction in *Giardia lamblia virus* (GLV), *Human immunodeficiency virus* type 1 (HIV-1), *Simian immunodeficiency virus* (SIV) and related lentiviruses (Brierley and Dos Ramos, 2006; Li et al., 2001; Marcheschi et al., 2007; Staple and Butcher, 2005). The requirement for a simple hairpin loop in frameshifting has been also demonstrated in *Human astrovirus* serotype-1 (HAst-1), *Human T-cell leukemia virus* type II (HTLV-II) and *Red clover necrotic mosaic virus* RNA-1 (RCNMV) (Falk et al., 1993; Kim and Lommel, 1994, 1998; Marczinke et al., 1994).

Cocksfoot mottle virus (CfMV, unassigned genus *Sobemovirus*) is a plant virus with a monopartite, single-stranded, positive-sense RNA genome (Mäkinen et al., 1995b). The polyprotein of CfMV encodes a viral protease, VPg and an RNA-dependent RNA polymerase (RdRp). It is translated from two overlapping open reading frames, ORF 2a and 2b, by a −1 PRF mechanism (Mäkinen et al., 1995a) (Fig. 1). The consensus signals for −1 PRF were identified at the beginning of the overlap region: the slippery sequence U UUA AAC (nucleotides 1634–1640, preframeshift codons are indicated) and a predicted stem-loop structure (nucleotides 1648–1676) starting seven nucleotides

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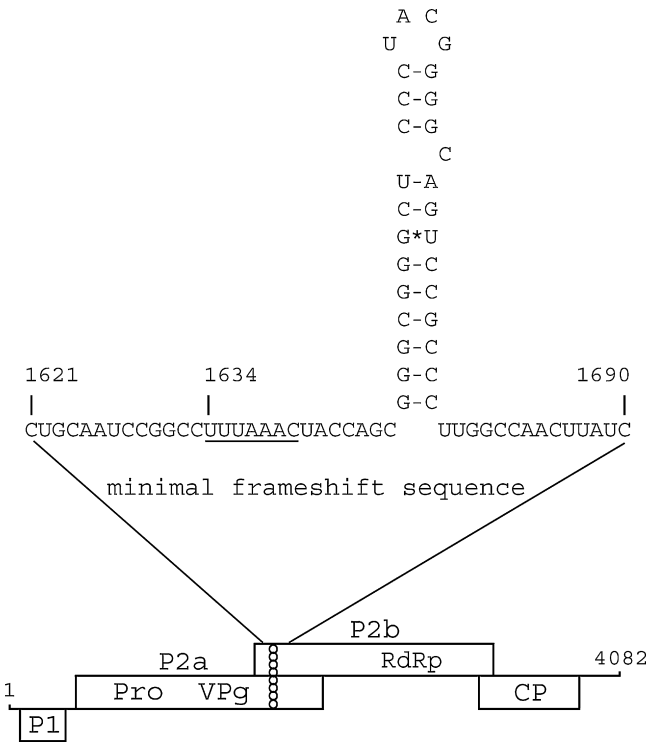

Fig. 1. Genomic organization of CfMV and *cis*-acting RNA elements involved in −1 ribosomal frameshifting. The coding regions of CfMV genome are shown by boxes. The serine protease (Pro), VPg and RNA-dependent RNA polymerase (RdRp) domains in the polyprotein are indicated. P1, ORF 1 encoded protein; P2a and P2b, ORF 2a and 2b encoded proteins; CP, coat protein. The chain label marks the position of the 70-nt minimal frameshift region required for efficient *in vitro* frameshifting. The sequence of the minimal frameshift region containing the *cis*-acting RNA elements is shown. The 7-nt slippery sequence is underlined. The downstream stem-loop secondary structure is predicted by MFOLD.

downstream. Both mutating the heptanucleotide sequence and deleting the putative secondary structure were shown to completely abolish the frameshifting activity, indicating that these *cis*-acting elements are absolutely required for −1 PRF to proceed (Lucchesi et al., 2000). The minimal frameshift sequence required for efficient *in vitro* frameshifting was mapped at the nucleotides 1621–1690 (Lucchesi et al., 2000) (Fig. 1). This 70 nucleotide sequence, containing both the slippery sequence and the predicted downstream secondary structure, was shown to direct −1 PRF in wheat germ extract (WGE) with an efficiency of 12.7 ± 1.4%. Similar *in vitro* frameshifting efficiency, 10.6 ± 1.4%, was determined for the entire ORF2a–2b encoding region (Lucchesi et al., 2000; Tamm et al., 1999).

