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In vitro Transcription and Translation of Potato Mop-Top Pomovirus RNA 3: Coat Protein and Read-through Cistron

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Abstract: RNA transcripts from Spe-1 linearised PMTV-T RNA 3 cDNA clone were translated in wheat germ extract system. Two major translation products of 19.7K and 65K correspond to the predicted translation products of RNA 3: 19.7K produced by coat protein and 65K by read-through of translation termination codon. The mean density of the coat protein band was 3.253 as determined by Whole Band analysis of different exposures of autoradiographs. Similarly, the mean density of the boat protein plus read-through protein band was 0.209. This gives a ratio of 0.064 for the density of the coat protein plus read-through protein: coat protein. However, there are three methionine residues in the coat protein and 8 in the coat protein plus read-through protein. Normalising for this gives a ratio of 0.024 for amount of coat protein plus read-through protein: coat protein. In other words -2.4 percent of the ribosomes which reach the coat protein gene termination codon suppress the termination and go on to produce coat protein plus read-through protein.

Key word: Potato mop-top furovirus, transcription translation, coat protein, red-through protein

Introduction

Potato mop-top furovirus (PMTV) has fragile tubular rod-shaped particles of two predominant lengths (100-150 nm and 250-300 nm), a tripartite, single-stranded plus RNA genome (Harrison and Jones, 1970; Scott *et al.,* 1994) and is a species of the genus *Pomovirus* (Torrance and Mayo, 1997). PMTV occurs in potato growing regions of Northern and Central Europe, the Andean region of South America, China, Japan (Jones, 1988) Canada (J. MacDonald, personal communication) and elsewhere in the world where its fungal vector is available. PMTV was considered to be transmitted by the plasmodiophorid fungus *Spongospora subterranea* f. sp. *subterranea* (Jones and Harrison, 1969) and confirmed by acquisition and transmission experiments using a virus-free fungus culture that was derived from single cystosorus (Arif *et al.,* 1995). The virus genome consists of three species of ssRNAs. RNA 2 contains four open reading frames (ORFs) which encodes proteins of 61K, 13K, 21K and 8K. The first three proteins resemble the triple gene block (TGB) of some other plant viruses particularly barley stripe mosaic Hordeivirus (Reavy *et al.,* 1993; Scott *et al.,* 1994). The 8K cysteine-rich protein is of unknown function. The RNA 3 contains single open reading frame (ORF) coding for the coat protein (20K) followed by an additional 47K read-through protein (Kashiwazaki *et al.,* 1995). Two other furoviruses, soil-borne wheat mosaic virus (SBWMV) and beet necrotic yellow vein virus (BNYVV), both have coat protein-readthrough genes but the location of this product is different. In both viruses, the coat protein is encoded by the 5'-proximal gene of RNA 2 (Bouzoubaa *et al.,* 1986; Shirako and Wilson, 1993) and the coat protein cistron is immediately followed by a long in-frame ORF which is expressed by translational read-through of the coat protein cistron termination codon to produce a read-through protein (Ziegler *et al.,* 1985; Schmitt *et al.,* 1992; Shirako and Wilson, 1993). In BNYVV, the read-through protein has been shown to be incorporated into virions as a minor component (Haeberle *et al.,* 1994) and to be essential for vector transmission (Tamada and Kusume, 1991) and particle assembly (Schmitt *et al.,* 1992). In this paper, we describe, *in vitro* transcription and translation of the coat protein/read-through domain of PMTV RNA 3 in a wheat germ extract system to determine if the putative leaky stop codon is suppressed during translation and to determine the frequency of this suppression event.

