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QUARTERLY

Studies on the translation mechanism of subgenomic RNA of potato leafroll virus*

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The expression of open reading frames located on the subgenomic RNA (sgRNA) has been studied in an *in vitro* transcription and translation system. The obtained results indicate: a) translation of sgRNA occurs according to the scanning model since the insertion of a palindrome (ΔG_0 = –61 kcal/mol) prevents the initiation of translation; b) ORF6 is translated by suppression of the stop codon separating ORF4 from ORF6 and the presence of suppressor tRNA is necessary for the readthrough; c) the presence of leader sequence of sgRNA (212 nucleotides) decreases the translation efficiency of ORFs located downstream and it affects the ratio of products of ORF4 and ORF5; d) 3'UTR does not influence on an expression of genes located on the sgRNA.

The genome of potato leafroll luteovirus (PLRV) is a single stranded (ss) nonpolyadenylated RNA of approx. 5.9 kb which has a small genome-linked protein (VPg) at its 5' end. Sequence analysis has revealed the presence of six open reading frames (ORF) in two gene clusters separated by a small intercistronic region (Fig. 1).

PLRV employs a variety of mechanisms to express its genetic information. Study of these mechanisms in naturally infected tissues is complicated by the restriction of PLRV to phloem tissue and various artificial systems have been used to overcome this limitation.

The first three ORFs, (ORF0, ORF1 and ORF2) are translated from the genomic RNA (gRNA), whereas the other three ORFs (ORF3, ORF4 and ORF5) are translated from the subgenomic RNA (sgRNA) which is detected only in infected cells [1, 2]. However, translation mechanisms of genes located on PLRV sgRNA have not been studied in *in vitro* systems. Bahner *et al.* [3] infected tobacco protoplasts with PLRV in order to investigate readthrough of the coat protein termination codon and Tacke *et al.* [4] used β-glucuronidase (GUS) reporter gene constructs to study translation of ORF3 (coat protein, CP) and ORF4 (17K protein) in protoplasts. Their constructs contained sequences with either the CP AUG or 17K AUG in frame.

The aim of this work is to study *in vitro* the expression of ORFs located on the sgRNA and to evaluate the influence of the leader

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Abbreviations: CP, coat protein; gRNA, genomic RNA; LS, leader sequence; ORF, open reading frame; PLRV, potato leafroll luteovirus; RRL, rabbit reticulocyte lysates; sgRNA, subgenomic RNA; 17K, M_r 17000 protein.

sequence (LS) of the sgRNA on the translation efficiency of the downstream ORFs.

MATERIALS AND METHODS

Enzymes, reagents and programmes. All reagents used for cloning were from Gibco BRL. Rabbit reticulocyte lysates (RRL) and wheat germ extracts (WGE) for *in vitro* translation were purchased from Boehringer Mannheim. [^{35}S]Methionine was from Amersham. Taq polymerase, T7 RNA polymerase, RNasin RNase inhibitor and RQ DNase were from Promega. The oligonucleotides were synthesised on a Pharmacia LKB Assembler Plus. Polymerase chain reactions (PCR) were performed using a Perkin Elmer Cetus Thermal Cycler. The quantification of translation products was performed using the ImageQuant programme.

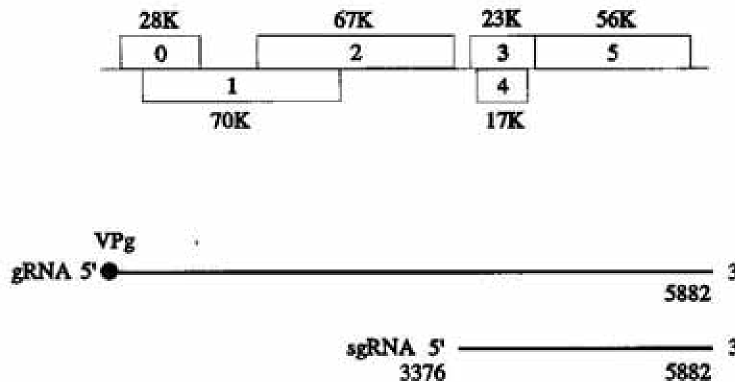


Figure 1. The PLRV genome organisation.

