Evidence for contribution of an internal ribosome entry site to intercellular transport of a tobamovirus

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Previously, it has been shown that tobacco mosaic virus (TMV) U1 and crucifer-infecting TMV contain a 75 nt internal ribosome entry site (IRES) upstream of movement protein (MP) gene (IRESU1MP,75 and IRESCRM,75, respectively). A movement-deficient TMV mutant, KK6, has been constructed previously [Lehto, K., Grantham, G. L. & Dawson, W. O. (1990). Virology 174, 145–157] by insertion of the second coat protein subgenomic promoter (CP SGP-2) upstream of the MP gene, in addition to the natural CP SGP-1. Here, the authors compare the efficiency of movement function expression by KK6 and a derivative, K86, obtained by insertion of IRESCRM,75 between the CP SGP-2 and MP genes resulting in restoration of IRESCRM,75 function in the 5′-untranslated sequence of the I2 subgenomic RNA of K86. The data indicate that the efficiency of K86 movement was largely restored by this insertion, which was apparently due to the translation-enhancing ability of IRESCRM,75.

The genome of tobamoviruses, of which tobacco mosaic virus (TMV) U1 is the type member, contains four genes. It has long been known that only the 5′-proximal region of the tobamovirus genomic RNA can be directly translated by ribosomes, producing two components of the replicase (the 130 kDa protein and its readthrough, the 183 kDa protein), whereas the other two proteins, the 30 kDa movement protein (MP) and 17 kDa coat protein (CP) are translated from two individual subgenomic RNAs (sgRNAs). The structurally bicistronic I2 sgRNA produces the 30 kDa MP upon translation, while its 3′-terminal CP gene is silent, and a monocistronic sgRNA encodes the CP (for a review, see Palukaitis & Zaitlin, 1986). The CP sgRNA is capped and contains a short (9 nt) leader sequence (Guilley et al., 1979; Lehto et al., 1990), while the 65 nt leader of the MP sgRNA is believed to be uncapped (Hunter et al., 1983; Joshi et al., 1983; Watanabe et al., 1984; Lehto et al., 1990). The transcription start sites of I2 sgRNA were previously mapped to nt 4838 (Watanabe et al., 1984) and 4828 (Lehto et al., 1990) of TMV OM and U1 RNAs, respectively. More recently, the transcription start was mapped to nt 4838 for strain U1 (Grdzelishvili et al., 2000), indicating that the 5′-untranslated leader region (5′UTR) of the I2 sgRNA contains 65 nt.

Recently, we reported that TMV U1 and a crucifer-infecting tobamovirus (crTMV) contain internal ribosome entry sites (IRESs) upstream of the MP gene (IRESU1MP,75 and IRESCLMP,75, respectively) (Skulachev et al., 1999; Dorokhov et al., 2002). It has been suggested that the IRESCRM,75 sequence is functionally active when located at or within the 5′UTR of the I2 sgRNA, but not in the full-length genomic RNA context. This could be due to the extensive secondary structure and inaccessibility of this region to ribosomes in full-length tobamovirus RNA. It is still unclear whether the TMV I2 sgRNA is capped, although the primer extension data of Grdzelishvili et al. (2000) presented indirect evidence for the capped nature of this RNA. Consequently, the question has arisen as to whether the 5′-proximal MP gene of the I2 sgRNA is translated by a ribosome-scanning mechanism or whether its 5′-terminal IRESMP,75 sequence mediates internal ribosome binding. It is possible that both mechanisms operate concurrently (Skulachev et al., 1999). Our recent data show that the IRESCRM,75 sequence acts as a translational enhancer, being located at the 5′ position of monocistronic mRNAs (M. V. Skulachev and others, unpublished observations).

The expression patterns of the MP and CP sgRNAs in TMV-infected cells are markedly different. The TMV MP is produced transiently, early in infection (Watanabe et al., 1984; Lehto et al., 1990), and the level of MP accumulation is relatively low (Ooshika et al., 1984). By contrast, the CP gene is expressed at later stages of TMV replication (Siegel et al., 1978). Little is known concerning the regulation of MP gene expression (for a review, see Lehto & Dawson, 1990).

Lehto et al. (1990) constructed the movement-deficient TMV mutant (KK6) by insertion of an additional subgenomic promoter (SGP) (CP SGP-2 in Fig. 1) upstream of the MP gene. This promoter was capable of mediating I2 sgRNA synthesis with the 24 nt 5′UTR transcribed from the new transcription start site. Consequently, KK6-produced I2 sgRNA was lacking the IRESU1MP,75 sequence of wild-type (wt) TMV304 (Fig. 1A). Remarkably, the KK6 mutant was
movement deficient, which correlated with the fact that production of the MP in KK6-infected cells was delayed in comparison with wt strain TMV304. This feature of KK6 could be due to reduction of the MP-coding I$_2$ sgRNA accumulation transcribed from CP SGP-2 or to reduction of this RNA translational efficiency caused by modification and shortening of its 5'$\text{UTR}$ (Fig. 1A).

