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Translation of the Second Gene of Peanut Clump Virus RNA 2 Occurs by Leaky Scanning *in Vitro*

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The two 5′-proximal open reading frames of peanut clump virus RNA 2, which encode the coat protein of 23 kDa and a protein of 39 kDa (P39), are both translated *in vitro* from genomic RNA 2. We have studied the translational strategy involved in the initiation at the second AUG of RNA 2, which is the initiation codon of P39. Mutation experiments with synthetic transcripts corresponding to the 5′-half of RNA 2 ruled out mechanisms of P39 translation initiation involving termination–reinitiation and internal ribosomes entry. The results were consistent, however, with a leaky scanning mechanism for P39 initiation, in which about one-third of the ribosomes fail to initiate translation of coat protein and scan along the template to initiate translation at the AUG of the P39 gene, more than 1000 residues in from the 5′-terminus of the RNA 2. © 1995 Academic Press, Inc.

INTRODUCTION

Peanut clump virus (PCV) is a rod-shaped soil-borne virus, probably transmitted by the plasmodiophoromycete fungus *Polymyxa graminis* (Thouvenel and Fauquet, 1981) and which has been classified as a furovirus (Brunt, 1991). The viral genome is composed of two plus-strand RNA molecules whose complete nucleotide sequences have recently been determined (Manohar *et al.*, 1993; Herzog *et al.*, 1994). PCV RNA 2 contains five open reading frames (ORFs) (Fig. 1A). The 5′-proximal ORF encodes the viral coat protein (P23). The second ORF encodes a 39-kDa protein (P39). The three following ORFs, coding for proteins with molecular weights of 51, 14, and 17 kDa, show homology to the triple gene block proteins, which are known to be involved in cell-to-cell movement in a number of rod-shaped plant viruses (Morozov *et al.*, 1989, 1991; Petty *et al.*, 1990; Beck *et al.*, 1991; Gilmer *et al.*, 1992).

In the two other furoviruses for which sequence information is available, beet necrotic yellow vein virus (BNYVV) and soil-borne wheat mosaic virus (SBWMV), the coat protein is encoded by the 5′-proximal gene of RNA 2 (Bouzoubaa *et al.*, 1986; Shirako and Wilson, 1993). In both BNYVV and SBWMV, the coat protein cistron is immediately followed by a long in-frame ORF which is expressed by translational readthrough of the coat protein cistron termination codon to produce a "readthrough protein" (Ziegler *et al.*, 1985; Schmitt *et al.*, 1992; Shirako and Wilson, 1993). In the case of BNYVV, the readthrough protein has been shown to be incorporated into virions as a minor component (Haeberlé *et al.*, 1994) and to be essential for vector transmission (Tamada and Kusume, 1991). The P39 ORF of PCV, on the other hand, is not in the same reading frame as the upstream coat protein cistron and so must be expressed by a mechanism other than translational readthrough.

The 5′-noncoding region of PCV RNA 2 is 390 nucleotides and the first potential initiation codon, AUG(391–393), marks the beginning of the coat protein (P23) ORF. Remarkably, there are no other AUG triplets in the sequence until AUG(1011–1013), which is the putative site of initiation of P39 translation and which overlaps the termination codon of the P23 ORF . . . **AUG**A . . . (termination codon is underlined and AUG(1011–1013) is in bold).

According to the generally accepted model for translation of eucaryotic messenger RNAs (Kozak, 1978), the initiation codon for protein synthesis is selected by the 40S ribosomal subunit as it scans the RNA beginning at the 5′-end. In most cases, initiation occurs at the first AUG encountered on the sequence but initiation can also occur at downstream initiation codons if the first AUG is in an unfavorable context for translation initiation ("leaky scanning") (Kozak, 1989). Internal initiation codons may also become accessible if, after termination of translation of the preceding cistron, some of the ribosomes remain associated with the messenger RNA and continue scanning until another AUG is encountered ("termination–reinitiation") (Kozak, 1989). Finally, a third mechanism in which ribosomes may access internal initiation codons is to bind directly to the messenger RNA at a sequence near the internal start codon ("internal ribosome entry"). Internal ribosome entry has been most extensively stud-

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ied for picornaviruses (Pelletier and Sonenberg, 1988) but has also been reported for other viral and cellular RNAs (Carrington and Freed, 1990; Thomas *et al.*, 1991; Verver *et al.*, 1991; Macejak and Sarnow, 1991; Oh *et al.*, 1992).

In order to determine the most likely mechanism for expression of P39, we have cloned a cDNA fragment containing the first two cistrons of PCV RNA 2 and performed *in vitro* translation experiments on synthetic transcripts carrying the wild-type or various mutated sequences. Our results indicate that translation of P39 is initiated *in vitro* by a leaky scanning mechanism.

MATERIALS AND METHODS

Virus and viral RNA

Purification of PCV isolate PCV2 from systemically infected *Nicotiana benthamiana* leaves and extraction of viral RNA was as described by Manohar *et al.* (1993).

Construction of plasmids pPC2-2223 and pPC2

The plasmid pPC2-2223 was prepared by insertion of a DNA fragment prepared by the polymerase chain reaction and containing nucleotides 1–2223 of PCV RNA 2 between the *Bam*HI and *Hin*dIII restriction sites of pUC18. The insert is flanked at the 5′-end by a *Bam*HI restriction site followed by a bacteriophage T7 promoter sequence and at the 3′-end by the *Hin*dIII restriction site (Manohar *et al.*, 1993).

The plasmid pPC2, the construction of which will be described elsewhere, is a pUC18 derivative containing full-length PCV RNA 2 cDNA with plus-stranded RNA synthesis under control of a bacteriophage T7 RNA polymerase promoter. The cDNA insert has an 80-residue poly(A) tail at its 3′-end and the promoter–cDNA–poly(A) cassette is flanked by *Bam*HI and *Hin*dIII restriction sites.

Oligonucleotide site-directed mutagenesis

The *Bam*HI–*Hin*dIII cDNA insert of pPC2-2223 was recloned between the *Bam*HI and *Hin*dIII sites of pBS(−) and oligonucleotide-directed mutagenesis was performed on single-stranded phagemid DNA (Kunkel *et al.*, 1987) to produce mutants pBPC2-2223b_1, pBPC2-2223b_2, 2MAUGS, 2MAUGW,S, 2MAUGΔ,S, 2MAUG +1, 2MAUG +2, and 2MAUG +3. For pBPC2-2223b_1 or pBPC2-2223b_2, modification of nucleotides 49 and 51 or 979, 980, and 982 in the RNA 2 sequence introduced a *Bgl*II restriction site at position 46 or 979, respectively. Clones containing the desired mutations were identified by sequence analysis and the *Bam*HI–*Hin*dIII fragment carrying the cDNA insert was cloned between the *Bam*HI and *Hin*dIII sites of pUC18. In each construct, the sequence in the vicinity of the mutation, the T7 promoter, and the initiation codons were verified by sequence analysis (Sanger *et al.*, 1977).

Construction of deletion mutants 2MΔ172 and 2MΔ21

2MΔ172. After digestion of pPC2-2223b_2 with *Bst*EII and *Bgl*II, a 172-bp fragment (nucleotides 808–979) was removed. The remaining 4.7-kb DNA fragment was isolated, and protruding ends were filled in by treatment with the Klenow fragment of DNA polymerase I from *Escherichia coli* and ligated with T4 DNA ligase.

