Expression of the coat protein of potato virus X from a dicistronic mRNA in transgenic potato plants

Kathleen L. Hefferon, Houri Khalilian, Huimin Xu and Mounir G. Abou-Haidar

Department of Botany, University of Toronto, 25 Willcocks Street, Toronto, Ontario, Canada M5S 3B2

Transgenic potato plants were generated which express a dicistronic mRNA corresponding to the 8 kDa protein and coat protein (CP) genes of potato virus X (PVX). Northern blot analysis of RNA isolated from these plants indicated that both the 8 kDa protein and the CP are translated from this dicistronic mRNA. Expression of both of these proteins in transgenic plants was demonstrated by Western blot analysis, and suggests that translation of CP takes place either by initiation by internal entry of ribosomes or by a termination/reinitiation mechanism. CP was detected in protoplasts electroporated with RNA transcripts of the dicistronic construct in the presence or absence of a stable hairpin structure at the 5′ terminus, suggesting that the former model is more likely. Similar results were obtained when a DNA fragment containing the PVX CP leader sequence was placed between two reporter genes in the transient assay system. These results suggest that potexvirus CP expression may take place from the genomic RNA as well as from the subgenomic RNA.

Introduction

The detailed mechanisms involved in the initiation of translation in eukaryotes are not well understood. In general, initiation of translation has been presented in the form of a scanning model, in which the small ribosomal subunit binds initially to the 5′ cap of the mRNA and scans along the message in a 5′–3′ direction until the first AUG codon is reached. If the first initiation codon lies in an optimal context, the subunit pauses, and a translational initiation complex is formed. At this point, translation can proceed (Kozak, 1986, 1989, 1990; Merrick, 1992).

Although the majority of mRNA species adheres strictly to the scanning model and initiates translation at the 5′ proximal initiation codon, a small but growing number of mRNAs have been identified which are capable of initiating translation in a cap-independent manner. Among the most extensively studied is poliovirus RNA, which can be translated in infected cells that have lost their ability to initiate cap-dependent translation (Pelletier & Sonenberg, 1985; Sonenberg & Meirovitch, 1990). This internal ribosome entry site (IRES) has been found to exist in similar regions of a number of other RNA viruses, including enteroviruses and rhinoviruses (Herman, 1986; Brown et al., 1991; Borman & Jackson, 1992; Le et al., 1992; Glass et al., 1993; Witherell & Wimmer, 1994). Recently, in vitro and in vivo translation studies have led to the identification of a number of host-derived proteins which are involved in binding internally to RNA and assist initiation of translation (Jang & Wimmer, 1990; Meirovitch et al., 1993; Sarnow, 1995). Evidence for internal initiation of translation has also been found in a number of other RNA viruses, including the middle component RNA (M-RNA) of cowpea mosaic virus (Thomas et al., 1991), and the 5′ untranslated regions (5′ UTRs) of tobacco etch virus (Carrington & Freed, 1990) and turnip mosaic virus (Basso et al., 1994). In many of these cases, the identification of both complex secondary structures and specific RNA-binding proteins has been demonstrated as playing an essential role in directing internal ribosome binding.

Potato virus X (PVX), the prototype member of the potexvirus family, is a rod-shaped virus containing single-stranded RNA which is capped, polyadenylated, and contains five open reading frames (ORFs) (Huisman et al., 1988). ORF 1 encodes a 166 kDa protein which functions as a replicase. ORFs 2, 3 and 4 encode proteins of 25, 12 and 8 kDa, respectively, and are known as the ‘triple gene block’. These gene products are thought to be involved in cell-to-cell
movement of the virus. The fifth ORF encodes the coat protein (CP), which is 25 kDa in size.