Open reading frames are designated by numbers in open boxes and the predicted relative molecular masses of these proteins are given. The viral genome-linked protein (●, VPg) is shown at the 5' end of the genomic RNA. The subgenomic RNA starts at position 3376.

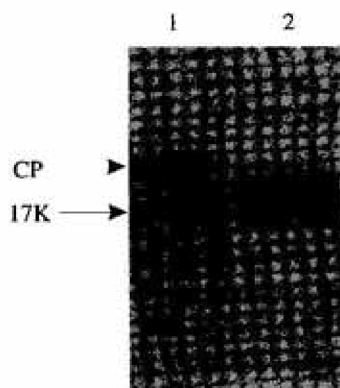


Figure 2. Translation products of tWT.

Lane 1 presents proteins synthesised from tWT/*Hind* III and lane 2 presents proteins synthesised from tWT/*Bam*HI. CP and 17K are coat protein and M_r 17000 protein, respectively.

Plasmid constructions. DNA manipulations were performed essentially as described by Sambrook *et al.* [5], except where indicated otherwise. All plasmids were cloned in *Escherichia coli* (strain DH5αF′). All nucleotide numbering refers to the PLRV-P genome [6]. In descriptions of all plasmids and transcripts the letters p and t, respectively were used. The pFCK plasmid containing the cDNA copy of PLRV-P from nucleotide 1 to 5882 was constructed previously [7] and served as a template for amplification in PCR reactions of viral cDNA fragments used for construction of plasmids. All primers used for amplification of viral cDNA fragments are listed in Table 1. The constructed plasmids are listed in Table 2.

The leader sequence of pWT was modified by two-step polymerase chain reaction mutagenesis by introduction of a palindrome containing a unique *Xho*I restriction site. Using two primers, P1 and P6, a 171 bp long DNA fragment was amplified and digested by *Kpn*I and *Xho*I restriction enzymes. Using two other primers, P7 and P4, a 972 bp long DNA fragment was amplified and digested by *Xho*I and *Sph*I restriction enzymes (see Table 1). Both fragments were gel purified and introduced into pUC118 vector, previously cleaved with *Kpn*I and *Sph*I. The resulting plasmid was designated **pWT** (Fig. 3A, B).

In vitro transcription and translation was performed as described by Kujawa *et al.* [8]. The products of translation were scanned by Phosphoimager from Molecular Dynamics Corporation.

RESULTS

Figure 3A shows the map of the constructed plasmid pWT from which transcripts encod-

Table 1. List of primers used for amplification of viral cDNA fragments

Primers	Coordinating numbers	Restriction sites	Sequence*
P1	3376-3395	*Kpn*I	5′-GG*GGTACC*TAATACGACTCACTATAG-**ACAAAAGAACACTGAAGGAG**-3′
P2	3476-3498	*Kpn*I	5′-GG*GGTACC*TAATACGACTCACTATAG-**TATAAATTCTTAGCGGGATTTG**-3′
P3	3572-3593	*Kpn*I	5′-GG*GGTACC*TAATACGACTCACTATAG-**GTGCGATCAATTGTTAATGAG**-3′
P4	4441-4457	*Sph*I	5′-**GACGGCTTGCATGCTCG**-3′
P5	5862-5882	*Cla*I, *Sca*I	5′-**GG*ATCGATAGTACT*ACACAACCCTGTAAGAGG**-3′
P6	3516-3499	*Xho*I	5′-GG*CTCGAG*CCGCCACCACGCGCCC*CGT-CGAC***GCGGATGAGAATCCTAAAG**-3′
P7	3517-3536	*Xho*I	5′GG*CTCGAG*CCGCCACCACGCGCCC*CGT-CGAC***AATCCCATTTTCAGTAGCC**-3′

*The sequences for the creation of the restriction sites (rs) are presented in italics, sequences added at the 5′ ends of primers to allow better cleavage by restriction enzymes are in normal letters, the PLRV-P sequences are indicated by bold letters and the T7 promoter is underlined.