2MΔ21. pPC2-2223b_2 was digested with *Bgl*II and *Apa*LI. *Bgl*II cleaves at nucleotide 983 of the insert and *Apa*LI cuts at nucleotide 1004 of the insert and at three sites in the vector. The two fragments containing, respectively, vector sequences plus the 5′-terminal portion of the insert up to the insert *Bgl*II site and vector sequences plus the 3′-terminal portion of the insert starting from the insert *Apa*LI site were purified and protruding extremities were filled in. The first fragment was then digested with *Bam*HI and the second fragment with *Hin*dIII. The insert-containing fragments were purified in each case and inserted into *Bam*HI–*Hin*dIII-digested pUC18 by three-point ligation. In the resulting construct (2MΔ21), insert nucleotides 984–1004 have been deleted.

Construction of mutant plasmids 2MUGA2 and 2MUGA3

2MUGA2. After digestion of pPC2-2223 by *Ava*I, which cleaves once in the vector DNA and once in the insert at nucleotide 635, the protruding ends of the two resulting fragments were filled in with Klenow fragment of DNA polymerase I from *E. coli* and religated. This resulted in the insertion of four nucleotides between nucleotides 635 and 636 and produced a frameshift so that the ORF terminated at UAA(642–644) instead of UGA(1012–1014).

2MUGA3. A 4-base insertion between nucleotides 979 and 980 was obtained by digesting pPC2-2223b_2 with *Bgl*II, filling in the protruding ends, and religating. This mutation extended the first ORF in the same frame as the second ORF.

Construction of 2Mhp, 2Mcs1, and 2Mcs2

pPC2-2223b_1 was linearized by *Bgl*II, dephosphorylated, and used as an acceptor for the introduction of two phosphorylated oligonucleotides, 5′-P-GATCCC-ACCACGGCCCA-3′ and 5′-P-GATCTGGGCCGT-GGTGG-3′. The sequences of these oligonucleotides are complementary to each other over 13 bp; the unpaired terminal sequences allow insertion into the *Bgl*II site of the acceptor DNA. Ligation of the oligonucleotides was performed as described by Kozak (1986b). Clones containing the two oligonucleotides in the complementary orientation (2Mhp), one copy of one of the oligonucleotides (2Mcs1), or two copies of the same oligonucleotide (2Mcs2) were identified by sequence analysis using a

kit (United States Biochemical Corp.) containing 7-deaza-dGTP.

In vitro transcription

Run-off transcripts were synthesized *in vitro* using T7 RNA polymerase (Greif *et al.*, 1990) from *Hin*dIII-linearized pPC2, pPC2-2223, and its various mutant derivatives. The resulting transcripts contain one extra G residue at the 5′-end compared to wild-type viral RNA. For preparation of capped transcripts the initial concentration of GTP was reduced from 500 to 50 μM, and 500 μM m^7GpppG (Pharmacia) was added to the medium. The amount and integrity of the synthesized transcripts were evaluated by formaldehyde–agarose gel electrophoresis (Gustafson *et al.*, 1982).

Cell-free translation

Transcripts were translated in the rabbit reticulocyte lysate (0.1 μg of transcript/10 μl of translation medium) as described by Hemmer *et al.* (1989) except that [^{35}S]-methionine was replaced by [^{3}H]leucine because of the low methionine content of P23. Potassium (100 mM) and magnesium (480 μM) concentrations were adjusted to obtain maximal amounts of full-length products. In some experiments, edeine (10 μg/ml) (Calbiochem) was added at various times after the start of incubation to prevent *de novo* translation initiation.

In vitro translation was also performed in the transcription–translation-coupled reticulocyte lysate system (TNT; Promega) as described by the supplier. 0.1 μg of circular DNA was introduced into 5 μl of incubation medium.

Translations were carried out at 30° and products analyzed by SDS–PAGE (Laemmli, 1970). Gels were fluorographed with EN^{3}HANCE (Dupont NEN) and visualized by autoradiography of the dried gel. For quantification, autoradiograms were scanned at 560 nm using a Shimadzu CS9000 densitometer (Shimadzu Corp.). Different exposures of the same gel were used to evaluate the relative intensity of the bands, i.e., the relative radioactivity incorporated into the synthesized proteins after normalization of the leucine content for each protein.

RESULTS

Analysis of PCV RNA translation kinetics

The genome organization of PCV RNAs 1 and 2 is illustrated in Fig. 1A. When PCV viral RNA was translated for 90 min in a rabbit reticulocyte lysate, four major translation products were observed (Fig. 1B, lane f). The two fastest migrating proteins correspond to P23 (CP) and P39, encoded by RNA 2 (see below). The two larger proteins, P131 and P191, correspond to the predicted translation products of RNA 1, P191 being produced by readthrough of the P131 translation termination codon (Manohar *et al.*, 1993; Herzog *et al.*, 1994). A time-course study of the translation of the PCV RNAs showed that the order of appearance of the four major products was correlated with their size; i.e., P23, P39, P131, and P191 first appeared, respectively, after 5, 10, 20, and 40 min of incubation (Fig. 1B, lanes a, b, c, and d). When accumulation of the products was maximal, at about 60 min, the relative molar amounts of P131 and P191 were typically about 4:1 and the relative molar amounts of P23 to P39 were about 2:1 (Fig. 1B, lane e).

In order to follow the kinetics of initiation of translation products, the translation reaction medium was supplemented with edeine 5, 10, or 20 min after the start of *in vitro* translation to block further initiation and then incubated for an additional 90 min to allow completion of nascent polypeptide chains. As shown in Fig. 1B, lanes g and h, synthesis of both P23 and P131 was initiated within the first 5 min of *in vitro* translation, although, as expected in view of their size, full-length chains of P131 were visible only when elongation was allowed to continue for an additional 90 min. P191, on the other hand, was detectable only when translation was allowed to proceed for at least 10 min before addition of edeine (Fig. 1B, lanes j and l). Since P131 and P191 are believed to have the same N-terminus, the failure to detect the readthrough product in lane h is probably a consequence of its lower relative accumulation compared to P131. Note that, after 90 min of incubation, the relative amounts of radioactivity incorporated in P131 and P191 were similar whether or not edeine was present in the medium (Fig. 1B, lanes j, l, and f). Like P191, P39 was not detectable when edeine was added to the medium after 5 min. But in this case, the amount of radioactivity incorporated in P39 relative to that incorporated in P23 (after 90 min of incubation) was significantly lower when edeine was added than when edeine was absent (Fig. 1B, compare lanes j, l, and f). It appears therefore that addition of edeine after a few minutes of incubation inhibits initiation of P39. This suggests that initiation of P39 translation is delayed relative to that of P23.