In potexviruses, translation of the downstream ORFs is believed to take place from a series of capped subgenomic RNAs which are synthesized from the genomic RNA. *In vitro* translation studies conducted with PVX RNA have indicated that the CP cannot be translated from genomic RNA; rather, it can be readily translated from a smaller, subgenomic RNA encoding the CP gene (Ricciardi et al., 1978; Karasev et al., 1988; Morozov et al., 1990, 1991). Similar observations have been made for a number of other potexviruses, including foxtail mosaic virus (Mackie & Bancroft, 1986), *Plantago asiatica* mosaic virus (Solovyev et al., 1994), viola mosaic virus (Bendena & Mackie, 1986), and daphne virus X (Short & Davies, 1983). However, *in vitro* studies of papaya mosaic virus, narcissus mosaic virus and, to a lesser extent, clover yellow mosaic virus have indicated that expression of the CP can take place from genomic as well as subgenomic RNAs, possibly by means of internal initiation of translation (Bendena et al., 1985, 1987; Bendena & Mackie, 1986; Karasev et al., 1988; Mackie & Bancroft, 1986; White & Mackie, 1990).

In this report, we demonstrate that a construct corresponding to the C-terminal half of the 12 kDa protein, the complete 8 kDa and CP genes, as well as the 3′ non-coding region of PVX RNA, is expressed from the cauliflower mosaic virus (CaMV) 35S promoter in transgenic plants. Analysis of plant RNAs with cDNA probes corresponding to the genes encoding the CP and 8 kDa protein, respectively, indicated the presence of a single, dicistronic mRNA. Western blot analysis of transgenic plants indicated expression of both the 8 kDa protein and CP, suggesting that, *in vivo*, internal initiation of translation may be taking place for the expression of the CP. Expression of the downstream cistron was demonstrated even after stable hairpin structures (shown in previous studies to suppress initiation of translation from downstream AUG codons) were placed in front of dicistronic constructs containing either the PVX CP gene or a reporter gene as the downstream cistron. This implies that initiation of translation of the PVX CP took place by internal ribosome binding, rather than by alternative mechanisms such as leaky scanning or termination/reinitiation. Possible translational strategies of PVX are discussed.

**Methods**

- **Plasmid construction and potato transformation.** cDNA was synthesized from PVX RNA by the method of Gubler & Hoffman (1983), and sequenced to identify ORFs. A 1.2 kb cDNA fragment encoding the 3′ half of the 12 kDa (180 nt) and 8 kDa (210 nt) protein genes and the CP (1.0 kb) gene was isolated by digestion with *Xba*I and *Sal*I. The *Sal*I site was blunt-ended using the Klenow fragment of DNA polymerase I and inserted into the *Xba*I and *Sal*I sites of the plant expression vector pBlN19 (Fig. 1). This vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 by standard procedures (Horsh et al., 1985). Transgenic plants were obtained from potato tuber discs of Russell Burbank and characterized by Northern and Western blot analyses.

- **Western blot analysis.** Protein extracts from transgenic leaf tissue were prepared and subjected to electrophoresis on an SDS–12.5% polyacrylamide gel (Laemmli, 1970). Following electrophoresis, proteins were electrotransferred onto nitrocellulose filters and immunoblotted with different rabbit antibodies specific for either the 8 kDa protein or the CP of PVX. Protein was detected enzymatically using goat anti-rabbit IgG conjugated with alkaline phosphatase (Gibco BRL) as the secondary antibody. Plant protein concentrations were determined by UV spectrophotometry. A serial dilution of known quantities of PVX CP spotted on the same filter was used to determine the quantity of PVX CP expressed in transgenic plants (data not shown). The percentage of CP expressed in transgenic plants was then determined by comparing the CP levels to known concentrations on a Western blot as a fraction of total plant protein added.

- **Extraction of RNA from leaf tissue.** Leaf tissue (0.4 g) from transgenic plants was frozen in liquid nitrogen and ground to a powder in a precooled mortar. This powder was resuspended in 3 ml extraction buffer containing 0.2 M Tris–HCl pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS and 0.1 M sodium acetate in DEPC-treated water. The solution was first extracted with an equal volume of Tris–HCl saturated phenol, followed by phenol–chloroform (1:1 v/v), and two extractions with chloroform. The RNA was precipitated in two volumes of 95% ethanol at +20°C for 1 h, then pelleted by centrifugation at 5800 g for 20 min. The pellet was washed with 70% ethanol, vacuum-dried for 15 min, and redissolved in 100 µl of 0.1 M Tris–EDTA pH 7.0.

