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# Non-canonical translation mechanisms in plants: efficient *in vitro* and *in planta* initiation at AUU codons of the tobacco mosaic virus enhancer sequence

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## ABSTRACT

**The 5′ untranslated leader (Ω sequence) of tobacco mosaic virus (TMV) genomic RNA was utilized as a translational enhancer sequence in expression of the 17 kDa putative movement protein (pr17) of potato leaf roll luteovirus (PLRV). *In vitro* translation of RNAs transcribed from appropriate chimeric constructs, as well as their expression in transgenic potato plants, resulted in the expected wild-type pr17 protein, as well as in larger translational products recognized by pr17-specific antisera. Mutational analyses revealed that the extra proteins were translated by non-canonical initiation at AUU codons present in the wild-type Ω sequence. In the plant system translation initiated predominantly at the AUU codon at positions 63–65 of the Ω sequence. Additional AUU codons in a different reading frame of the Ω sequence also showed the capacity for efficient translation initiation *in vitro*. These results extend the previously noted activity of the TMV 5′ leader sequence in ribosome binding and translation enhancement in that the TMV translation enhancer can mediate non-canonical translation initiation *in vitro* and *in vivo*.**

## INTRODUCTION

Translational efficiencies of eukaryotic mRNAs are influenced by various factors, such as primary (5′-cap) and secondary (hairpin) structures, the sequence context of the start codon or upstream regulatory elements, such as enhancer sequences or small upstream open reading frames (uORFs) (1–7). While for their specific interaction with ribosomes and for start codon recognition prokaryotic mRNAs make use of the Shine–Dalgarno sequence (8), the lack of a corresponding sequence in eukaryotic mRNAs upstream of the start codon has led to various models for pre-initiation complexes binding to the RNA 5′-end and then scanning along the mRNA for recognition of the translational start codon(s) (7,9,10).

An additional facet of eukaryotic mRNA translation has come from the identification of 5′ untranslated sequences which largely enhance translation. Such regulatory translational enhancer sequences have been primarily documented to exist in the 5′ leader sequences of RNAs from plant and animal viruses, such as potato virus X, rous sarcoma virus, brome mosaic virus and tobacco mosaic virus (TMV) (11,12). In the case of the TMV translational enhancer (Ω) sequence (consisting of the 5′-terminal 68 nt) it has been proposed that the absence of extended secondary structures in this region causes the increase in translational efficiency (13). In fact, a detailed analysis of the TMV (strain U1) Ω sequence pointed to the importance of the primary structure by identifying two elements, a direct repeat of 8 nt and a CAA-rich region, as being responsible for translation enhancement (14). In line with previous observations that the Ω sequence is capable of promoting binding of two ribosome molecules (disome formation) when elongation is blocked in the presence of sparsomycin (15,16), it was proposed that the core regulatory elements of the Ω sequence allow specific binding of a protein factor(s) required for efficient initiation (14). In the disome complex one of the two ribosomes occupies the AUG start codon of the replicase gene and the second was postulated to bind further upstream in the Ω sequence (TMV strain SPS) at an AUU codon in position 14 ($AUU_{14}$). Translation initiation at this AUU codon was proposed to occur (16), but with appropriate chimeric constructs consisting of the TMV (strain U1) translational enhancer in-frame with the AUG start codon of a reporter gene putative initiation at the corresponding AUU codon ($AUU_{15}$) did not contribute to increased reporter gene activity (14).

Initiation at non-AUG codons was originally proposed from experiments using synthetic oligonucleotides (17). Furthermore, usage of AUU as a translational start was postulated for human mitochondrial mRNA (18), but the first evidence for involvement of AUU as a start codon was described for the *Escherichia coli* gene encoding initiation factor IF3 (19). Since then further evidence for eukaryotic translation initiation at AUU and other codons has accumulated for animal and plant systems (10,20–22). Here we show that the TMV translational enhancer sequence can promote alternative translation initiation at AUU codons.

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## MATERIALS AND METHODS

### Construction of plasmids for plant transformation

The potato leaf roll luteovirus (PLRV) *pr17* (ORF4) gene was amplified by PCR from clone pCPL1 (23). The primers were designed to give unique restriction sites for *Spe*I and *Xba*I at the 5′- and 3′-ends respectively. A plasmid previously constructed for high level expression of the PLRV capsid protein CP (ORF3) controlled by the Ω sequence and the 35S promoter of cauliflower mosaic virus (CaMV) (24) was cut with *Spe*I and *Xba*I to remove the ORF3 N-terminal sequence. Subsequently the amplified ORF4 fragment was cut with *Spe*I and *Xba*I and cloned into the linearized plasmid pRT17/NIV. A *Hin*dIII fragment isolated from pRT17/NIV was cloned into the binary vector pBIN19 (25). Plasmids containing the ORF4 expression cassette were designated p17/NIV.

### Transformation of *Solanum tuberosum* and Western blot analysis

P17/NIV was transformed into *Agrobacterium tumefaciens* strain LBA 4404 (26) and stable transformation of *Solanum tuberosum* var. Desirée was performed according to published procedures (27), with the resulting agrobacteria carrying plasmid p17/NIV. Western blot analysis of regenerated plants was carried out as described in Tacke *et al.* (28).