P23 (CP) and P39 expression from synthetic transcripts

To facilitate investigation of the mechanism of expression of P39, we placed a bacteriophage T7 RNA polymerase promoter upstream of a cDNA corresponding to the 5′-proximal 2223 nucleotides of RNA 2 (pPC2-2223) (Fig. 2). The coding capacity of this plasmid was studied either by introducing it directly into a coupled transcription–translation system (TNT System) or by translating capped or uncapped transcripts prepared from the *Hin*dIII-linearized plasmid in reticulocyte lysate. As shown in Fig. 3, P23 and P39 were synthesized in both the TNT system (Fig. 3, lane a) and the transcript-primed lysate (Fig. 3, lanes c and d) in amounts comparable to those observed

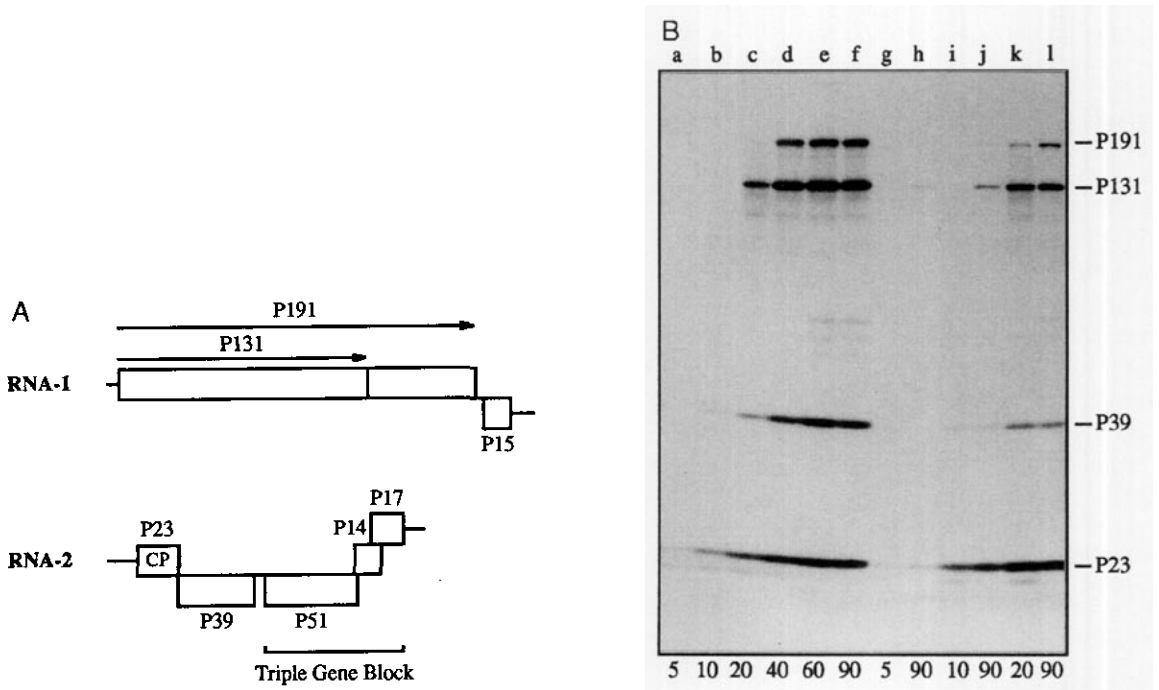


FIG. 1. (A) Genome organization of PCV RNAs 1 and 2. The boxes represent ORFs and the arrows the two translation products of RNA 1. (B) *In vitro* translation of PCV RNA 1 and 2. PCV RNA was incubated in a rabbit reticulocyte lysate in presence of [^{3}H]leucine. The medium contained no edeine (a–f) or was supplemented with edeine (10 μg/ml) at 5 min (g–h), at 10 min (i–j), or at 20 min (k–l) after the start of incubation. Samples were analyzed after the indicated time of incubation by 8% SDS–PAGE and products were detected by fluorography. Position of synthesized products and their M_r are indicated.

with wild-type RNA (Fig. 3, lane b). This finding rules out the possibility that an undetected subgenomic RNA in the viral RNA preparation was responsible for synthesis of P39 (Figs. 1B and 3, lane b). The fact that similar translation patterns were observed with pPC2 and pPC2-2223 transcripts (Fig. 3) indicates that 5′-distal sequences on the full-length RNA are not required for translation of P23 and P39. The origin of the two bands of M_r evaluated at 58 and 60 kDa, visible after translation of full-length RNA 2 (Fig. 3, lanes b and f), is unknown.

The relative accumulation of P23 and P39 in this and in the following experiments was evaluated by scanning the corresponding bands in the autoradiogram. After normalization of leucine content for each protein, the relative efficiency of initiation at the first and the second AUG was expressed as the ratio of radioactivity incorporated into P23 or P39, respectively, divided by the amount of radioactivity incorporated into both proteins. When transcripts were translated in the reticulocyte lysate, 20–30% of the initiation events occurred at the second AUG when either full-length transcript or 3′-truncated transcript was used as template and regardless of whether the transcript was capped (Fig. 3, lanes c, d, e, and f). Slightly more efficient translation initiation at the second AUG (30–40%) was consistently observed in the coupled transcription–translation system (Fig. 3, lane a). This difference may reflect the superior integrity of the template in the coupled system where, during translation, the ribosomes are thought to closely follow the bacteriophage T7 polymerase on the nascent RNA strand.

Initiation at AUG(1011–1013) does not require prior termination at the neighboring termination codon

The manner in which the P39 initiation codon (1011–1013) is embedded within the UGA termination codon of the P23 ORF raises the possibility that ribosomes which terminate translation of P23 (CP) remain attached to the mRNA and reinitiate at the proximal AUG (termination–reinitiation mechanism) (Peabody and Berg, 1986). To determine if termination of translation at UGA(1012–1014) is a prerequisite for initiation of P39 synthesis, four mutant plasmids were constructed in which the stop codon of P23 was eliminated or displaced in different ways (Fig. 2).

In 2MΔAUG, the P23 initiation codon, AUG(391–393), was eliminated. In mutant 2MUGA1, a point mutation changed UGA(1012–1014) to UGG. This extends the P23 ORF by 15 amino acids. In mutants 2MUGA2 and 2MUGA3, 4 nucleotides were inserted after nucleotides 635 and 979, respectively, to produce frameshifts. In 2MUGA2, the frameshift results in appearance of an in-frame stop codon at nucleotide 642, to give a predicted truncated translation product of 9.3 kDa, while in 2MUGA3 the P23 ORF is fused to the P39 ORF to give a predicted product of 62 kDa (P62).