- **Northern blot analysis.** Total RNA (40 µg) extracted from leaf tissue of both transgenic and non-transgenic plants was loaded onto a 1% agarose gel containing 10% formaldehyde in MOPS buffer pH 7.0, and subjected to electrophoresis. The RNA was transferred onto nitrocellulose filters overnight and incubated in a prehybridization solution containing 1 M NaCl, 5 mM EDTA, 100 mM Tris–HCl pH 7.5, 0.1% SDS and 100 mg/ml Homomix I (containing 2 g yeast RNA, 0.3 M KCl and 8.4 g urea in 20 ml total volume) for 4 h at 55°C. Filters were hybridized overnight with denatured DNA probes containing genes encoding either the 8 kDa protein or the CP of PVX. Probes were labelled with [α-32P]dATP (3000 Ci/mmol) by random priming, according to the protocol of Feinberg & Vogelstein (1983). Filters were washed once in 0.1 × SSC and 1% SDS at 55°C for 20 min, and twice in 1 × SSC and 0.1% SDS at 55°C for 20 min. They were dried and exposed to X-ray film (Amersham RPN 30) for 3–5 days. To determine the amount of PVX RNA which could be detected by Northern blot analysis, a serial twofold dilution of a known quantity of PVX RNA (1 µg) was spotted onto the same nitrocellulose filter and used to estimate the amount of PVX RNA expressed in transgenic plants.

- **Production of antibodies to the 8 kDa–IFN fusion protein.** Synthetic primers with *Bgl*II sites (5′ CGATCTATGGAAGTAAATAC 3′ and 5′ CGATCTTCAATGGAAAC 3′) and corresponding to the 5′ and 3′ termini of the gene encoding the 8 kDa protein, respectively, were designed and used to amplify the gene by PCR. The gene encoding the 8 kDa protein was cloned into the *Bgl*II site of the human β-IFN gene in the plasmid *P*.R.JIFN (Ivanov et al., 1987). Bacterial clones containing the gene encoding the 8 kDa protein in the correct orientation and in frame (fusion protein) with β-IFN were selected by colony hybridization using the 5′ primer as a probe. Total bacterial proteins were subjected to electrophoresis on an SDS–12.5% polyacrylamide gel, and the band corresponding to the fusion protein was cut from the gel and electroeluted in 0.1 M Tris–HCl pH 7.5. The fusion protein (8 kDa–β-IFN) was concentrated by lyophilization to 1 mg/ml, emulsified with Freund’s incomplete adjuvant and injected into rabbits. Two booster injections at
3 week intervals were carried out. Antisera were collected after 6 weeks and tested for reactivity with the fusion protein.

**Plasmid constructions for the transient assay system.** Two cDNAs, one similar to the dicistronic construct expressed in the transgenic plants (PVX2), and the other representing an artificial subgenomic mRNA of the PVX coat protein (PVX3), were cloned into pBS+ under the transcriptional control of the T7 promoter. The 5’ terminus of PVX2 is located approximately 180 bases upstream of the ATG codon of the gene encoding the 8 kDa protein. PVX3 was derived from a Scat and HindIII double digest of the original PVX cDNA. The 5’ terminus of the corresponding RNA transcript is 155 bases upstream of the AUG codon of the CP gene. The true 5’ terminus of this subgenomic RNA is located approximately 35 nt upstream of the initiation codon of the CP gene (Dojla et al., 1987).

A 0.4 kb Xbal and TaqI cDNA fragment (nt 5250–5643) containing the sequence upstream of the ATG codon of the PVX CP gene (including the 8 kDa ORF and 177 nt upstream of this ORF) was placed between β-Gal and CAT reporter genes by blunt-end ligation, and is referred to as βPVXCAT. The TaqI restriction site is located 7 nt upstream of the AUG codon of the CP gene. An Xba–TaqI cDNA fragment was also inserted in the reverse orientation to generate XVPCAT, used in this study as a negative control. A sequence expected to form a stable hairpin (5’—GCTATGGCGAGGTTACG—3’) was incorporated in front of both dicistronic constructs, PVX2 and βPVXCAT, to generate PVX2+ and βPVXCAT+, respectively. The βPVXCAT+ construct was generated by Accl and TaqI digestion of the PVX fragment. All reporter gene constructs were under the transcriptional control of the T7 promoter.