### Plasmid construction for *in vitro* analysis of the Ω sequence

A *Hin*dIII fragment comprising the Ω sequence and ORF4 was isolated from plasmid p17/NIV and cloned into plasmid pSP64 (Promega) under the control of the SP6 promoter (pS17N). Mutations in the Ω sequence were carried out by PCR using synthetic oligonucleotides (synthesized on a DNA/RNA synthesizer 392; Applied Biosystems, Darmstadt, Germany) and plasmid pS17N as the template. The upstream primer was located 5′ of the SP6 promoter (position 2105 of pSP64), comprising a unique *Ssp*I restriction site. This oligonucleotide was combined with a set of downstream primers complementary to the Ω sequence and bearing different point mutations and a *Ksp*I restriction site. Amplified fragments covered the SP6 promoter and the mutated Ω sequence. These PCR fragments were subsequently cut with *Ssp*I and *Ksp*I and cloned into pS17N. Plasmids containing mutated forms of the Ω sequence were sequenced on a DNA Sequencer 373A (Applied Biosystems).

### *In vitro* transcription/translation

All pSP64-based plasmids were linearized downstream of ORF4 with *Eco*RI prior to *in vitro* transcription with SP6 RNA polymerase in the presence of the cap analogue $m^7$GpppG (29). RNAs were translated either in a wheat germ extract or rabbit reticulocyte lysate (Amersham Buchler) in the presence of [$^{35}$S]methionine under conditions recommended by the supplier. *In vitro* products were analysed on 12.5% SDS–polyacrylamide gels and detected by fluorography (30).

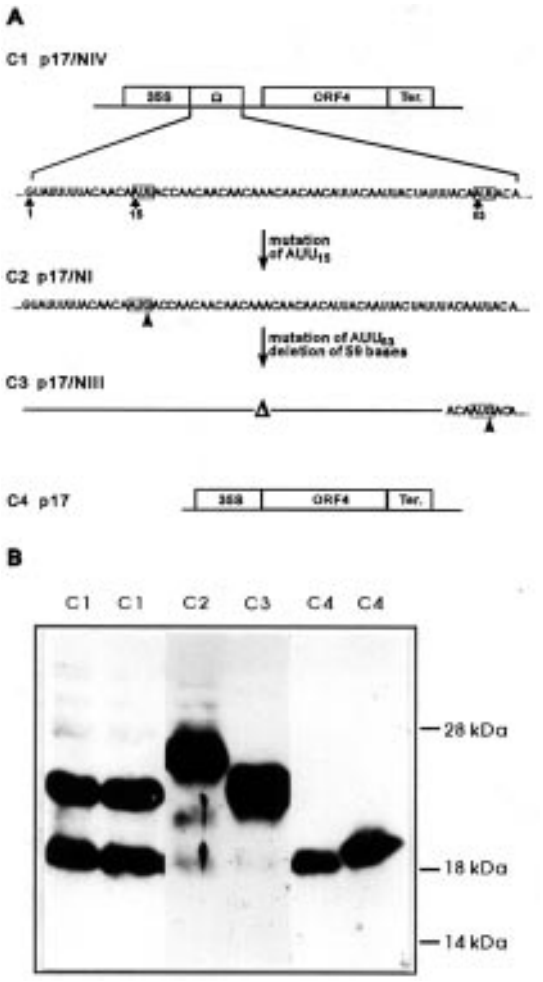


**Figure 1.** Western blot analysis of potato plants transformed with ORF4 from potato leaf roll virus. (**A**) Schematic representation of the expression cassette used for high level expression of ORF4 *in planta*. Two AUU codons ($AUU_{15}$, $AUU_{63}$) of the Ω sequence which are located in-frame with the ORF4 start codon are indicated by open boxes. Mutation of $AUU_{15}$ and $AUU_{63}$ to AUG in constructs p17/NI and p17/NIII are indicated by arrowheads. In construct p17/NIII the first 59 nt of the Ω sequence were deleted to avoid initiation upstream of $AUG_{63}$. Construct p17 without the Ω sequence was taken as a control, indicating the molecular weight of wild-type pr17. (**B**) Western analysis of protein extracts from two independent transgenic potato lines transformed with construct p17/NIV (loaded in lanes indicated with C1), p17/NI (C2) and p17/NIII (C3). In C4 protein extracts of plants expressing ORF4 without the Ω sequence are loaded. Proteins were separated by PAGE and processed for immunological detection as described before (28).

## RESULTS

### Analysis of ORF4 transgenic plants

Potato leaf discs were transformed with construct p17/NIV (Fig. 1A) and transgenic lines carrying two or more copies of the transgene were recovered. Western blot analysis of extracts from all independent transformants detected the wild-type pr17 and an additional immunoreactive protein (pr17/n) with an apparent molecular weight of 24 kDa (Fig. 1B, C1). This pr17/n protein was not detected in PLRV-infected plants (28) nor in transgenic plants expressing ORF4 without the Ω sequence (Fig. 1A and B,