ing ORFs located on sgRNA can be transcribed (the pWT plasmid was linearised by *Hin*dIII, this restriction site is in the polylinker). The resulting transcript has the precise 5′ end of the sgRNA that mapped to base 3376 at the PLRV genomic RNA and it has the truncated 3′ end at 4457 nucleotide position. Upon translation in RRL, both products of ORF3 (CP) and ORF4 (17K) were synthesised. To verify which bands correspond to the coat protein (23K) and 17K protein, pWT was linearised with *Bam*HI. Translation of the resulting tWT/*Bam*HI was expected to yield the CP protein shorter by 15 amino acids since the restriction site of this enzyme is located behind 17K gene but inside the coat protein gene. The size of the 17K protein remained unchanged Fig. 2, whereas the shortened 23K protein migrated faster. Consequently the band migrating as a 23K protein corresponds to the coat protein, and the 17K band corresponds to the 17K protein.

The effect of a palindrome on the translation of genes from sgRNA

To determine whether sgRNA is translated due to scanning model [9] or the internal

Table 2. List of constructs used to study expression of genes located on the sgRNA PLRV

Plasmid name	A	B	C	D
pWT	P1–P4	1081 nt	3376–4457	*Kpn*I, *Sph*I
pHWT	P2–P4	981 nt	3476–4457	*Kpn*I, *Sph*I
pAWT	P3–P4	885 nt	3572–4457	*Kpn*I, *Sph*I
pSG	P1–P5	2506 nt	3376–5882	*Kpn*I, *Cla*I
pHSG	P2–P5	2406 nt	3476–5882	*Kpn*I, *Cla*I
pASG	P3–P5	2310 nt	3572–5882	*Kpn*I, *Cla*I
pPWT*	P1–P6, P7–P4	1120 nt	3376–4457	*Kpn*I, *Xho*I, *Sph*I

A, primers used for amplification of cDNA; B, length of amplified cDNA fragments; C, coordinates of nucleotides according to PLRV-P isolate; D, restriction sites used for cloning cDNA; *, construction of pPWT is described in "Materials and Methods".

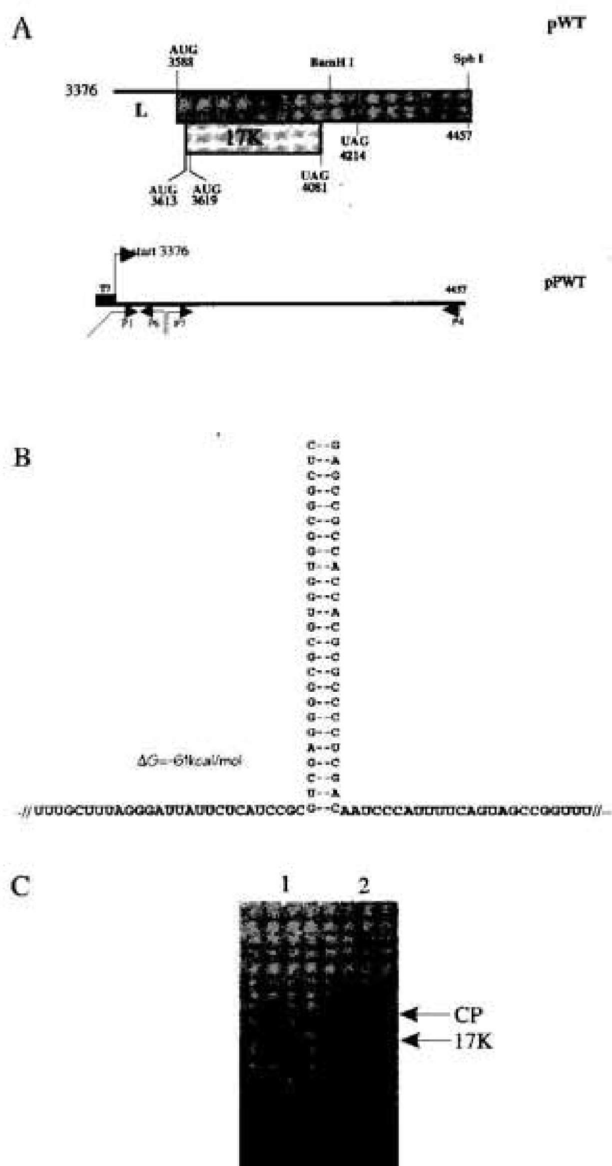


Figure 3. The effect of the palindrome on the translation of genes from sgRNA.