**In vitro transcription.** Virion RNA was prepared by phenol–chloroform extraction of purified PVX, and capped *in vitro* transcripts were synthesized by T7 RNA polymerase as described by the manufacturer (Promega).

**Preparation and electroporation of protoplasts.** Protoplasts were prepared from uninoculated *Nicotiana glutinosa* leaves according to the method of Otsuki & Takebe (1972), and electroporation carried out as described by Luciano *et al.* (1987). A 450 µl suspension of protoplasts (1 × 10^6) in electroporation buffer (330 mM sorbitol, 1 mM potassium phosphate pH 7.0, 150 mM KCl) was electroporated using a Pro-Generator II electroporation unit (Hoefer Scientific Instruments) at 950 mF, 130 V pulse amplitude and 3.5 mm electrode gap; 6 µg of each *in vitro* transcript as well as full-length PVX RNA were used. Protoplasts were incubated for 24 h in incubation media as described by Luciano *et al.* (1987). Protoplasts (5 × 10^4) were collected by centrifugation at 5000 g for 10 min, ground on ice in 2 × cold Laemmli buffer, and centrifuged at 4 °C and 10000 g for 10 min. Five independent experiments were carried out for each construct, using approximately 1 × 10^5 protoplasts per sample. PVX 8 kDa and CP expression were determined by Western blot analysis. RNA stability of each construct in protoplasts was assessed by Northern blot analysis, using RNA isolated at the same time as protein was extracted and prepared in the RNA extraction buffer described previously. β-Gal and CAT activities were determined by standard procedures (Miller, 1972; Gorman *et al.*, 1982).

**Results**

**Expression of PVX CP in transgenic plants**

Eleven lines of transgenic plants were generated which express the dicistronic construct consisting of both the 8 kDa and CP ORFs of PVX (Fig. 1). Western blot analysis using antibodies specific for the PVX CP resulted in the detection of a protein comparable in size to the control PVX CP for some lines (Fig. 2a, compare lanes 1, 2 and 4 with lane 8). No CP could be detected for the lines shown in lanes 3, 5 and 6, or in the non-transformed plant used as a control (lane 7). The level of accumulation of CP in transgenic plants was determined by comparison of protein samples from transgenic lines with serial dilutions of known quantities of PVX coat protein on an immunoblot. The CP level varied within a range of 0.05–0.2% of total soluble protein among different transgenic plants. One typical transgenic plant line, designated 304, was chosen for further studies (Fig. 2a, lane 1).

**Expression of the 8 kDa protein in transgenic plants**

To determine whether the first ORF of the 8 kDa/CP dicistronic construct is also expressed in these transgenic plants, it was necessary to raise antibodies specific for the 8 kDa protein. Since this protein is relatively small and hydrophobic, it was fused with γIFN and overexpressed in

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**Fig. 1.** Construction of PVX I containing the dicistronic 8 kDa/CP construct used to produce transgenic plants. Xb, Xbal; Ac, Accl; Sa/Sm, destroyed Sall/Smal site; TER nos, terminator nos; neo, neomycin resistance gene; Pnos: promoter nos; LBorder, left border; RBorder, right border. Xbal and Sall sites are located at nt 5250 and 6453, respectively (Huisman *et al.*, 1988). The C-terminal portion of the 12 kDa gene is indicated by the white box located upstream of the 8 kDa gene. Initiation codons of the 8 kDa and CP genes are indicated.
Fig. 2. Analysis of PVX CP and RNA in transgenic plants. (a) Detection of PVX CP expressed in transgenic plants by Western blot of a 12-5% acrylamide gel. Lanes 1–6 contain 50 µg total protein extracted from leaf tissue of putative transgenic plant lines. Lane 7 contains 50 µg protein extracted from a non-transformed plant. Lane 8 contains 100 ng PVX native CP used as a positive control. (b) Northern blot analysis of total RNA isolated from transgenic and non-transgenic plants, hybridized with a 32P-labelled cDNA fragment corresponding to the CP gene of PVX. Lane 1 contains an in vitro transcript of the PVX construct which is also expressed in transgenic plants. Lanes 2 and 3 contain 40 µg total RNA extracted from leaf tissue of non-transformed and transgenic plants, respectively. Level of transcript is 100 ng for lane 1; 75 ng for lane 3.