Coupled transcription–translation of the various mu-

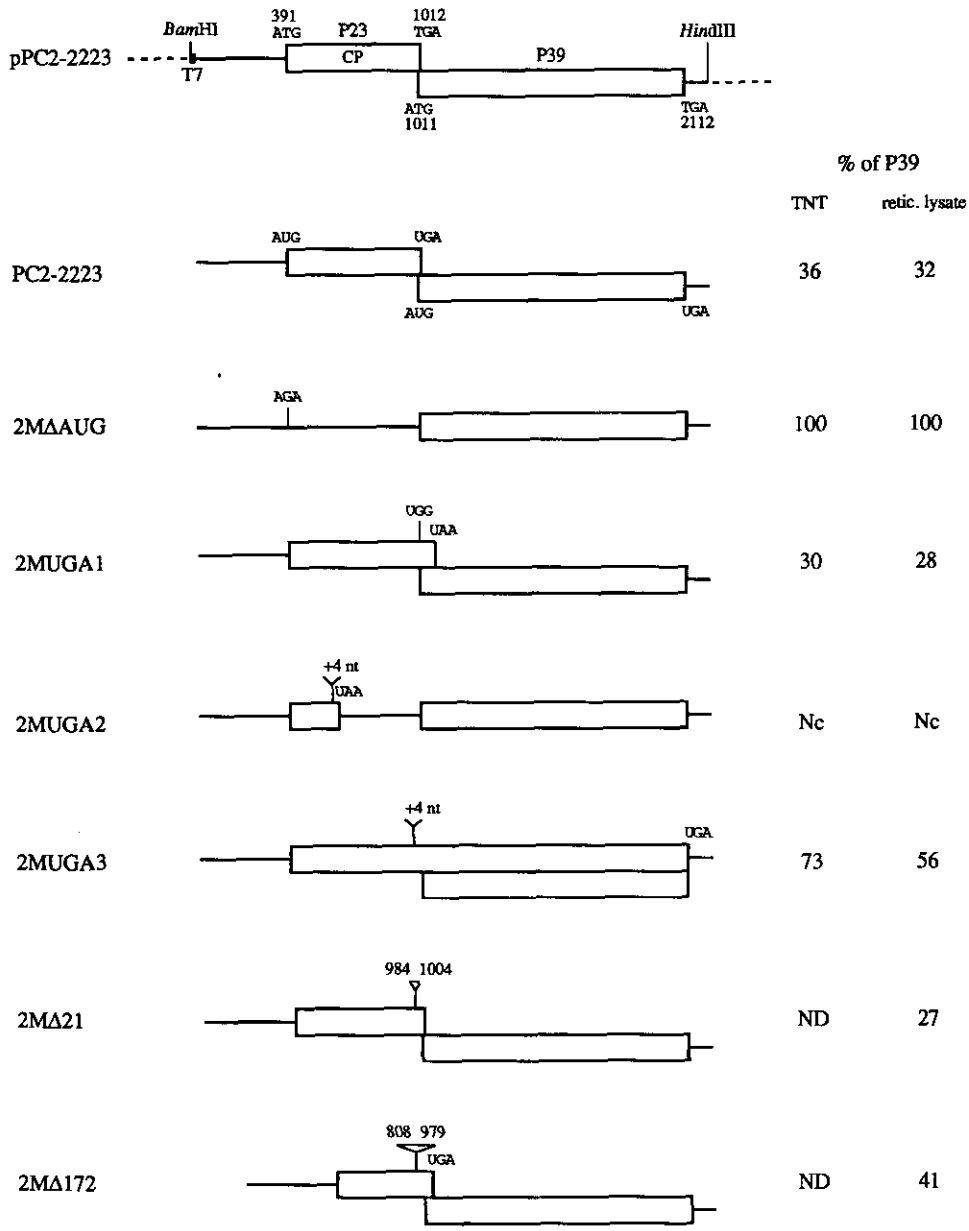


FIG. 2. Effect of different mutations introduced in the P23 (CP) gene on the expression efficiency of P39. Schematic representation of the cDNA clone pPC2-2223 containing the first two ORFs of PCV RNA 2 and of transcripts obtained from the wild-type clone or from different mutants. For pPC2-2223, relevant enzyme restriction sites are indicated and the T7 promoter is represented as a black square. Nucleotides are numbered according to wild-type PCV RNA 2, although the transcripts contain an extra G residue at their 5′-end. The values given at the right correspond to the relative yield of P39 synthesized in the TNT system and in the reticulocyte lysate, calculated as described in the text. Nc, not calculated; ND, not done.

tants (Fig. 4A) or translation of uncapped transcripts in the reticulocyte lysate (Fig. 4B) gave similar results. P39 was produced from 2MΔAUG although P23 was not synthesized (Fig. 4). A modified CP of about 25 kDa was produced from 2MUGA1 and a product of 62 kDa, the size predicted for the P23–P39 fusion protein, was produced from 2MUGA3. In both cases, P39 was efficiently synthesized (Fig. 4), with yields comparable to those of the wild-type for 2MUGA1 and even in relatively higher amounts from 2MUGA3 (Fig. 2). P39 was produced from 2MUGA2 (Fig. 4). The predicted 9.3-kDa species corresponding to the truncated CP was not detected, however, because of its small size.

The fact that all of the above mutants produce abundant quantities of P39 argues strongly that translation termination at the UGA neighboring the P39 initiation codon is not required for efficient initiation. It may be argued that, in mutant 2MUGA1, ribosomes could scan backward 46 nucleotides after termination (Peabody and Berg, 1986) and reinitiate translation of P39 at the up-

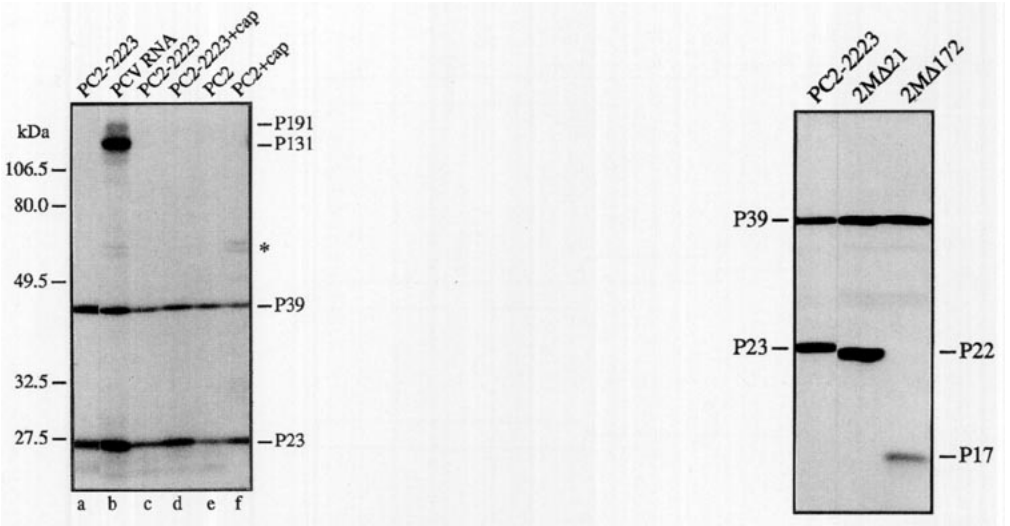


FIG. 3. *In vitro translation of PCV RNA and of transcripts from pPC2-2223 and from pPC2.* pPC2-2223 was translated in the TNT system (a). PCV RNA (b) and capped or uncapped transcripts of pPC2-2223 (c, d) and of pPC2 (e, f) were translated in the rabbit reticulocyte lysate. Products synthesized after 60 min of incubation at 30° in the presence of [^{3}H]leucine were analyzed by 12% SDS–PAGE and detected by fluorography. Sizes are indicated on the left. Positions of translation products are indicated at the right. The pair of faint bands (*) correspond to proteins of M_r 58 and 60 kDa, discussed in the text.

FIG. 5. Effect of deletions upstream of AUG(1011–1013) on translation of P39. *Translation of transcripts 2MΔ21 and 2MΔ172 was carried* out for 60 min at 30° in the rabbit reticulocyte lysate. Translation products were analyzed as described in Fig. 3.

stream AUG, but such backward scanning does not seem plausible for 2MUGA3, in which the termination codon of the P23–P39 fusion product is 1099 residues downstream of the P39 initiation codon.

Initiation at AUG(1011–1013) does not require a *specific upstream sequence*

The above experiments do not eliminate the possibility that translation of P39 could occur via an internal initiation mechanism similar to that described for picornaviruses (Pelletier and Sonenberg, 1988; Herman, 1989). To test this possibility, two deletion mutants were constructed to remove possible internal ribosome entry sites upstream of AUG(1011–1013) (Fig. 2). In 2MΔ21, a small deletion of 21 nucleotides was introduced 6 nucleotides upstream of AUG(1011–1013). In 2MΔ172, a deletion of 172 nucleotides between positions 808 and 979 deleted 58 amino acids of P23 and also produced a frameshift so that the P23 was extended by 10 amino acids in another reading frame at its C-terminus. As shown in Fig. 5, similar amounts of P39 were produced by the mutants and the wild-type transcripts, indicating that no particular sequence upstream of AUG(1011–1013) serves as a "ribosomal landing pad" (Pelletier and Sonenberg, 1988) for internal translation initiation at this AUG.