Materials and Methods

***In vitro* transcription:** PMTV RNA 3 cDNA plasmid clone, pPMTV-21 (Kashiwazaki *et al.,* 1995) was linearised by digestion with Spe-1. RNA transcripts were produced with a T3 RNA polymerase using an *in vitro* transcription kit (Stratagene). The transcription reaction contained 5 μl of 5 × transcription buffer (200 mM Tris-pH 8.0; 40 mM $MgCl_2$; 10 mM spermidine; 250 mM NaCl), 1 μg restricted plasmid (pPMTV-21) DNA template, 1 μl 10 mM rATP, 1 μl 10 mM rCTP, 1 μl 10 mM rGTP, 1 μl 1 mM rUTP, 1 μl 'CAP' (m^7GpppG), 1 μl 0.75 M dithiothreitol (DTT), 1 μl RNase inhibitor (RNAguard™, Pharmacia) and 10 units of 13 DNA polymerase (1 μl of 1/5 dilution). The final volume was adjusted at 25 μl with DEPC-treated water. The transcription reaction was incubated at 37°C for 30 min in a water bath. After incubation, the RNA transcript was extracted with phenol:chloroform (1:1), precipitated with ethanol and resuspended in DEPC-treated water.

***In vitro* Translation:** Two microlitres of transcription reaction (above) were used for translation in a wheat germ extract *in vitro* translation kit (Promega), The translation reaction contained 25 μl wheat germ extract; 2 μl reaction buffer; 1 μl 1 mM amino acid mixture (minus methionine), 4 μl ^{35}S-methionine (1,000 Ci/mmol at 10m Ci/m1); 1 μl RNasin ribonuclease inhibitor and 2 μl transcription reaction. The final volume was maintained at 50 μl with nuclease-free water and incubated at 30°C for 120 min.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis: The translated protein was denatured with 2 percent SOS, 1 percent 2-mercaptoethanol, 50 mM Tris-HCI, pH 8.8, 15 percent sucrose at 100°C for 5 min and separated by 12.5 percent PAGE as described by Franssen *et al.* (1982). Gels were stained in 0.2 percent Coomassie Brilliant Blue R in 50 percent methanol, 7 percent acetic acid and destained in 50 percent methanol, 3.5 percent acetic acid.

Autoradiography and gel analysis: After staining, the gels were soaked twice in water for 15 min and dried on filter paper (Whatman 3 MM) using a gel dryer (Hetodryer, Heto Lab Equip., Denmark). Multiple exposures were made to X-ray film (Fuji RX) at room temperature. Analysis of autoradiographs was performed using a Kodak Megaplus CCD high-resolution Video Camera linked

to a Sun IPC Workstation (Sun Microsystems, Mouentain View, CA). Images were analyzed using Millipore BioImage System 'Whole Band Analyser' Software.

Results

***In vitro* transcription of coat protein and coat protein read-through gene:** A cDNA plasmid clone of PMTV RNA 3 linearised with Spe was transcribed with T3 RNA polymerase. Figure 1 shows the production of RNA transcripts at time zero (T0) and after 1 h (T60) at 37°C.

Analysis of coat protein and coat protein/read-through gene *in vitro* translation product: RNA 3 transcripts were translated in wheat germ extract system for 90 min and analysed by SDS-PAGE followed by autoradiography. Two major translation products were observed. Fig. 2 shows the two major proteins, 19.7K and 65K correspond to the predicted translation products of RNA 3, 19.7K product produced by coat protein and 65K by read-through of translation termination codon.

The mean density of the coat protein band was 3.253 as determined by whole band analysis of different exposures of autoradiographs. Similarly, the mean density of the coat protein plus read-through protein band was 0.209. This gives a ratio of 0.064 for the density of the coat protein plus read-through protein: coat protein. However, there are three methionine residues in the coat protein and 8 in the coat protein plus read-through protein. Normalising for this gives a ratio of 0.024 for amount of coat protein plus read-through protein: coat protein. In other words -2.4 percent of the ribosomes which reach the coat protein gene termination codon suppress the termination and go on to produce coat protein plus read-through protein.