Fig. 3. Analysis of PVX 8 kDa protein and RNA transcripts in transgenic plants. (a) Expression of the 8 kDa protein in transgenic plant line 304 on a 12-5% acrylamide gel. Protein extracts (50 µg) from: leaf tissue of non-transformed and non-infected potato plants (lanes 1 and 2); from transgenic potato plant (lane 3); and from PVX-infected tobacco plants (lanes 4 and 5). Antibodies were preadsorbed with non-transformed plant proteins prior to use, except in lanes 1 and 2. Lane 6 contains purified fusion protein (γIFN and 8 kDa protein, molecular mass 17-3 kDa) extracted from E. coli. (b) Northern blot of total RNA isolated from transgenic and non-transformed plants, hybridized with a 32P-labelled cDNA fragment corresponding to the gene encoding the 8 kDa protein of PVX. Lanes are labelled as in Fig. 2(b).

E. coli. Antibodies were raised against the purified 8 kDa–γIFN fusion protein and utilized to detect the presence of 8 kDa protein in transgenic plant line 304. A band, 7-8 kDa in size, is seen only in protein extracts from transgenic or PVX-infected plants (Fig. 3a, lanes 3, 4 and 5) but not in non-transgenic or non-infected plants (Fig. 3a, lanes 1 and 2). An extract containing the 8 kDa–γIFN fusion protein is depicted in lane 6, and was included as a positive control. Since the 8 kDa protein was prepared as a fusion protein in E. coli, the antibodies obtained are highly specific to the 8 kDa protein and did not
cross-react with any plant host proteins (Fig. 3a). These antibodies were also preadsorbed to the extracts from non-transformed plants as an additional precaution.

Analysis of RNA from transgenic plants

To determine whether the in vivo translation of both 8 kDa and CP takes place from full-length dicistronic mRNA transcripts or from fragmented but functional monocistronic mRNAs, transcript sizes were determined by Northern blot analysis. Two non-overlapping cDNA probes corresponding to the genes encoding the 8 kDa and CP, respectively, were used in this study. A single prominent band of 1–3 kb, identical in size to the full-length in vitro transcript (1–26 kb), was detected in total RNA extracted from leaf tissue of transgenic plants using either cDNA probe (Figs 2b and 3b, lanes 1 and 3). No other PVX-specific bands were detected in these lanes or from RNA extracted from non-transformed plants (Figs 2b and 3b, lane 2). PVX control RNA (1–10 ng) can be detected under the same hybridization conditions.

Analysis of protein expression in protoplasts

To determine whether the 8 kDa and CP are expressed from this dicistronic construct by internal ribosome binding or by an alternative mechanism such as leaky scanning or termination/reinitiation of translation, tobacco protoplasts were electroporated with transcripts derived from various cDNA constructs of PVX (Fig. 4a). Expression of both 8 kDa and CP was determined by Western blot analysis using antibodies specific to the 8 kDa and CP (Fig. 4b, c). Protoplasts electroporated either with purified PVX RNA or in the absence of RNA were included as positive and negative controls, respectively (Fig. 4b, lanes 1 and 4, Fig. 4c, lanes 1 and 5). Expression of both 8 kDa and CP was detected in protoplasts electroporated with the dicistronic 8 kDa/CP transcript PVX2 (Fig. 4b, c, lane 2). While no 8 kDa protein could be detected from protoplasts electroporated with RNA from PVX2 containing the hairpin structure (Fig. 4b, lane 3), the level of CP expressed from the same construct was virtually unaffected (Fig. 4c, lane 3). As expected, the CP is expressed in protoplasts electroporated with the artificial subgenomic transcript for the CP PVX3 (Fig. 4c, lane 4). CP expression was also detected even when the in vitro transcripts used were uncapped; however, 8 kDa protein expression was not detected (data not shown).