The objective of the present work was to gain insight into the functional role (if any) played in virus infection by the IRES$^{\text{CR,MP,75}}$ sequence of the I$_2$ sgRNA. To this end, the 75 nt IRES$^{\text{CR,MP,75}}$ sequence was inserted into the genome of movement-deficient KK6, downstream of the CP SGP-2 and upstream of the MP gene start codon, to produce the K86 mutant virus (Fig. 1A). Both mutants used the same start site (located within the 253 nt CP SGP-2) for I$_2$ sgRNA transcription. Therefore, the 5'$\text{UTR}$-untranslated sequence of I$_2$ sgRNA produced by K86 contained the 75 nt IRES$^{\text{CR,MP,75}}$ sequence, in addition to the 24 nt sequence of KK6 (Fig. 1A).

Plasmid KK6, which is a derivative of wt TMV304 (Fig. 1A),
was kindly provided by K. Lehto (University of Turku, Finland). The TMV MP gene was amplified by PCR to introduce the NcoI site including the initiation codon at the 5' terminus of the MP gene. To insert the IRES<sub>MP</sub><sup>CR,75</sup> just upstream of the MP gene (Fig. 1A), the KK6 plasmid vector was digested with XhoI/PstI and ligated together with three
inserts: the IRES\textsuperscript{CR, MP,75} (Skulachev et al., 1999; Xhol/Ncol), the MP fragment (Ncol/HindIII) and the 3′-terminal part of the TMV genome (MP–CP–3′ UTR; HindIII/PstI). Plasmid KS4 was used as a negative control and contained a non-physiological polylinker-derived nucleotide sequence (PL80) upstream of the MP gene referred to as a control sequence (CS; Fig. 1A). The plasmid construction was verified by sequencing.

Comparison of the time-course of symptom development on \textit{Nicotiana tabacum} cv. Samsun plants infected with TMV304, KK6 and K86 RNA transcripts showed that all the viruses induced systemic infection of tobacco plants, whereas KS4 infection was symptomless. However, systemic symptoms (yellow spots) produced by KK6 could not be detected earlier than 15–17 days post-inoculation (p.i.), while systemic symptoms of K86 and wt TMV304 (yellow spots and mild mosaic) were induced 10 and 5–7 days p.i., respectively (data not shown).

Accumulation of genomic and sgRNAs in total RNA extracts from \textit{N. tabacum} cv. Samsun plants inoculated with \textit{in vitro}-produced T7 RNA transcripts of TMV304, KK6 and K86 was analysed by a Northern blot hybridization assay using a probe containing sequences of the CP gene, as described previously (Dorokhov et al., 2002). Only negligible amounts of viral RNA could be detected in KS4-inoculated leaves (Fig. 1B), due to a very low level of infection produced by this mutant. Fig. 1(B) shows that 7 days after inoculation, the wt TMV304 and K86 produced similar amounts of RNA, whereas the level of viral RNA production by the KK6 mutant was significantly reduced. This reduction reflected the delay in the spread of the KK6 mutant throughout the infected plant.

This suggestion was consistent with time-course Western blot analysis of CP (Fig. 2A) and MP (Fig. 2B) accumulation in tobacco leaves inoculated with wt TMV304 and with the KK6 and K86 mutants. The amount of CP and MP produced by wt virus increased from 3 to 10 days p.i. As shown by Lehto et al. (1990), production of MP by KK6 was considerably delayed. Relatively low amounts of KK6 CP and MP could be detected only at 7 and 10 days p.i. The efficiency and time course of CP and MP production by the K86 mutant were intermediate between the wt virus and the movement-deficient KK6 (Fig. 2A and B). This observation suggested that insertion of the IRES\textsuperscript{CR, MP,75} element into the 5′ UTR of the KK6 genome resulted in a significant restoration of K86 mutant cell-to-cell movement ability.

To examine this effect more closely, we compared the size of the local lesions produced by the wt and the mutant

**Fig. 2.** Comparative characteristics of wt TMV and its chimeric mutants, KK6 and K86. (A, B) Time course of CP and MP production in tobacco leaves inoculated with T7 RNA transcripts of the mutant viruses shown in Fig. 1(A). (C, D) The size of local lesions at 3 days after infection with wt TMV304 and with KK6 and K86 mutants on the leaves of MP-transgenic \textit{N. tabacum} cv. Xanthi nc (C) and \textit{N. tabacum} cv. Xanthi nc, line 2005 plants (D). The mean diameter (±SE) of local lesions was calculated for at least 50 lesions.
viruses in non-transformed *N. tabacum* cv. Xanthi nc plants (Fig. 2C) and in *N. tabacum* cv. Xanthi nc, line 2005, which is transgenic for the TMV MP gene (Fig. 2D), kindly provided by R. Beachy (Deom et al., 1991). Our data indicated that the size of local lesions produced by the K86 mutant in non-transgenic plants was intermediate between that of wt virus and the KK6 mutant of Lehto et al. (1990). It was particularly noteworthy that the difference in the size of lesion produced by all three viruses in MP transgenics was not significant: the minor differences were within the limits of standard error (Fig. 2D). This indicated that the movement deficiency could either be restored partially by insertion of the IRES<sup>CR</sup><sub>MP,75</sub> element into the 5'UTR of the KK6 genome or restored completely by trans-complementation in MP-producing transgenics.