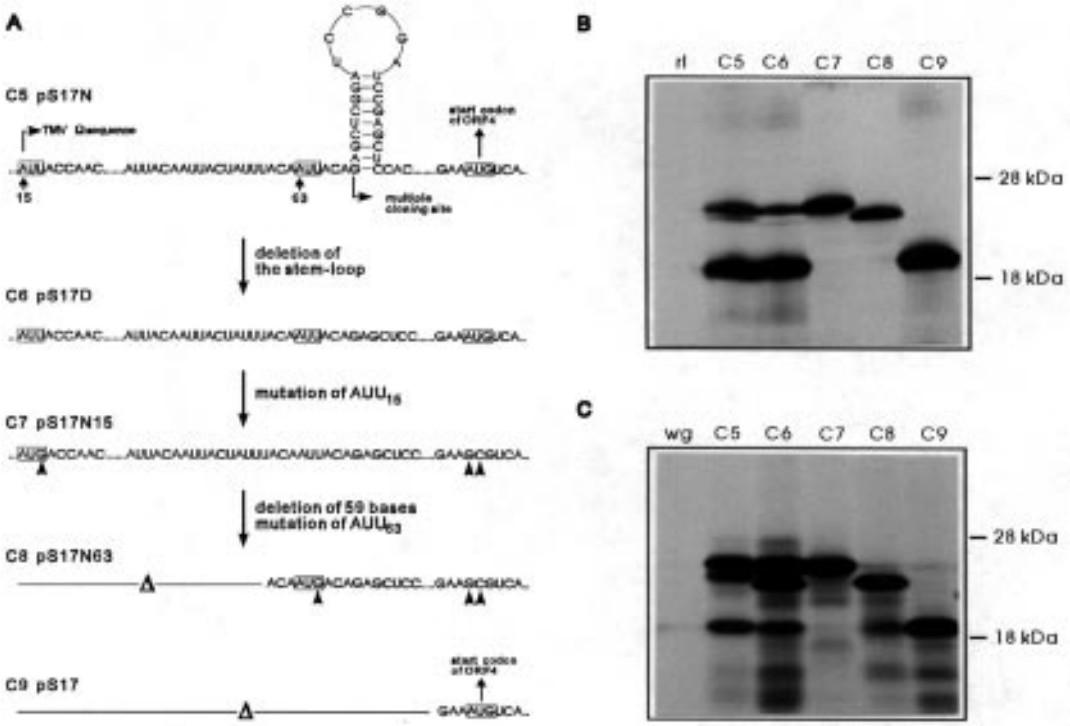


**Figure 2.** Influence of a putative stem–loop structure on translation initiation. (**A**) Part of the Ω sequence and a potential stem–loop structure 3 nt downstream of $AUU_{63}$ in the multiple cloning site is shown. The AUG start codon of ORF4 and two potential sites of translation initiation ($AUU_{15}$ and $AUU_{63}$) in the Ω sequence are marked by open boxes. In construct pS17D the stem–loop was deleted. Arrowheads indicate the mutation of $AUU_{15}$, $AUU_{63}$ and of the ORF4 AUG start codon in constructs pS17N15 and pS17N63. The 5′ located 59 nt of the Ω sequence were deleted in construct pS17N63. Construct pS17 without the Ω sequence served as a control for the synthesis of wild-type pr17. (**B** and **C**) *In vitro* translation of chimeric RNAs. RNAs from all constructs were translated *in vitro* using a reticulocyte lysate (B) or a wheat germ extract (C) and separated by PAGE. Control lanes are designated rl (reticulocyte lysate) or wg (wheat germ extract) respectively and represent*in vitro* translation products in the absence of externally added RNAs. C5–C9 correspond to the constructs shown in (A).

C4). Recloning and sequencing of the transgenes from a potato line containing two transgene copies revealed identical sequences for the transcribed and translated regions (data not shown). Together with the fact that a single copy line established at later stages also showed the same two immunoreactive proteins and that, moreover, transgenic lines expressing ORF4 without translational enhancer did not show the larger immunoreactive protein pr17/n, these data indicate that formation of pr17/n was possibly a result of alternative translation initiation at a non-AUG codon in the Ω wild-type sequence, thereby giving rise to an N-terminally elongated protein.

Inspection of the transgene sequence revealed that two AUU codons ($AUU_{15}$ and $AUU_{63}$) of the Ω sequence were in-frame with the ORF4 AUG start codon (Fig. 1A). To assess the size of a protein that would initiate in the Ω sequence two constructs were synthesized by site-directed mutagenesis in which the pr17 start codon was converted to GCG and the AUU codons $AUU_{15}$ and $AUU_{63}$ of the TMV Ω sequence were mutated to $AUG_{15}$ and $AUG_{63}$ respectively (constructs p17/NI and p17/NIII; Fig. 1A). Both constructs were used for transformation of *S.tuberosum* and protein extracts from leaves of regenerated plants were subjected to Western blot analysis (Fig. 1B). Plants transformed with construct p17/NI expressed a protein larger than pr17/n (Fig. 1B, C2), whereas p17/NIII transgenic plants showed a protein corresponding in size to pr17/n, as detected in p17/NIV transgenic plants (Fig. 1B, C3). It appears that expression of p17/NI and p17/NIII in transgenic plants (Fig. 1B, C2 and C3) resulted in much higher protein levels as compared with p17 transgenic plants (Fig. 1B, C4). As p17 and p17/NIII did not contain the Ω sequence, this observation was explained by the unfavourable context of the pr17 initiator codon (GGAAAUGUCA). These data provided the first evidence that translation initiation can occur in the Ω sequence and suggested a preferential translation initiation at $AUU_{63}$. A further, more detailed analysis of potential translation start codons was carried out *in vitro*.

## A stem–loop structure does not contribute to translation initiation in the Ω sequence

The 5′ leader of construct p17/NIV consisted of the TMV Ω sequence and an additional 63 nt derived from the multiple cloning site. Due to the cloning strategy part of this cloning site was inversely repeated, allowing the formation of a stable stem–loop structure (Fig. 2A). This stem–loop is located 3 nt downstream of codon $AUU_{63}$ and could have made a substantial contribution to the signal for translation initiation. In order to investigate the possible effect of this stem–loop on translation efficiency a *Hin*dIII fragment released from construct p17/NIV and comprising the complete 5′ leader and ORF4 (pr17) sequence was cloned under the control of the SP6 promoter into vector pSP64 (construct pS17N). Furthermore, the stem–loop was deleted to yield plasmid pS17D. RNAs from both constructs were transcribed *in vitro* and translated in a rabbit reticulocyte lysate, as well as in a wheat germ extract.