A. Schematic representation of sgRNA truncated from the 3′ end and the strategy of construction of pPWT. Shaded boxes represent open reading frames; black box represents T7 promoter; L, leader sequence of sgRNA; P1, P4, P6 and P7, primers used for amplification (see Table1). The Figure is not in scale. B. Nucleotide sequence and structure of the palindrome introduced into leader sequence. Letters in bold represent nucleotides of palindrome. Energy of palindrome was calculated according to the Zuker programme [25]. C. Products of translation of tPWT (lane 1) and tWT (lane 2).

initiation model [10, 11] a palindrome with a strong secondary structure ($\Delta G_0 = -61$ kcal/mol) was inserted into the leader sequence (see Methods). Some papers show that introduction of a palindrome before the initiation codon may prevent translation of genes located downstream if translation occurs according to the scanning model [9]. The schematic construction of the pPWT is presented in Fig. 3.

After linearisation by *Hin*dIII pWT and pPWT were transcribed and translated *in vitro*.

The lack of translation products of tPWT indicates (Fig. 3C, lane 1) that the presence of the palindrome prevents translation of genes located downstream.

Mechanism of the ORF5 expression

This lack of an initiator codon in ORF5 and its separation from ORF3 by stop codon UAG suggests that the ORF5 is expressed by a readthrough mechanism [1]. The sequence context around the termination codon of CP gene which separates ORF3 from ORF5 in luteoviruses is highly conserved (CCAAA-UAGGUAGAC) and resembles these around several other virus termination codons which are misread by naturally occurring suppressor tRNAs to generate readthrough proteins. The suppression of the stop codon of ORF5 was identified by western blots in tobacco protoplasts and plant tissues infected with PLRV [3, 4] and in *Chenodium quinoa* protoplasts infected with beet western yellow virus, [12]. *In vitro* suppression of the UAG stop codon of the coat protein gene of barley yellow dwarf virus has been demonstrated [13].

However, no *in vitro* studies of readthrough of the stop codon of the PLRV CP gene were performed. The cDNA of sgRNA (pSG) was transcribed and translated *in vitro* with or without suppressor tRNA. Translation products were analysed by PAGE. The results of these experiments are presented in Fig. 4.

In vitro translation without suppressor tRNA of the tSG transcript resulted only in the synthesis of two proteins of 23K and 17K (Fig. 4, lane 2 and 4). The addition of total tRNA from a suppressor strain of yeast to the translation reaction resulted in the synthesis of a third protein having M_r about 79000 (Fig. 4, lane 3). The same situation was observed when tRNA from a SUQ5-a suppressor (UAG, serine inserting) yeast strain was added to the translation reaction mixture (Fig. 4, lane 5).

This result indicates that ORF5 is expressed due to the suppression of the UAG codon in the *in vitro* system and the presence of suppressor tRNA is necessary.

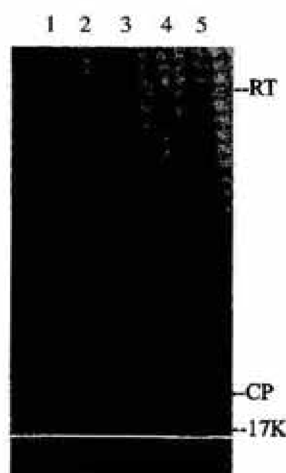


Figure 4. Mechanism of ORF5 expression.

Products of translation tSG in RRL without suppressor tRNAs are presented in lanes 2 and 4. Lane 3 represents translation products when total tRNAs was added. Lane 5 represents translation products when tRNA from SUQ5-a suppressor yeast strain (UAG, serine inserting) was added. The same results were obtained when tRNA from SUP4-a suppressor yeast strain was added (data not shown on the Figure). Lane 1 represents translation products of tobacco yellow mosaic virus RNA as control of translation. RT, readthrough protein; CP, coat protein, and 17K, 17000 protein.

The effect of the leader sequence on the efficiency of sgRNA translation

Plant viral 5´ untranslated regions (5´ UTR), designated also as leader sequence have been shown to enhance translation in plant cells [14]. By analogy with transcriptional enhancer sequences, plant leaders have been denoted as translational enhancers.