RNA stability was assessed from the protoplast system 24 h post electroporation by Northern blot analysis (Fig. 4d, e). Again, no fragmented RNA transcripts were detected from protoplasts expressing CP from dicistronic transcripts in the presence or absence of the hairpin structure (Fig. 4d, e, lanes 1 and 2). RNA corresponding to the artificial subgenomic construct (PVX3) was found to be the appropriate size (0.9 kb) (Fig. 4e, lane 3). No PVX-specific RNA transcripts could be detected from non-electroporated protoplasts used as controls (Fig. 4d, lane 3, e, lane 4).

Fig. 4. Expression of CP and 8 kDa protein in tobacco protoplasts. (a) Schematic representation of transcripts derived from cDNA constructs used. Arrows represent predicted sites for initiation of translation. Positions of the 5′ terminus of transcripts, 8 kDa and CP genes are included. Xb, XbaI; Sc, ScI; Sa, Sall; H3, HindIII. (b) Western blot analysis of the 8 kDa protein expression in tobacco protoplasts. Protoplast extracts were prepared as described under Methods. One fifth of the total extraction volume was loaded in each lane. Antibodies against the fusion protein were preadsorbed with non-infected, non-transformed tobacco leaf extracts prior to immunoblotting. Protein extracts from protoplasts electroporated with full-length PVX RNA (lane 1), PVX2 RNA (lane 2), PVX2′ RNA (lane 3) and no RNA (lane 4) are included. (c) Western blot analysis of CP expression in tobacco protoplasts. Protein extracts from protoplasts electroporated with full-length PVX RNA (lane 1), PVX2 RNA (lane 2), PVX2′ RNA (lane 3), PVX3 RNA (lane 4), no RNA (lane 5). (d) RNA stability assay. One fourth of total RNA extraction volume was loaded in each lane. RNA extracts from protoplasts electroporated with PVX2 RNA (lane 1), PVX2′ RNA (lane 2), and no RNA (lane 3). A cDNA corresponding to the 8 kDa gene was used as a probe. (e) RNA stability assay. RNA extracts from protoplasts electroporated with PVX2 RNA (lane 1), PVX2′ RNA (lane 2), PVX3 RNA (lane 3), no RNA (lane 4). Probe used corresponded to the CP gene.

The ability of PVX RNA derived from the non-coding region upstream of the CP ORF to facilitate internal translational initiation of a downstream cistron was examined and is
depicted in Fig. 5. Insertion of a sequence expected to form a stable hairpin upstream of the β-Gal gene suppressed β-galactosidase activity in βPVXCAT to background levels, indicating that this structure had effectively blocked translation (Fig. 5b, compare constructs βPVXCAT and βΔPVXCAT to βPVXCAT). However, CAT activity was retained both in the presence or absence of the hairpin structure (Fig. 5c, compare constructs βPVXCAT and βΔPVXCAT to PVXCAT). Deletion of a portion of the PVX sequence in construct βΔPVXCAT resulted in a dramatic reduction in CAT activity to background levels (Fig. 5c). No CAT activity was observed from the construct XVPCAT containing the PVX fragment in the reverse orientation.

**Discussion**

In this paper, we have generated transgenic plants which express the CP gene of PVX from the downstream cistron of a dicistronic mRNA under in vivo conditions, suggesting that cap-independent initiation of translation may be taking place.
A DNA construct containing the genes encoding the 8 kDa protein, CP, and the 3' half of the 12 kDa protein of PVX was transformed into potato plants. Expression of the CP in several lines of transgenic plants was detected at levels (0.2% of total soluble protein) comparable to other transgenic plants expressing the monocistronic CP gene under the control of the 35S promoter of CaMV (Hemenway et al., 1988). This result contradicts the scanning model of initiation of translation, in which ribosomes scan from the 5' end of the message and initiate translation at the first AUG codon they encounter.