Translation in both cell-free systems resulted in expression of wild-type pr17 and two additional proteins, one identical in size to the pr17/n protein detected *in planta* (Fig. 2B, C; see above). The two additional proteins corresponded in size to polypeptides synthesized by initiation at the $AUG_{15}$ or $AUG_{63}$ codons respectively of RNAs from constructs pS17N15 and pS17N63 (Fig. 2). Thus *in vitro* expression of construct pS17N permitted translation initiation at more than one non-AUG codon, probably at $AUU_{15}$ and $AUU_{63}$. *In vitro* translation of construct pS17D also showed expression of two additional proteins. It was concluded, therefore, that the putative stem–loop structure was not necessary *in vitro* for translation initiation in the Ω sequence (Fig. 2B and C).

The expression level for the two extra proteins differed in the animal (reticulocyte lysate) and plant (wheat germ extract) *in vitro* translation systems. The 24 kDa protein was more prominently expressed in the wheat germ system, which thereby reflected the actual *in planta* situation (Fig. 1B). The reticulocyte lysate predominantly expressed the 26 kDa protein. Therefore, the wheat germ system was selected for further *in vitro* experiments.

## Translation in the wheat germ system initiates at $AUU_{63}$ of the TMV Ω sequence

*In vitro* expression of the chimeric Ω–ORF4 construct (constructs pS17N and pS17D) indicated that translation initiation occurred at two non-AUG codons of the Ω sequence upstream of the ORF4 AUG start codon. In addition, translation of pS17N15 and pS17N63 RNAs provided circumstantial evidence for initiation at codons $AUU_{15}$ and $AUU_{63}$ respectively. Further analyses were directed at unequivocally identifying the non-AUG initiator codon in the Ω sequence utilized *in planta*. The most likely non-AUG codon recognized by the plant ribosomal initiation complex was $AUU_{63}$, as transgenic plants transformed with construct p17/NIII ($AUG_{63}$) expressed a protein corresponding in size to pr17/n.

The $AUU_{63}$ codon of plasmid pS17D was mutated to $AGG_{63}$ in order to inhibit translation initiation at this codon (construct pS17D3; Fig. 3A). In fact, *in vitro*-translated RNA of plasmid pS17D3 did not result in a product corresponding in size to pr17/n, demonstrating that *in vitro* translation initiated at $AUU_{63}$ of the Ω sequence (Fig. 3B, C12). Based on the results of Gordon (21) and Peabody (22), $AUU_{63}$ was further mutated to ACG or CUG (constructs pS17D1 and pS17D2 respectively; Fig. 3A). These codons are known to permit translation initiation with high efficiency in mammalian cells and plant protoplasts. Similar results were obtained with the mutated $AUU_{63}$ codon, as *in vitro* translation of pS17D1 and pS17D2 RNAs in the wheat germ system allowed expression of pr17/n by initiation at both $ACG_{63}$ and $CUG_{63}$ (Fig. 3B).

The potential mechanism by which translation initiated at both $AUU_{63}$ (to yield pr17/n) and at the wild-type ORF4 AUG start codon (pr17 synthesis) was examined by converting $AUU_{63}$ into $AUG_{63}$ (pS17D4; Fig. 3B, C13). The fact that $AUG_{63}$ directed almost exclusive synthesis of pr17/n, with scarcely any pr17 formed, was taken as an indication that in the wild-type situation internal initiation at the pr17 AUG, as opposed to $AUU_{63}$ initiation, occurred by a leaky scanning mechanism.

## Influence of AUU flanking sequences on translation initiation

The flanking sequences at the $AUU_{63}$ codon largely conformed to the consensus context for plant AUG initiation codons (Fig. 4A).

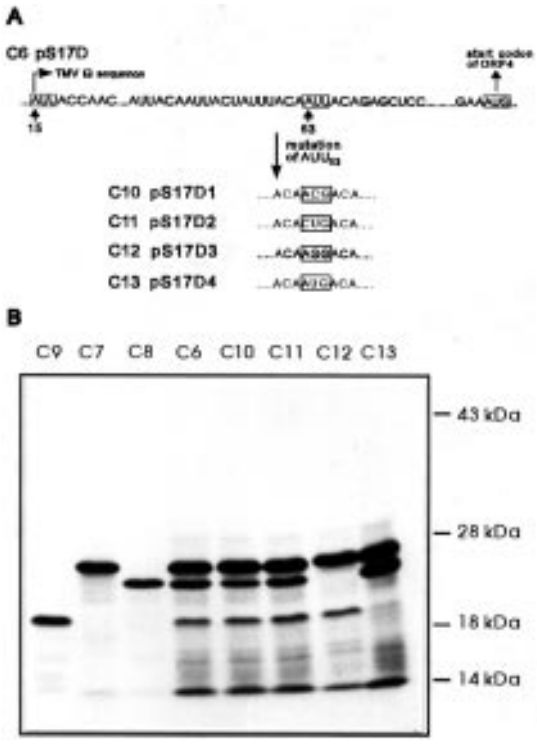


**Figure 3.** Mutational analysis of the $AUU_{63}$ translation initiation codon of the Ω sequence. (**A**) The Ω sequence and the start codon of ORF4 are shown. Based on construct pS17D four different point mutations were introduced in the $AUU_{63}$ codon to create constructs pS17D1–pS17D4. (**B**) PAGE analysis of *in vitro* translation products from RNAs of constructs ps17D (C6) and ps17D1–pS17D4 (C10–C13). Lanes indicated with C9, C7 and C8 contain *in vitro* translation products from RNAs of constructs pS17, pS17N15 and pS17N63 (see Fig. 2).