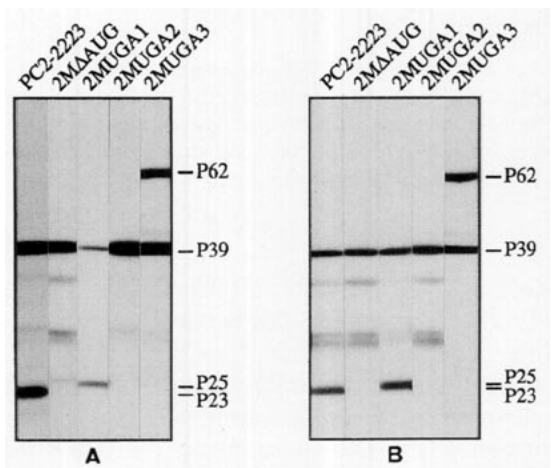
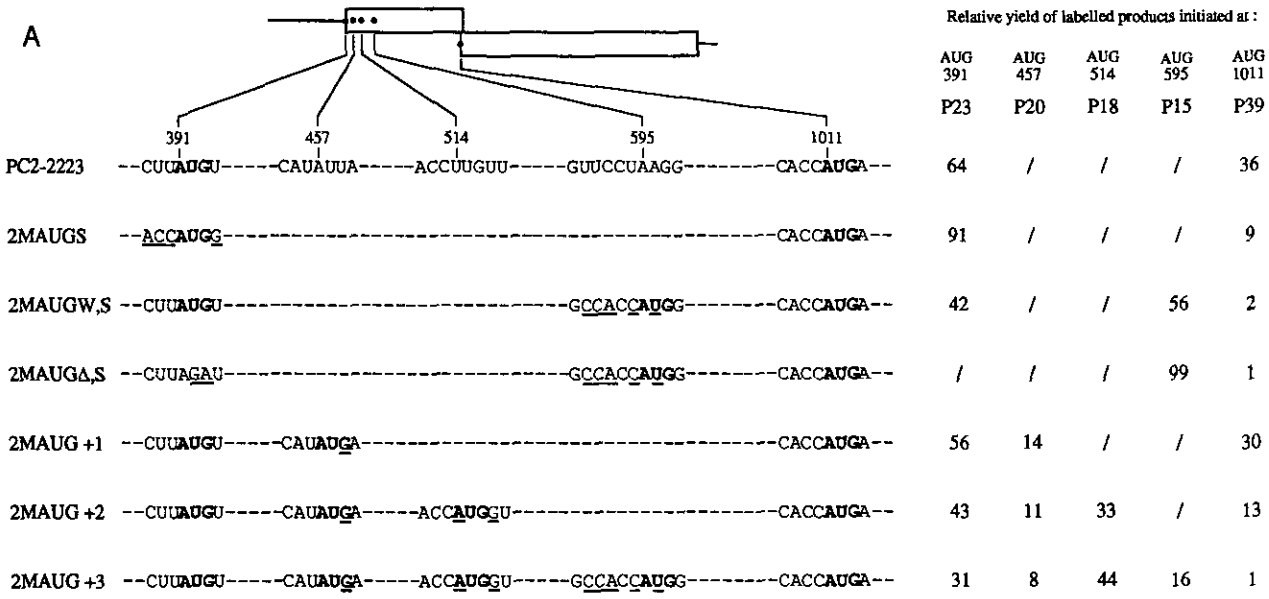


FIG. 4. Expression of P39 from transcripts of mutants 2MΔAUG, 2MUGA1, 2MUGA2, and 2MUGA3. Products obtained by translation in the TNT system (A) or in reticulocyte lysate (B) were analyzed as described in Fig. 3.

Evidence for leaky scanning

The optimal sequence context for efficient translation initiation at an AUG of an eukaryotic messenger RNA is A/GCCAUGG (Kozak, 1984, 1986c), with the purines in the −3 and +4 positions being of particular importance (Lütcke *et al.*, 1987; Cavener and Ray, 1991). If initiation at AUG(1011–1013) results from inefficient initiation at the upstream AUG(391–393) (leaky scanning), our *in vitro* translation experiments indicate that about one-third of the scanning ribosomes ignore the upstream initiation codon, which is in a weak context, and continue scanning until the P39 initiation codon. To provide direct evidence for this hypothesis, we analyzed the effect on translation of mutants (Fig. 6A) in which (1) AUG(391–393) was itself placed in a better sequence context (2MAUGS), (2)



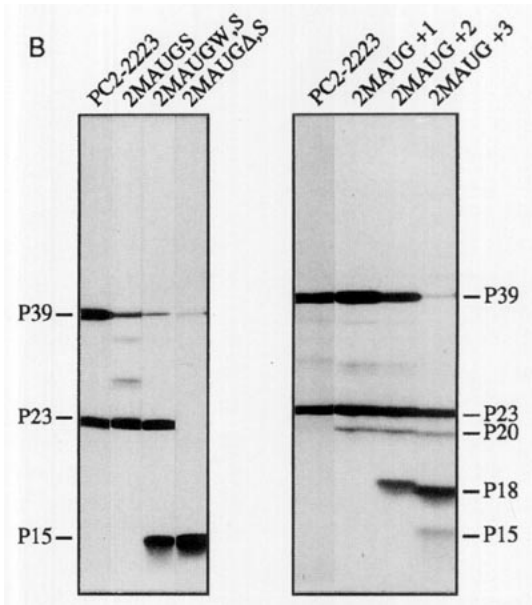


FIG. 6. Effect of modifications of the sequence flanking the initiation codon of P23 (CP) and of the insertion of supplementary AUG codons. (A) Representation of the sequence flanking the AUG codons of P23 and P39 ORFs and at three positions chosen for insertion of new AUGs. Mutations are underlined and AUGs are in bold. The yields of the proteins initiated at the different AUGs (right) correspond to the radioactivity incorporated into each protein relative to the radioactivity incorporated into all translation products. (B) Translation of the different mutants in the TNT system. Products were analyzed as described in Fig. 3. The mobility of proteins (P23, P20, and P18) initiated at the same AUG in 2MAUG +1, 2MAUG +2, and 2MAUG +3 varies slightly, probably because the proteins differ by one to six amino acids. The difference is particularly visible for P18, synthesized from 2MAUG +2 and from 2MAUG +3.

AUG(391–393) was suppressed while another AUG in a favorable context was inserted downstream (2MAUGΔ,S), or (3) one or more additional in-frame AUGs in suboptimal or optimal contexts were inserted downstream of AUG(391–393) (2MAUGW,S and 2MAUG +1, +2, and +3). The coupled transcription–translation system was used for translation. As predicted by the leaky scanning model, optimization of the context of AUG(391–393) in mutant 2MAUGS reduced the relative abundance of P39 from 36 to 9% (Figs. 6A and 6B) and an AUG in good context inserted in-frame at position 595 (in 2MAUGW,S and 2MAUGΔ,S) trapped almost all of the scanning ribosomes so that P39 synthesis dropped to 1–2% (Figs. 6A and 6B). Creation of additional AUGs downstream of AUG(391–393) in mutants 2MAUG +1, 2MAUG +2, and 2MAUG +3 gave rise to N-terminally truncated P23 polypeptides as well as full-length P23 and resulted in a decrease in the relative abundance of

P39 as the number of extra AUGs was increased (Figs. 6A and 6B). For these mutants the amount of P23 produced was relatively constant.