In this study, we report that the −1 PRF signal of CfMV indeed includes a stem-loop structure as a downstream element. We have mapped the structure of the frameshifting site of CfMV by chemical probing. Several mutations were introduced to study the importance of particular structural elements in determining the frameshifting efficiency. The impact of these mutations was quantitatively analyzed by measuring the frameshifting efficiencies in a WGE *in vitro* translation system. The effects of mutating the frameshifting region on the local and systemic infection of CfMV was examined in oat plants.

2. Materials and methods

2.1. Plasmid construction

The base numbering used in this study refers to the genome of the CfMV Norwegian isolate as in Mäkinen et al. (1995b). The construction of pAB-21, containing CfMV polyprotein region (nucleotides 418–3265), has been described earlier (Lucchesi et al., 2000). This plasmid was used as a template to create all the other constructs for *in vitro* studies.

For the construction of pRF2, a fragment containing the slippery sequence and the stem-loop region (nucleotides 1604–1898) was amplified by PCR and cloned into pGEM-T Easy (Promega) under the control of T7 RNA polymerase promoter. This plasmid was used for the *in vitro* transcription experiments.

The following mutations in pAB-21 were introduced by PCR-based mutagenesis (Fig. 3A). pAB(ΔCUU) contains a deletion of a conserved CUU triplet (nucleotides 1685–1687). In the case of pAB(ΔC), the C nucleotide at position 1667 was deleted and a restriction site for ApaI introduced (nucleotides 1679–1684) to retain the reading frame. In pAB(+G), an additional G nucleotide was inserted at position 1657 and nucleotides 1683–1799 were deleted in order to restore the reading frame. Plasmids pAB(ΔCUU), pAB(ΔC) and pAB(+G) were obtained using Excite site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. pAB(+UUU) was generated by inserting UUU at position 1662–1664. In pAB(G → U), the G nucleotide at position 1649 was mutated to U. In pAB(C → A), the C nucleotide at position 1675 was mutated to A. In pAB(G → U, C → A), the G at position 1649 and C at position 1675 were changed to U and A, respectively. These mutations were introduced as described by Meier et al. (2006). The PCR fragments carrying the mutations were cut with KpnI and Cfr42I and inserted into pAB-21 cut with the same enzymes.

The construction of the infectious cDNA of CfMV (CfMV icDNA) and replicase-deficient CfMV cDNA clone (CfMV RdRp(−)) have been described earlier (Meier et al., 2006). Three CfMV cDNA clones were constructed carrying the following mutations: in CfMV mRF(C → A), the C nucleotide at position 1675 was mutated to A; in CfMV mRF(+UUU), the UUU were inserted at position 1662–1664; in CfMV mRF(ΔC,+C), the C nucleotide at position 1667 was deleted and one extra C nucleotide inserted at position 1677 (Fig. 4A). The mutations were introduced by PCR-based mutagenesis (Meier et al., 2006). The obtained PCR fragments were cut with Cfr42I and Eco147I and inserted into an icDNA construct cut with the same enzymes.

All mutations were verified by sequencing.

2.2. RNA in vitro transcription, chemical probing and primer extension analysis

pRF2 was linearized with SalI for run-off transcription with bacteriophage T7 RNA polymerase. *In vitro* transcribed RNA was purified on a Sephadex S400 (GE Healthcare) spin column as described (Liiv et al., 1998). Modification reactions with dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CMCT) or kethoxal were done in a 50 μl reaction volume containing 20 pmol of RNA according to Liiv and Remme (2004). The unpaired bases are accessible to alkylation by these agents. DMS modifies the N3 position of cytosine and the N1 position of adenine. CMCT modifies the N3 group of uracil and N1 of guanine. Kethoxal modifies guanine residues at positions N1 and N2. Modification sites were determined by primer extension using reverse primer 117,864 (complementary nucleotides 1777–1757) and reverse transcriptase. Primer extension with [α-^{32}P]dCTP labelling was done as described earlier (Maiväli et al., 2002; Stern et al., 1988). Control experiments with untreated RNA were carried out to detect natural pauses of reverse transcription. Primer extension products were separated by 7% urea–polyacrylamide gel electrophoresis (PAGE).