To further analyse the influence of bases neighbouring $AUU_{63}$ several point mutations were introduced into this region (Fig. 4A). Single point mutations did not alter translation efficiency at $AUU_{63}$ (the total amount of protein synthesized from construct pS17A3 RNA and loaded in lane C16 is lower as compared with total protein in the other lanes). Even the replacement of a purine by a pyrimidine at the mutation-sensitive position –3 did not inhibit expression of pr17/n (construct pS17A6, Fig. 4, C19). Only when the entire context of the $AUU_{63}$ codon was disrupted, as in pS17A7, expression of pr17/n was reduced (Fig. 4, C20). On the other hand, adaptation of the flanking sequences according to the consensus sequence did not increase translation initiation at $AUU_{63}$ as compared with the wild-type sequence (Fig. 4B, C6, C14 and C15). These results indicate that the $AUU_{63}$ flanking sequences have only a minor effect on pr17/n translation efficiency *in vitro*.

## Interaction of a triple AUU block with translation initiation at $AUU_{63}$

As the flanking sequences exhibited little activity in modulating the efficiency of translation initiation at codon $AUU_{63}$, sequences located further upstream of $AUU_{63}$ (positions 44–58 of the Ω sequence) were examined for their influence on translation initiation. An element composed of three AUU codons separated from each other by one codon ('triple AUU block') is located 4 nt upstream of $AUU_{63}$ in a different reading frame (Fig. 5A).

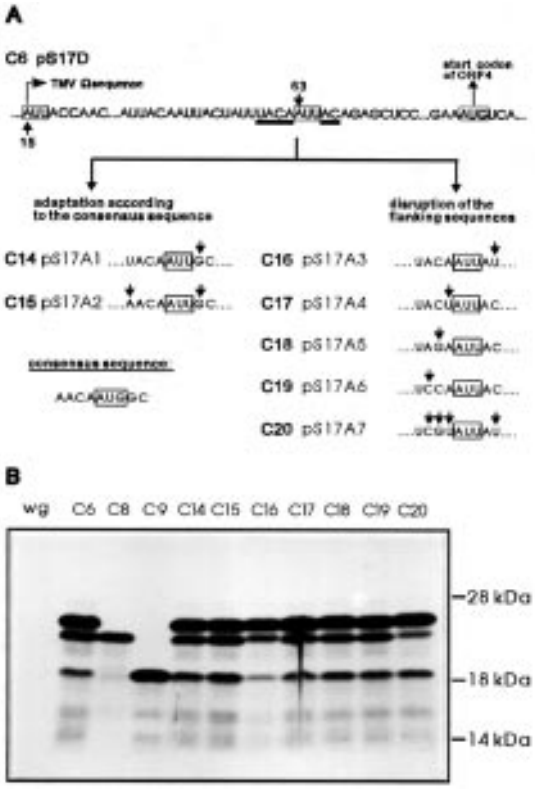


**Figure 4.** Influence of flanking sequences on translation initiation. (**A**) The flanking sequences of the $AUU_{63}$ codon are underlined. Two lines of mutations were carried out by disrupting or adapting the flanking sequences according to the consensus sequence for plant translation initiation codons (2). Single base substitutions are indicated by arrows. (**B**) PAGE analysis of *in vitro* translation products from RNAs of constructs pS17A1–pS17A7 (C14–C20). As a negative control the wheat germ extract incubated without external RNA was loaded (wg). *In vitro* translation products of construct pS17D, p17N63 and pS17 RNAs (see Fig. 2) were loaded in lanes indicated with C6, C8 and C9 respectively.

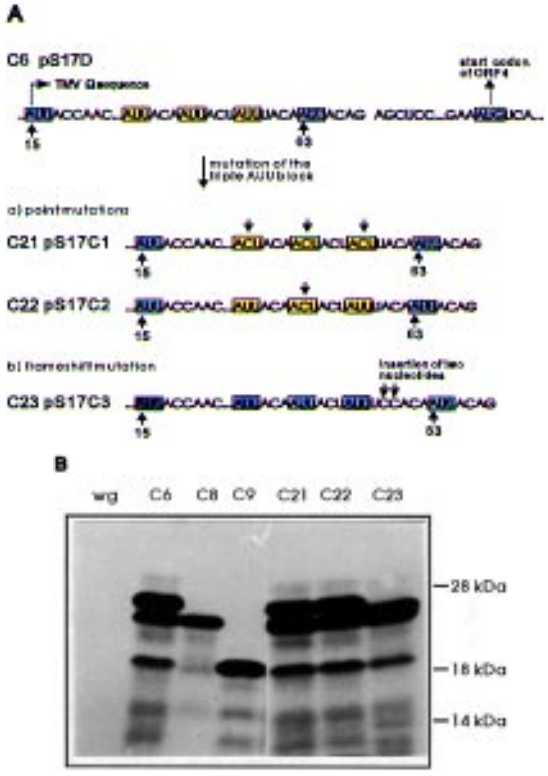


**Figure 5.** Translation initiation at a triple AUU block of the Ω sequence. (**A**) The AUG codon of ORF4 and $AUU_{15}$, as well as $AUU_{63}$, are highlighted by blue boxes, indicating the same reading frame. Different reading frames are represented by different colours. The triple AUU block is located 4 nt upstream of $AUU_{63}$, marked by yellow boxes. Point mutations and insertion of nucleotides are indicated by arrows. The altered reading frame of the triple AUU block in construct pS17C3 is represented by blue boxes. In the same construct $AUU_{15}$ is in the third reading frame, shown by a pink box. (**B**) PAGE analysis of *in vitro* translation products from RNAs of constructs pS17C1–pS17C3 (C21–C23). The controls pS17D, pS17N63 and pS17 (see Fig. 2) were loaded in lanes indicated with C6, C8 and C9.