These experiments support our earlier contention that translation initiation at AUG(1011–1013) does not occur by internal entry of ribosomes as the efficiency of such a process would not be expected to be influenced by the insertion of upstream initiation codons or by creation of a favorable context around AUG(391–393). Not unexpectedly, the efficiency of initiation at a new downstream AUG depends on the sequence context. Only 8–14% of the ribosomes initiated at the second AUG in mutants 2MAUG +1, +2, and +3, in which its context was poor, whereas 56% initiated at AUG(595–597), which is in a good context in mutant 2MAUGW,S (Fig. 6A). In the latter mutant and in mutant 2MAUG +3, which also contains this codon, only about 1% of the ribosomes reach AUG(1011–1013). It should be noted that AUG(514–516), although in a favorable initiation context, was less efficient in capturing scanning ribosomes than AUG(595–597), in which the presence of GCC in front of ACCAUGG creates an even more favorable context (Kozak, 1987a,b) (compare 2MAUG +2 with 2MAUGΔ,S or 2MAUGW,S in Fig. 6A).

The above results support a leaky scanning model for initiation at AUG(1011–1013) as they clearly establish that ribosomes can ignore the first AUG(391–393) to initiate at a downstream AUG(1011–1013). Experiments in which transcripts were translated in the reticulocyte lysate led to the same conclusion although there were some differences in the relative amounts of the proteins synthesized from the extra AUGs (data not shown). The presence of significant amounts of incomplete translation products in the transcript-driven system may be responsible in large part for these apparent differences as no account was taken of such products in the calculations.

Influence of secondary structure in the 5′-noncoding region on initiation at AUG(1011–1013)

The previous observations indicate that translation initiation at AUG(1011–1013) primarily, if not exclusively, involves ribosomes which have scanned past AUG(391–393). Under these circumstances, we would predict that an obstacle that hinders access of scanning ribosomes to AUG(391–393) would also interfere with access to AUG(1011–1013). Kozak (1986b) has shown that a stable stem–loop secondary structure in the 5′-noncoding region can block the movement of scanning ribosomes toward a downstream AUG. We have therefore tested the consequences of inserting a hairpin loop upstream of AUG(391–393) on the translation of P23 and P39.

The hairpin loop structure in mutant 2Mhp (Fig. 7A) was created by inserting complementary heptadecamers in the complementary orientation at the *Bgl*II site (position 46) of pPC2-2223b$_1$, a derivative of pPC2-2223. As controls, mutants were obtained by inserting a single copy of one of the heptadecamers (2Mcs1) or two copies (2Mcs2), neither of which can form a hairpin independently (Fig. 7A). The yield of both P23 and P39 from translation of 2Mhp was very low in the coupled transcription–translation system (Fig. 7B), whereas the amounts of the two products synthesized from 2Mcs1 and 2Mcs2 were similar to that obtained with the wild-type pPC2-2223 (Fig. 7B). Note in particular that the inefficient synthesis of P23 and P39 with 2Mhp can be attributed to the presence of the hairpin structure, as insertion of the same number of residues which do not form a hairpin in 2Mcs2 had no effect. Similar results were obtained when transcripts 2Mhp, 2Mcs1, and 2Mcs2 were used as templates in the reticulocyte lysate system (not shown). These experiments thus provide additional evidence that ribosomes which translate P39 reach that cistron primarily if not exclusively by leaky scanning of P23 gene.

DISCUSSION

In this paper we demonstrate that the *in vitro* expression of the second cistron on the PCV RNA 2 occurs by internal initiation. This involves a mechanism in which a portion of the ribosomes or initiation complexes bypass the first AUG (leaky scanning). Leaky scanning has been reported in many cases in which two overlapping open reading frames are expressed from the same messenger RNA (Kozak, 1986a; Samuel, 1989; Riechmann *et al.*, 1991; Schwartz *et al.*, 1992; Dinesh-Kumar and Miller, 1993), but PCV is exceptional in that the distance between the two initiation codons is unusually long (620 nt). For this reason we have sought to exclude other possible mechanisms which could account for P39 initiation.

Our results show that translation of P39 is initiated even when the first ORF is absent (2MΔAUG) or when the first ORF extended to the stop codon of the second ORF (2MUGA3). These experiments exclude a mechanism of termination–reinitiation which may occur if a new start codon is separated from a stop codon by a short distance (Peabody and Berg, 1986; Levine *et al.*, 1991; Fütterer and Hohn, 1991). Our experiments also show that the efficiency of initiation at the second AUG(1011–1013) is directly related to the efficiency of initiation at the first AUG(391–393); insertion of a stable hairpin in the 5′-noncoding region abolished translation of both P23 (CP) and P39, and an increase in initiation at AUG(391–393) produced by changing the sequence context is coupled with decreased initiation at AUG(1011–1013). These results show that ribosomes do not reach AUG(1011–1013) by internal entry. They do not, however, rule out the possibility that a portion of the ribosomes enter at the 5′-end, scan past the first AUG,