The results of Northern blot analysis, using cDNA probes specific to the 8 kDa and CP genes of PVX, demonstrated that total RNA extracted from transgenic plants contains only a single full-length dicistronic mRNA of 1.3 kb in length and identical in size to the full-length PVX construct. No other bands corresponding to the PVX CP mRNA were detected, even after prolonged exposure, reducing the possibility that any smaller fragmented monocistronic RNAs may be responsible for CP expression in these transgenic plants. In addition, a control experiment using serial dilutions of PVX RNA revealed that a specific degradation product representing as little as 5% of the dicistronic transcript could be detected if it were present.

The CP was expressed in protoplasts electroporated with transcripts corresponding to the dicistronic construct of PVX, both in the presence or absence of a hairpin structure (Fig. 4c). The 8 kDa protein, however, was detected only in the absence of the hairpin structure (Fig. 4h). The possibility that CP expression is taking place from degraded transcripts was ruled out by the detection of intact PVX transcripts from an RNA stability assay performed on PVX-specific transcripts isolated from protoplasts (Fig. 4d, e). Finally, when a 0.4 kb cDNA fragment containing the sequence upstream of the AUG codon of the PVX CP gene was placed between two reporter genes, expression of the downstream cistron was retained at comparable levels even when expression of the upstream cistron was blocked by a hairpin structure (Fig. 5). The significance of this viral sequence in initiating downstream gene expression was confirmed by the elimination of CAT activity when a large deletion was generated within the PVX sequence, as well as when the sequence was present in the reverse orientation. The results of this transient assay demonstrate that the PVX sequence is capable of initiating translation of the downstream cistron in a cap-independent manner.

The existence of a true IRES element in Picornaviridae is based upon certain criteria which imply true internal initiation; specifically, a dicistronic mRNA assay in which insertion of the putative IRES between two reporter genes should result in an increase in expression of a downstream cistron to a level comparable to that of the upstream cistron. Levels of downstream expression less than 10% of levels of expression of the upstream cistron would therefore be considered artefacts (Sarnow, 1995). In this study, relative levels of CAT activity remained consistent whether this cistron was part of a monocistronic construct or the downstream cistron of a dicistronic construct (Fig. 5c). Degradation or cleavage of dicistronic transcripts by nuclease activity was unlikely, as a full-length single RNA transcript was detected on Northern blots.

In addition, an out-of-frame AUG was identified upstream of the CP gene, making translation of the CP gene by leaky scanning unlikely. The start codon of the PVX CP gene was in a suboptimal context (TCTATGG for 8 kDa protein; AAGATGT for CP), arguing against ribosomes bypassing the 8 kDa gene start site in favour of the CP gene start site (Kozak, 1989).

The most likely mechanism, which would explain the ability of this PVX cDNA fragment to initiate downstream gene expression, is initiation by internal ribosome entry. No IRES-specific elements present in Picornaviridae could be found in PVX, suggesting that the structure and/or sequence directing internal initiation is/are novel. Other IRES-like elements which do not resemble picornavirus IRES have been shown to exist in both BiP (immunoglobulin heavy-chain-binding protein) and Antennapedia (Macejak & Sarnow, 1991; Oh et al., 1992). Another alternative to the ribosome scanning model which involves the direct transfer, or shunt, of ribosomes from the 5' terminus to an internal site located further downstream on the RNA transcript has been identified in CaMV (Futterer et al., 1993). In this case, however, expression of the downstream cistron is abolished when a stable hairpin structure is introduced at the 5' terminus of the transcript.

The results presented here demonstrate the need for a re-evaluation of the translation strategy described for potexviruses, and perhaps other viruses as well. The fact that the dicistronic transcript of PVX was expressed from a transgenic plant system suggests that alternative translation initiation strategies can take place. The results suggest that in potexviruses, the CP can be expressed in vivo from genomic RNAs. The common belief that the CP and presumably other viral proteins are solely expressed from a subgenomic mRNA should be reassessed. This dual translational strategy may be universal in all polycistronic viruses and assist in the virus replication cycle by enabling the virus to accumulate maximal levels of required proteins. However, the relative importance of internal initiation and translation from subgenomic RNAs for PVX, and possibly other viruses, in the normal infection process remains to be determined.

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