Simultaneous mutation of all three AUU codons to ACU slightly increased expression of pr17/n (Fig. 5B, C21), whereas a point mutation of the central AUU codon to ACU had no effect on translation initiation at $AUU_{63}$ (Fig. 5B, C22). Thus the triple AUU block in the wild-type Ω sequence obviously decreased translation initiation at $AUU_{63}$ to some extent.

This observation could have resulted from translation initiation at the triple AUU block, thereby competing for ribosomal initiation complex formation with codon $AUU_{63}$. To test this possibility the triple AUU block was placed in-frame with ORF4 by the insertion of 2 nt upstream of $AUU_{63}$ (Fig. 5A, pS17C3). *In vitro* translation of the frame-shift mutant RNA resulted in a double band at 24 kDa, indicating that translation initiation had taken place at the triple AUU block as well as at $AUU_{63}$ (Fig. 5B, C23). Another frame-shift mutant was created to unequivocally demonstrate translation initiation at the triple AUU block (Fig. 6A, pS17B1). In this frame-shift mutation the triple AUU block was placed in-frame with ORF4 by inserting 2 nt into the multiple cloning site such that $AUU_{15}$ as well as $AUU_{63}$ were out-of-frame with respect to ORF4. *In vitro* translation of construct pS17B1 RNA resulted in expression of pr17 and a second protein with an apparent molecular weight of 25 kDa, as expected from translation initiation at the triple AUU block (Fig. 6B, C24). Thus the negative regulatory effect of the triple AUU block on translation initiation at the $AUU_{63}$ codon was due to competition for the scanning complex and initiation complex formation. The proteins translated from the triple AUU block (in the wild-type construct) would have calculated molecular weights of 2 kDa and were, therefore, not visible by SDS–PAGE analysis.

An additional frame-shift mutant was constructed as a negative control with all AUU codons of the Ω sequence out-of-frame with ORF4 (Fig. 6A, construct pS17B2). Translation of RNA from this construct showed an additional protein with an apparent molecular weight of 22 kDa (Fig. 6B, C25). The extra protein had a smaller apparent molecular weight than pr17/n and was possibly synthesized by initiation within the multiple cloning site (Fig. 6A). A GUG codon, the most likely initiator codon in this region, was mutated to GAG (pS17B3). Absence of the extra protein confirmed that initiation on pS17B2 RNA had occured at the GUG codon (Fig. 6B, C26). Whether GUG and the triple AUU block direct translation initiation *in planta* remains to be determined.

## DISCUSSION

Potato plants transformed with PLRV ORF4 under the translational control of the TMV Ω sequence expressed two immunoreactive

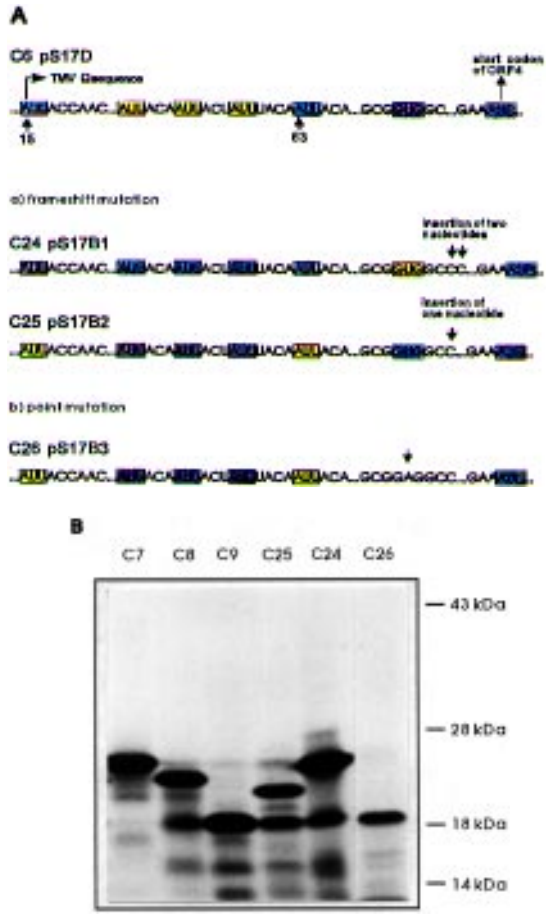


**Figure 6.** Translation initiation at a GUG codon of the 5′ untranslated leader. (**A**) Part of the Ω sequence and multiple cloning site are shown. Differently coloured boxes represent different reading frames. Blue indicates the reading frame of ORF4. Insertion or mutation of single bases are indicated by arrows. The frame-shift of the triple AUU block and initiation codons in constructs pS17B1 and pS17B2 are indicated by different colours. (**B**) PAGE analysis of *in vitro*-translated products from constructs pS17B3, pS17B1 and pS17B2 were loaded in lanes C25, C24 and C26 respectively. Controls pS17N15, pS17N63 and pS17 (see Fig. 2) were loaded in lanes C7, C8 and C9 respectively.

proteins, wild-type pr17 and mutant protein pr17/n. We were able to show that initiation at the internally located translational start codons proceeded by leaky scanning of pre-initiation complexes and that a non-canonical translation mechanism was responsible for pr17/n formation by alternative translation initiation at a non-AUG codon of the TMV translational enhancer. *In planta* and during *in vitro* translation in a cell-free plant system (wheat germ) initiation occured efficiently at the ORF4 AUG start codon, as well as some 25 codons upstream at $AUU_{63}$ of the Ω sequence. When $AUU_{63}$ was replaced by $AUG_{63}$ (construct p17/NIII) a protein corresponding in size to pr17/n was expressed in transgenic plants, but mutation of $AUU_{63}$ to $AGG_{63}$ prevented expression of this N-terminally elongated pr17 (pr17/n).