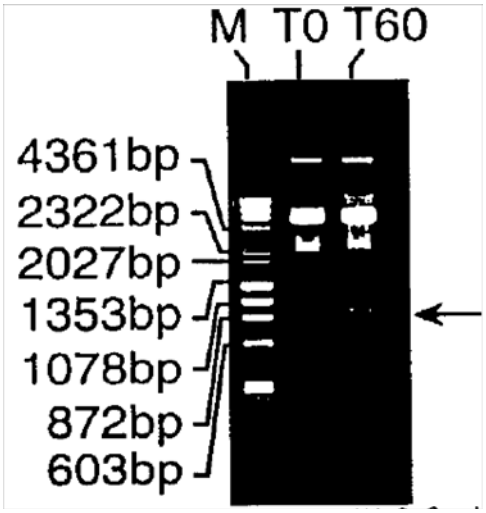

Fig. 1: *In vitro* transcription of PMTV RNA 3, Spe I linearised cDNA plasmid clone (pPMTV-21) with T3 RNA polymerase. RNA transcripts were separated in 1 percent agarose gel and stained with ethidium bromide (0.5 µg/ml). Lanes: M, DRIgest III molecular size marker (Pharmacia); T_0, PMTV RNA3 transcripts at time zero; T_{60}, PMTV RNA 3 transcripts after incubation of transcription reaction mixture at 37°C for 60 min

Discussion

In this paper, we present results of the *in vitro* translation of PMTV RNA 3 coat protein and coat protein/read-through protein genes. It is assumed that a common initiation codon is used for the viral coat protein gene and for the coat protein/read-through gene. Two major translation products of 19.7K and 65K correspond to the predicted translation products of RNA 3: 19.7K produced by the coat protein and 65K by read-through of the coat protein termination codon. Measurement of the relative intensities of these two products on autoradiographs indicated that -2.4 percent of the ribosomes which reach the coat protein gene termination codon suppress the termination and produce coat protein plus read-through protein. Similar strategy of genorne expression involving a leaky termination codon has been reported for number of plant viruses (Joshi and Haenni, 1984). RNA 2 of SBWMV, like BNYVV RNA 2 (Ziegler *et al.,* 1985) and PMTV RNA 3, can undergo translational suppression of the termination codon of its 5'-terminally situated coat protein cistron to produce longer read-through polypeptides in addition to the viral coat protein (Hsu and Brakke,1985; Shirako and Ehara, 1986).

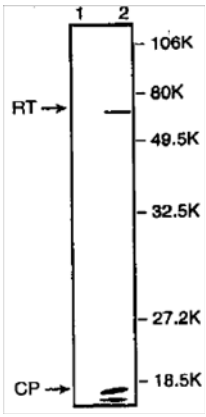

Fig. 2: *In vitro* translation of PMTV RNA 3: coat protein and coat protein read-through cistron. Fluorogram of SDS-PAGE of translation products of PMTV RNA 3 in wheat germ extracts system. Lanes: 1, negative control; Lane 2 Translation products of PMTV RNA 3 from Spe I linearized cDNA plasmid clone (pPMTV-21). Arrows indicate the position of the translated PMTV coat protein and coat protein/read-through protein

Cell-free translation of peanut clump virus (PCV; Indian peanut clump virus, IPCV), another furovirus showed that RNA 2 encodes the viral coat protein but does not undergo read-through to produce a longer polypeptide (Mayo and Reddy, 1985) indicating that it may be different from BNYVV, SBWMV and PMTV. Sequence analysis of PCV RNA 2 indicated that the 39K protein (ORF 2) is encoded in a different frame from the coat protein gene (ORF 1) with the AUG initiation codon of ORF 2 overlapping the UGA termination codon of the coat protein gene (Manohar *et al.,* 1993). *In vitro* translation experiments of PCV RNA 2 indicated that expression of the 39K protein is initiated *in vitro* by a leaky scanning mechanism (Herzog *et al.,* 1995), It is believed that 39K protein may be involved in vector transmission (Manohar *et al.,* 1993; Herzog *et al.,* 1995) because it undergoes deletions when the virus is propagated by mechanical inoculation as does the read-through domain of the SBWMV (Shirako and Ehara, 1986; Chen *et al.,* 1994) and that of BNYVV (Bouzoubaa *et al.,* 1986; Tamada and Kusume, 1991). We detected 543nt deletion in read-through domain of PMTV RNA 3 and this mutated isolate (PMTV-T, Harrison and Jones, 1970) could not be acquired and transmitted by the single cystosoral culture of *S. subterranea f. sp. subterranea* (Reavy *et al.,* 1998; Arif *et al.,*

1999), while a wild-type isolate (PMTV-S, Arif *et al.,* 1994) was successfully acquired and transmitted by the same fungal culture (Arif *et al.,* 1995; 1999).

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