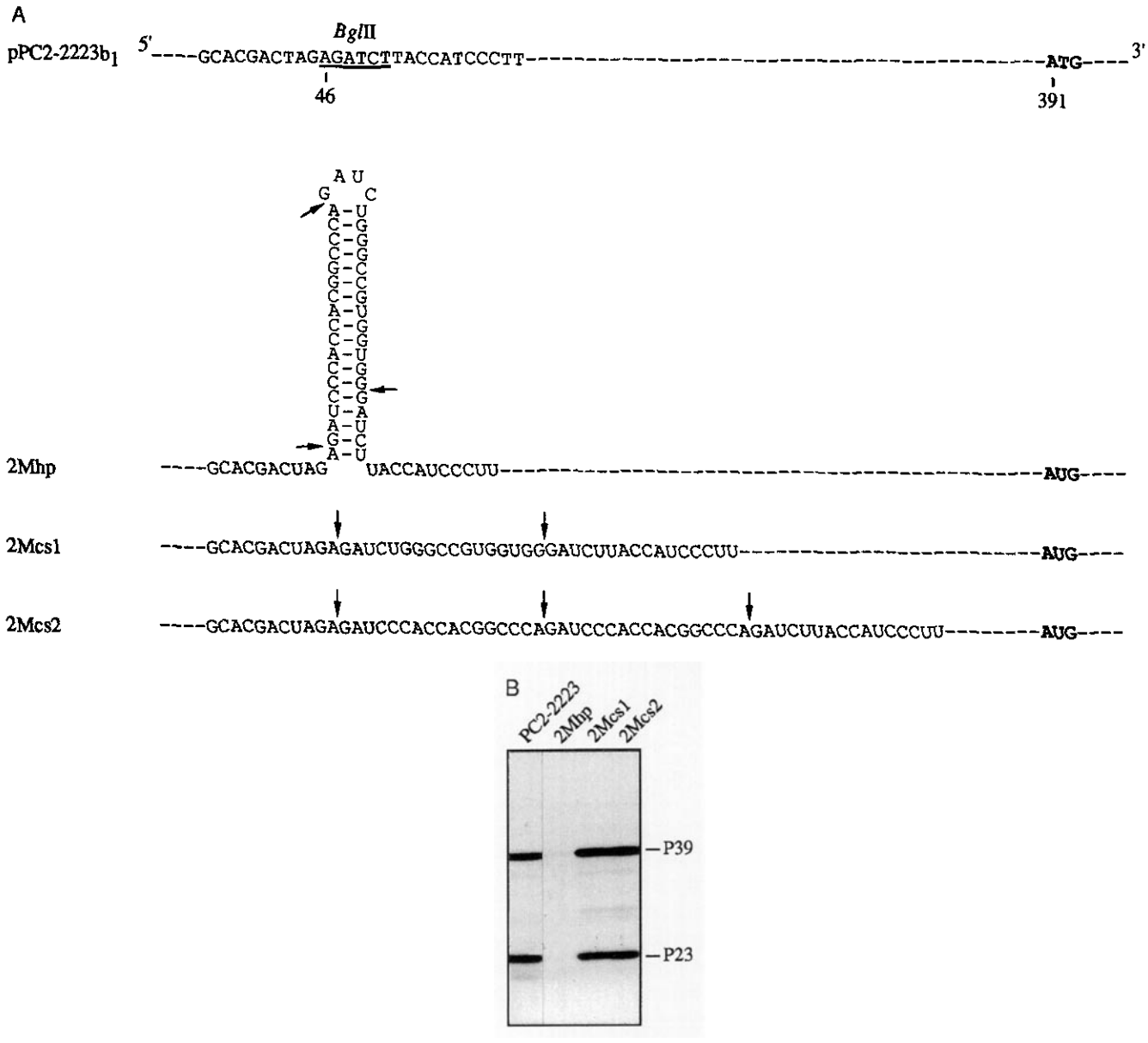


FIG. 7. Effect of insertion of a hairpin structure in the 5'-noncoding region on translation of P23 (CP) and P39. (A) Schematic representation of a mutant of pPC2-2223 containing a *Bgl*II site (pPC2-2223b$_1$) and used for insertion of the two 17-mer oligonucleotides in the complementary orientation (2Mhp) or a single copy (2Mcs1) or two successive copies of the same oligonucleotide (2Mcs2). The sequences of resulting transcripts are shown with the limits of the 17-nt insert or inserts indicated by arrows. The predicted free energy of the hairpin structure is −50 kcal/mol. (B) Analysis by 12% SDS–PAGE of the translation products obtained in the TNT system from the different mutants.

and then jump directly to the second AUG without need to scan the entire upstream sequence, similar to the process leading to initiation of translation of sendai virus X protein (Curran and Kolakofsky, 1988) and translation of cauliflower mosaic virus 35S RNA (Fütterer *et al.*, 1993). However, in such a model, insertion of additional AUGs at positions 457, 514, and 595 would not be expected to influence the expression of P39, which is contrary to our observations. The fact that P39 is normally synthesized in rather large amounts is probably related to the fact that, in wild-type RNA 2, only one AUG, AUG(391–393), is present upstream of AUG(1011–1013). However, the percentage of initiation occurring at the three inserted AUGs in mutant 2MAUG +3 is much higher than that occurring at AUG(1011–1013) in wild-type (Fig. 6). This may indicate that, in wild-type RNA 2, a significant portion of the scanning ribosomes which bypass AUG(391–393) leave the RNA before having reached AUG(1011–1013).

Ribosomal frameshifting is another possible means by which P39 could be expressed although such a mechanism would be expected to produce only low levels of P39 and as a CP–P39 fusion protein. Gramstat *et al.*

(1994) have shown that about 0.3% frameshifting occurs at the junction between the CP cistron and the ORF of the adjacent 12-kDa protein of potato virus M, a junction which is identical to the sequence at the PCV CP–P39 boundary (. . . AUGA . . .). It should be pointed out, however, that there is no upstream "shifty" heptanucleotide sequence such as observed in PVM or in other frameshift sites (Brault and Miller, 1992; Prüfer *et al.*, 1992; Kim and Lommel, 1994) at the CP–P39 junction. In the translation experiments shown in Fig. 3 there are a pair of minor bands of about 60 kDa (indicated by an asterisk in the figure), one of which could represent a CP–P39 frameshift fusion product. Neither of these species comigrated, however, with the translation product of 2MUGA3, which is an authentic CP–P39 fusion (data not shown). Because of the low abundance of the two indicated translation products in the translation medium, immunoprecipitation experiments using CP and P39 antisera did not produce conclusive results. However, P39 (and no other species) was readily immunodetected with a P39-specific antiserum in PCV-infected *N. benthamiana* leaves or *Chenopodium quinoa* protoplasts (data not shown), establishing that a mechanism exists for abundant P39 production in infected tissue. Based upon our *in vitro* translation experiments, we feel it highly likely that leaky scanning accounts for the synthesis of this product.

The role of P39 is still unknown, but there is circumstantial evidence that P39 may be involved in vector transmission since its ORF undergoes deletions when the virus is propagated by mechanical inoculation on leaves (Manohar *et al.*, 1993), as does the "readthrough domain" of the SBWMV readthrough protein (Shirako and Ehara, 1986) and that of BNYVV (Tamada and Kusume, 1991). As noted in the introduction, a portion of the readthrough domain downstream of the BNYVV coat protein cistron has been shown to be essential for vector transmission (Tamada and Kusume, 1991). It will be recalled that BNYVV readthrough protein is expressed by a translational readthrough of the coat protein cistron termination codon, a mechanism which should ensure that the rates of CP and readthrough protein synthesis are correlated. For PCV, the strategy involved in the expression of P39 is different (leaky scanning), but also provides a mechanism to correlate the rate of expression of P39 and coat protein. It will be interesting to determine if P39, like the readthrough protein of BNYVV, is also a minor virion component and if it also has a role in vector transmission.

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REFERENCES

Beck, D. L., Guilford, P. J., Voot, D. M., Andersen, M. T., and Forster, R. L. S. (1991). Triple gene block proteins of white clover mosaic potexvirus are required for transport. *Virology* **183,** 695–702.

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K., and Jonard, G. (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA-2. *J. Gen. Virol.* **67,** 1689–1700.

Brault, V., and Miller, W. A. (1992). Translational frameshifting mediated by a viral sequence in plant cells. *Proc. Natl. Acad. Sci. USA* **89,** 2262–2266.

Brunt, A. A. (1991). Furovirus. *In* "Classification and nomenclature of viruses. Fifth Report of the International Committee on Taxonomy of Viruses" (R. I. B. Francki, C. M. Fauquet, D. L. Knudson, and F. Brown, Eds.), Vol. Archives of Virology Supplementum 2. Springer-Verlag, Wien/New York.

Carrington, J. C., and Freed, D. D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. *J. Virol.* **64,** 1590–1597.

Cavener, D. R., and Ray, S. C. (1991). Eukaryotic start and stop translation sites. *Nucleic Acids Res.* **19,** 3185–3192.

Curran, J., and Kolakofsky, D. (1988). Scanning independent ribosomal initiation of the sendai virus X protein. *EMBO J.* **7,** 2869–2874.

Dinesh-Kumar, S. P., and Miller, W. A. (1993). Control of start codon choice on a plant viral RNA encoding overlapping genes. *Plant Cell* **5,** 679–692.

Fütterer, J., and Hohn, T. (1991). Translation of a polycistronic mRNA in the presence of the cauliflower mosaic virus transactivator protein. *EMBO J.* **10,** 3887–3896.

Fütterer, J., Kiss-Laszlo, Z., and Hohn, T. (1993). Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. *Cell* **73,** 789–802.

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K., and Jonard, G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3′ proximal genes located on RNA 2. *Virology* **189,** 40–47.