*In vitro* translation of ORF4 under the control of the Ω sequence resulted in expression of three proteins instead of the two detected in transgenic plants. This was observed both in the reticulocyte lysate and wheat germ extract: translation initiated additionally at $AUU_{15}$, as is obvious from a mutant RNA in which $AUU_{15}$ had been replaced by $AUG_{15}$. Differences in the expression patterns for both cell-free systems could probably reflect conditions of the *in vitro* translation systems which allow translation initiation at a non-AUG codon upstream of $AUU_{63}$ not recognized *in planta*. In addition, it is noteworthy that the animal and plant *in vitro* systems show different affinities for the two codons $AUU_{15}$ and $AUU_{63}$. The fact that $AUU_{15}$ is predominantly used by the reticulocyte lysate for translation intiation does not reflect preferences of the animal system for a different consensus context of this AUU start codon, as the flanking sequences for $AUU_{15}$ and $AUU_{63}$ are identical. Although artefacts of the conditions of the *in vitro* translation cannot be excluded, animal-specific protein factors may be involved in mRNA interaction and specific recognition of the first initiator codon, a phenomenon recently discussed in detail for eukaryotic gene expression (7).

Further analyses of the Ω sequence focused on elements contributing to translation initiation at $AUU_{63}$. Optimal initiation of protein biosynthesis depends on the sequence context for the start codon (1–4), which is different in plant and animal consensus sequences. However, in both systems positions –3 and +4, with reference to the +1 adenosine of the AUG start codon, require purine residues for efficient translation initiation (31). According to Cavener and Ray (32) the flanking sequences of mono- and dicotyledonous plants differ substantially. As the experiments described here were carried out in a wheat germ system we cannot exclude that in *S.tuberosum* the point mutations in the flanking sequences would exert a more prominent effect on translational efficiency. The data presented here on the AUU flanking sequences confirm their importance for optimal protein initiation, but mutation of the entire consensus sequence did not completely inhibit translation initiation. While mutation of a purine to a pyrimidine residue at position –3 did not apparently alter initiation efficiency at $AUU_{63}$, the triple AUU block preceding this codon reduces its efficiency in initiation. As was shown by site-directed mutagenesis, the triple AUU block may itself interact with the scanning complex, forming initiation complexes and thereby competing with $AUU_{63}$. In fact, leaky scanning is obviously the mechanism by which recognition of start codons occurs in the TMV Ω sequence. When $AUU_{63}$ was mutated to $AUG_{63}$ expression of pr17 at the AUG of ORF4 was barely detectable, indicating that the canonical AUG start codon at position 63 was now almost exclusively used for formation of initiation complexes.

The translation initiation at AUU codons described here is a novel feature of the Ω sequence, in addition to its function as a translational enhancer. Previously a detailed analysis of the Ω sequence had identified two motifs necessary for translation enhancement, a $(CAA)_n$ region and a direct repeat of 8 nt (12). Both the $AUU_{15}$ and $AUU_{63}$ codons are part of this direct repeat ACAAUUAC. Based on the observation of disome formation in the Ω sequence, translation initiation at $AUU_{15}$ was proposed (16,33) as contributing to enhancement of translation. However, mutation of $AUU_{15}$ to $CUU_{15}$ in the 5′ located direct repeat or the introduction of two stop codons further downstream demonstrated that translation initiation at $AUU_{15}$ did not contribute to enhancement by the Ω sequence (14,34). The stop codons were introduced in a construct where the downstream direct repeats (comprising $AUU_{63}$ and part of the triple AUU block) were deleted. Thus a potential contribution to enhancement by translation initiation at $AUU_{63}$ could not be assessed.

Alternative translation initiation at $AUU_{63}$ of the TMV Ω sequence, as well as leaky scanning and initiation at the canonical ORF4 AUG start codon from the identical mRNA, resulted in

expression of two proteins. Such bifunctional mRNAs are known for a number of other viruses and eucaryotic mRNAs (6,20) and may lead to N-terminally altered proteins with modified functions, as for example in the expression of two N-terminally different serine-threonine protein kinases encoded by the mouse *pim-1* oncogene or the translation of three proteins (C′, C and Y) from Sendai virus RNA by exploiting an ACG and two different AUG codons (35,36). Our results indicate that translation initiation at $AUU_{63}$ of the Ω sequence takes place with high efficiency *in planta*, with both pr17/n and wild-type pr17 accumulating to similar levels in transgenic plants. The question remains whether the TMV Ω sequence directs expression of two N-terminally different proteins from TMV RNA. In TMV RNA the Ω sequence is followed by the polymerase gene and initiation at $AUU_{63}$ of TMV strain U1 would extend the viral polymerase by only two amino acid residues. The Ω sequences of other TMV strains (U2, L and Dahlemense) are slightly different and the AUU codons corresponding in position to $AUU_{63}$ of TMV U1 are not in-frame with the polymerase gene. Hence, eventual expression of an N-terminally modified polymerase protein would not be a conserved feature of different TMV strains.

As an alternative to the production of an N-terminally modified viral replicase the small uORFs starting at AUU codons of the Ω sequence could represent a regulatory mechanism for TMV gene expression, as they would decrease the number of ribosomes initiating at the AUG start codons of the polymerase gene, either by direct competition through the formation of initiation complexes or as a consequence of poor re-initiation of eukaryotic ribosomes subsequent to termination at the uORF stop codons. The efficiency of uORF translation may be regulated during TMV replication by interaction of this sequence with protein factors of the host cell, like eIF-2, which is involved in initiation site recognition and stabilization of tRNA–mRNA interactions (37). However, virus encoded proteins, like CaMV *trans*-activator protein (38), may also function in modulation of translational efficiency and it remains to be determined whether TMV proteins make use of this mechanism for regulation of TMV gene expression during the late stages of replication, when genomic TMV RNA is preferentially assembled into progeny virus particles.