To check the stability of the K86 mutant virus genome, the RNA preparations isolated from progeny virions of K86 accumulated in inoculated or systemically infected tobacco leaves were examined by primer-extension assay and RT-PCR with specific oligonucleotide primers. Our data indicated that the genome of K86 was stable and retained the IRES<sup>CR</sup><sub>MP,75</sub> insertion upon replication in inoculated and systemically infected leaves (data not shown).

It is reasonable to suggest that partial restoration of the K86 movement function was due to a peculiar feature of the IRES<sup>CR</sup><sub>MP,75</sub> sequence mentioned above, i.e. its ability to serve as a translational enhancer when inserted into the 5'UTR of mRNA (M. V. Skulachev and others, unpublished observations). To test this suggestion, *in vivo* model experiments were undertaken using agroinjection of *N. benthamiana* leaves with 35S RNA-based monocistronic cDNA constructs containing the U1, KK6, K86 and KS4 I<sub>2</sub> sgRNA-derived leaders cloned upstream of the reporter green fluorescent protein (GFP) gene. The GFP gene contained a normal intron in its coding region (GFP-int) to prevent GFP production in *Agrobacterium tumefaciens*.

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**Fig. 3.** Monocistronic analysis of the translational enhancer activity of the IRES<sup>CR</sup><sub>MP,75</sub> in agroinjection experiments. (A) Schematic representation and nomenclature of cauliflower mosaic virus 35S RNA promoter-based monocistronic GFP-expressing constructs containing different 5'UTR sequences. 35S, Cauliflower mosaic virus 35S promoter. The GFP-int chimeric gene included an intron in the coding sequence, which prevented GFP expression in *Agrobacterium tumefaciens* cells. 3'UTR, TMV 3'-untranslated region; Tnos, terminator of nopaline synthase. (B) Representative Western blot analysis of GFP protein in extracts from *N. benthamiana* cells agroinjected with the constructs described in (A). Western blotting was performed using rabbit anti-GFP (Boehringer) and goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) as the secondary antibody; the reaction was visualized by ECL (Amersham Pharmacia). (C) Bar graph showing the mean ± SE of the Western blot bands for five separate agroinjection experiments. The GFP band density was measured in arbitrary units (AU) taking the transmittance value mediated by the TMV U1 leader as 100 AU.
cells (Fig. 3A). The intron sequence was taken from the actin 2 gene of Arabidopsis thaliana (cv. Columbia). The natural intron was modified to get the optimal context for donor-acceptor splicing sites and to remove some restriction sites. Specific primers were designed for both the intron and gene sequences. The 77 nt synthetic intron was inserted into the 5'-proximal region of the GFP gene using a PCR that started from the internal part of the gene and amplified the whole plasmid.

Four separate spots on each N. benthamiana leaf were injected with Agrobacterium tumefaciens containing 35S–U1L–GFP-int, 35S–KK6L–GFP-int, 35S–K86L–GFP-int or 35S–KS4L–GFP-int cDNA constructs (Fig. 3A). Fig. 3(B) shows the results of Western blot analysis of the GFP in extracts from N. benthamiana leaves agroinjected with these constructs. Visual comparison of the GFP band density indicated increased efficiency of the K86 I2 sgRNA leader compared with that of KK6 and, in particular, with the KS4 leader used as the negative control. This conclusion was supported when light transmittance of autoradiographed bands was quantified using a computerized imaging system (Imaging Research) and the NIH Image program (http://rsb.info.nih.gov/nih-image). The density of GFP bands revealed by Western blotting was expressed in arbitrary units (AU) taking the transmittance value of the GFP bands produced under the control of the TMV U1 5' leader as 100 AU (Fig. 3C).

Thus, the results of in vivo experiments showed that insertion of the IRES<sub>CR,MP,75</sub> sequence into the 5'UTR of KK6 I2 sgRNA resulted in significant translational enhancement of the reporter GFP gene expression in agroinjected leaves. Taken together, our data indicate that insertion of the IRES<sub>CR,MP,75</sub> sequence corresponding to the 5'-untranslated leader of I3 sgRNA into the movement-deficient TMV KK6 genome conferred an increased efficiency of MP production and cell-to-cell movement in the resulting K86 mutant. We suggest that the contribution of the IRES<sub>CR,MP,75</sub> sequence to TMV movement is due to its translation-enhancing function.

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**References**