Gramstat, A., Prüfer, D., and Rohde, W. (1994). The nucleic acid-binding zinc finger protein of potato virus M is translated by internal initiation as well as by ribosomal frameshifting involving a shifty stop codon and a novel mechanism of P-site slippage. *Nucleic Acids Res.* **22,** 3911–3917.

Greif, C., Hemmer, O., Demangeat, G., and Fritsch, C. (1990). *In vitro* synthesis of biologically active transcripts of tomato black ring virus satellite RNA. *J. Gen. Virol.* **71,** 907–915.

Gustafson, G. D., Milner, J. J., McFarland, J. E., Pedersen, K., Larkins, B. A., and Jackson, A. O. (1982). Investigation of the complexity of barley stripe mosaic virus RNAs with recombinant DNA clones. *Virology* **120,** 182–193.

Haeberlé, A. M., Stussi-Garaud, C., Schmitt, C., Garaud, J. C., Richards, K. E., Guilley, H., and Jonard, G. (1994). Detection by immunogold labelling of P75 readthrough protein near an extremity of beet necrotic yellow vein virus particles. *Arch. Virol.* **134,** 195–203.

Hemmer, O., Demangeat, G., Greif, C., and Fritsch, C. (1989). Detection of a proteolytic activity in the micrococcal nuclease used for preparation of messenger-dependent reticulocyte lysates. *FEBS Lett.* **250,** 357–361.

Herman, R. C. (1989). Alternatives for the initiation of translation. *Trends Biochem. Sci.* **14,** 219–222.

Herzog, E., Guilley, H., Manohar, S. K., Dollet, M., Richards, K., Fritsch, C., and Jonard, G. (1994). Complete nucleotide sequence of peanut clump virus RNA 1 and relationships with other fungus-transmitted rod-shaped viruses. *J. Gen. Virol.* **75,** 3147–3155.

Kim, K. H., and Lommel, S. A. (1994). Identification and analysis of the site of −1 ribosomal frameshifting in red clover necrotic mosaic virus. *Virology* **200,** 574–582.

Kozak, M. (1978). How do eucaryotic ribosomes select initiation regions in messenger RNA? *Cell* **15,** 1109–1123.

Kozak, M. (1984). Selection of initiation sites by eucaryotic ribosomes:

Effect of inserting AUG triplets upstream from the coding sequence for preproinsulin. *Nucleic Acids Res.* **12,** 3873–3893.

Kozak, M. (1986a). Bifunctional messenger RNAs in eukaryotes. *Cell* **47,** 481–483.

Kozak, M. (1986b). Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proc. Natl. Acad. Sci. USA* **83,** 2850–2854.

Kozak, M. (1986c). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44,** 283–292.

Kozak, M. (1987a). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15,** 8125–8148.

Kozak, M. (1987b). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol.* **196,** 947–950.

Kozak, M. (1989). The scanning model for translation: An update. *J. Cell Biol.* **108,** 229–241.

Kunkel, T. A., Roberts, J. A., and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154,** 367–382.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Levine, F., Yee, J. K., and Friedmann, T. (1991). Efficient gene expression in mammalian cells from a dicistronic transcriptional unit in an improved retroviral vector. *Gene* **108,** 167–174.

Lütcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A. (1987). Selection of AUG initiation codons differs in plants and animals. *EMBO J.* **6,** 43–48.

Macejak, D. G., and Sarnow, P. (1991). Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. *Nature* **353,** 90–94.

Manohar, S. K., Guilley, H., Dollet, M., Richards, K., and Jonard, G. (1993). Nucleotide sequence and genetic organization of peanut clump virus RNA 2 and partial characterization of deleted forms. *Virology* **195,** 33–41.

Morozov, S. Y., Dolja, V. V., and Atabekov, J. G. (1989). Probable reassortment of genomic elements among elongated RNA-containing plant viruses. *J. Mol. Evol.* **29,** 52–62.

Morozov, S. Y., Miroshnichenko, N. A., Solovyev, A. G., Fedorkin, O. N., Zelenina, D. A., Lukasheva, L. I., Karasev, A. V., Dolja, V. V., and Atabekov, J. G. (1991). Expression strategy of the potato virus X triple gene block. *J. Gen. Virol.* **72,** 2039–2042.

Oh, S. K., Scott, M. P., and Sarnow, P. (1992). Homeotic gene *Antennapedia* mRNA contains 5′-noncoding sequences that confer translational initiation by internal ribosome binding. *Genes Dev.* **6,** 1643–1653.

Peabody, D. S., and Berg, P. (1986). Termination-reinitiation occurs in the translation of mammalian cell mRNAs. *Mol. Cell. Biol.* **6,** 2695–2703.

Pelletier, J., and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334,** 320–325.

Petty, I. T. P., French, R., Jones, R. W., and Jackson, A. O. (1990). Identification of barley stripe mosaic virus genes involved in viral RNA replication and systemic movement. *EMBO J.* **9,** 3453–3457.

Prüfer, D., Tacke, E., Schmitz, J., Kull, B., Kaufmann, A., and Rohde, W. (1992). Ribosomal frameshifting in plants: A novel signal directs the −1 frameshift in the synthesis of the putative viral replicase of potato leafroll luteovirus. *EMBO J.* **11,** 1111–1117.

Riechmann, J. L., Lain, S., and Garcia, J. A. (1991). Identification of the initiation codon of plum pox potyvirus genomic RNA. *Virology* **185,** 544–552.

Samuel, C. E. (1989). Polycistronic animal virus mRNAs. *Prog. Nucleic Acid Res. Mol. Biol.* **37,** 127–153.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

Schmitt, C., Balmori, E., Jonard, G., Richards, K. E., and Guilley, H. (1992). *In vitro* mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: Evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly. *Proc. Natl. Acad. Sci. USA* **89,** 5715–5719.

Schwartz, S., Felber, B. K., and Pavlakis, G. N. (1992). Mechanism of translation of monocistronic and multicistronic human immunodeficiency virus type 1 mRNAs. *Mol. Cell. Biol.* **12,** 207–219.

Shirako, Y., and Ehara, Y. (1986). Comparison of the *in vitro* translation products of wild-type and a deletion mutant of soil-borne wheat mosaic virus. *J. Gen. Virol.* **67,** 1237–1245.

Shirako, Y., and Wilson, T. M. A. (1993). Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. *Virology* **195,** 16–32.

Tamada, T., and Kusume, T. (1991). Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus *Polymyxa betae*. *J. Gen. Virol.* **72,** 1497–1504.

Thomas, A. A. M., Ter Haar, E., Wellink, J., and Voorma, H. O. (1991). Cowpea mosaic virus middle component RNA contains a sequence that allows internal binding of ribosomes and that requires eukaryotic initiation factor 4F for optimal translation. *J. Virol.* **65,** 2953–2959.

Thouvenel, J. C., and Fauquet, C. (1981). Further properties of peanut clump virus and studies on its natural transmission. *Ann. Appl. Biol.* **97,** 99–107.

Verver, J., Le Gall, O., van Kammen, A., and Wellink, J. (1991). The sequence between nucleotides 161 and 512 of cowpea mosaic virus M RNA is able to support internal initiation of translation *in vitro*. *J. Gen. Virol.* **72,** 2339–2345.

Ziegler, V., Richards, K., Guilley, H., Jonard, G., and Putz, C. (1985). Cell-free translation of beet necrotic yellow vein virus: Readthrough of the coat protein cistron. *J. Gen. Virol.* **66,** 2079–2087.