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## REFERENCES

1 Kozak,M. (1986) *Cell*, **44**, 283–292.
2 Lütcke,H.A., Chow,K.C., Mickel,F.S., Moss,K.A., Kern,H.F. and Scheele,G.A. (1987) *EMBO J.*, **6**, 43–48.
3 Grünert,S. and Jackson,R.J. (1994) *EMBO J.*, **13**, 3618–3630.
4 Boeck,R. and Kolakofsky,D. (1994) *EMBO J.*, **13**, 3608–3617.
5 Kozak,M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2850–2854.
6 Gallie,D.R. (1993) *Annu. Rev. Plant Physiol. Plant Biol.*, **44**, 77–105.
7 McCarthy,J.E.G. and Kollmus,H. (1995) *Trends Biochem. Sci.*, **20**, 191–197.
8 Shine,J. and Dalgarno,L. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 1342–1346.
9 Kozak,M. (1980) *Cell*, **22**, 7–8.
10 Kozak,M. (1983) *Microbiol. Rev.*, **47**, 1–45.
11 Smirnyagina,E.V., Morozov,S.Y., Rodionova,N.P., Miroshnichenko,N.A., Solovyev,A.G., Fedorkin,O.N. and Atabekov,J.G. (1991) *Biochimie*, **73**, 587–598.
12 Gallie,D.R., Sleat,D.E., Watts,J.W., Turner,P.C. and Wilson,T.M.A. (1987) *Nucleic Acids Res.*, **15**, 8693–8711.
13 Lawson,T.G., Ray,B.K., Dodds,J.T., Grifo,J.A., Abramson,R.D., Merrick,W.C., Betsch,D.F., Weith,H.L. and Thach,R.E. (1986) *J. Biol. Chem.*, **261**, 13979–13989.
14 Gallie,D.R. and Walbot,V. (1992) *Nucleic Acids Res.*, **20**, 4631–4638.
15 Konarska,M., Filipowicz,W., Domdey,H. and Gross,J. (1981) *Eur. J. Biochem.*, **114**, 221–227.
16 Tyc,K., Konarska,M., Gross,J. and Filipowicz,W. (1984) *Eur. J. Biochem.*, **140**, 503–511.
17 Both,G.W., Furuichi,Y., Muthukrishnan,S. and Shatkin,A.J. (1976) *J. Mol. Biol.*, **104**, 637–658.
18 Montoya,J., Ojala,D. and Attardi,G. (1981) *Nature*, **290**, 465–470.
19 Sacerdot,C., Fayat,G., Dessen,P., Springer,M., Plumbridge,J.A., Grunberg-Manago,M. and Blanquet,S. (1982) *EMBO J.*, **1**, 311–315.
20 Rohde,W., Gramstat,A., Schmitz,J., Tacke,E. and Prüfer,D. (1994) *J. Gen. Virol.*, **75**, 2141–2149.
21 Gordon,K., Fütterer,J. and Hohn,T. (1992) *Plant J.*, **2**, 809–813.
22 Peabody,D.S. (1989) *J. Biol. Chem.*, **264**, 5031–5035.
23 Tacke,E., Sarkar,S., Salamini,F. and Rohde,W. (1989) *Arch. Virol.*, **105**, 153–163.
24 Tacke,E., Kull,B., Prüfer,D., Reinold,S., Schmitz,J., Salamini,F. and Rohde,W. (1995) In Bills,D.D. and Kung,S.D. (eds), *Viral Pathogenesis and Disease Resistance*. World Scientific Publishing, River Edge, in press.
25 Bevan,M. (1984) *Nucleic Acids Res.*, **12**, 8711–8721.
26 Hoekema,A., Hirsch,P., Hooykaas,P. and Schilperoort,R.A. (1983) *Nature*, **303**, 179–180.
27 Horsch,R.B., Fry,J.E., Hoffmann,N.C., Eichholtz,D., Rogers,S.G. and Fraley,R.T. (1985) *Science*, **227**, 1229–1231.
28 Tacke,E., Schmitz,J., Prüfer,D. and Rohde,W. (1993) *Virology*, **197**, 274–282.
29 Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
30 Bonner,W.M. and Laskey,R.A. (1974) *Eur. J. Biochem.*, **46**, 83–88.
31 Kozak,M. (1989) *J. Cell Biol.*, **108**, 229–241.
32 Cavener,D.R. and Ray,S.C. (1991) *Nucleic Acids Res.*, **19**, 3185–3192.
33 Filipowicz,W. and Haenni,A.L. (1979) *Proc. Natl. Acad. Sci.* USA, **76**, 3111–3115.
34 Gallie,D.R., Sleat,D.E., Watts,J.W., Turner,P.C. and Wilson,T.M.A. (1988) *Nucleic Acids Res.*, **16**, 883–893.
35 Saris,C.L.M., Domen,J. and Berns,A. (1991) *EMBO J.*, **10**, 655–664.
36 Curran,J. and Kolakofsky,D. (1988) *EMBO J.*, **7**, 245–251.
37 Donahu,T.F., Cigan,A.M., Pabich,E.K. and Valavicius,B.C. (1988) *Cell*, **54**, 621–632.
38 De Tapia,M., Himmelbach,A. and Hohn,T. (1993) *EMBO J.*, **12**, 3305–3314.