However, the effect of LS of PLRV, neither genomic nor subgenomic RNA has been studied. LS of sgRNA is 212 nucleotides long and it begins at a position equivalent to 3376 nucleotides from the 5´ end of the PLRV genome. To analyse the role of LS of sgRNA in translation of genes located downstream, plasmids encoding coat protein and 17K with different lengths of LS joined to the coding sequence of sgRNA were constructed (Fig. 5A). *In vitro* synthesised transcripts of pWT, pHWT and pAWT were translated with RRL and products were analysed by PAGE. The results presented in Fig. 5 indicate that the shortening or lack of LS not only stimulate translation of both genes but also affect the ratio of these two proteins. In the case of tWT the ratio CP/17K seems to be 1:1, whereas removing LS (translation of tAWT) caused a significant increase of 17 K protein level. The transcripts of pWT, pHWT and pAWT are truncated versions of sgRNA since they ended at 4457 nucleotide from 5´ end of the PLRV genome.

The influence of the 3´UTR on the sgRNA translation

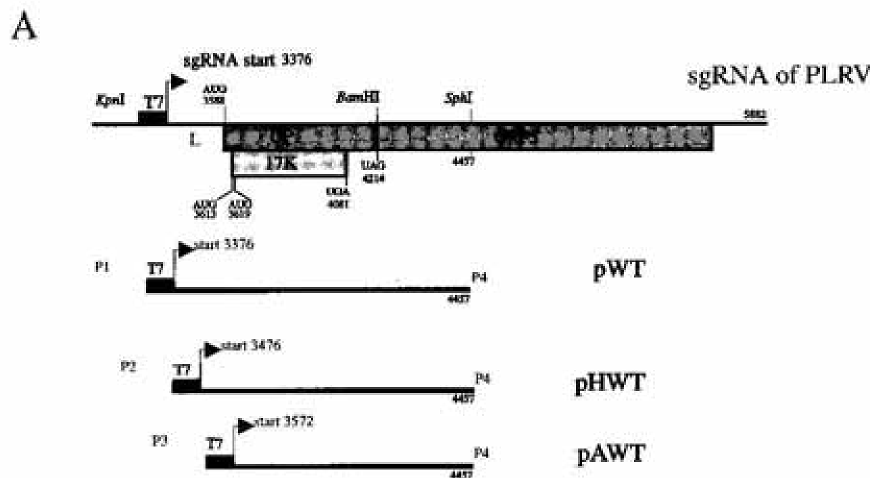
In order to check if the presence of the 3´ untranslated region (3´UTR) has some effect on translation efficiency plasmids carrying the full length of cDNA sgRNA (Fig. 6A), pSG, pHSG and pASG were constructed and their transcripts were translated. The PAGE analysis of translation products is presented in Fig. 6. A similar pattern of LS influence on translation efficiency has been obtained, the shortening of LS enhances translation of downstream located genes and changes the ratio ORF3 to ORF4 products.

DISCUSSION

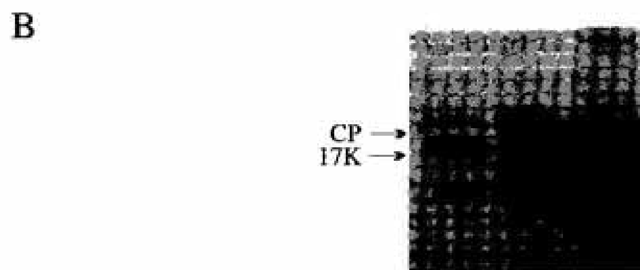
Translation, rather than transcription often serves as a major step in regulation of gene expression of RNA viruses. The luteoviruses utilise a variety of unusual translational control mechanisms to express their genes [1, 2].

Many plant viruses express genes *via* subgenomic RNAs, making it possible to translate the genes located in the 3´ region of genomic RNA [1, 2]. These RNAs are 5´ truncated forms of the genomic RNA. By bringing the 5´ end in proximity with the start codon of genes located in the 3´ end of the genome, sgRNAs act as efficient messages for these genes. The sgRNAs can encode one (monocistronic RNA) or several proteins (polycistronic RNA). The sgRNAs of tombusviruses [15] and luteoviruses [3, 13, 16, 17], have been precisely mapped and shown to act as mRNAs for translation of overlapping genes

A



B



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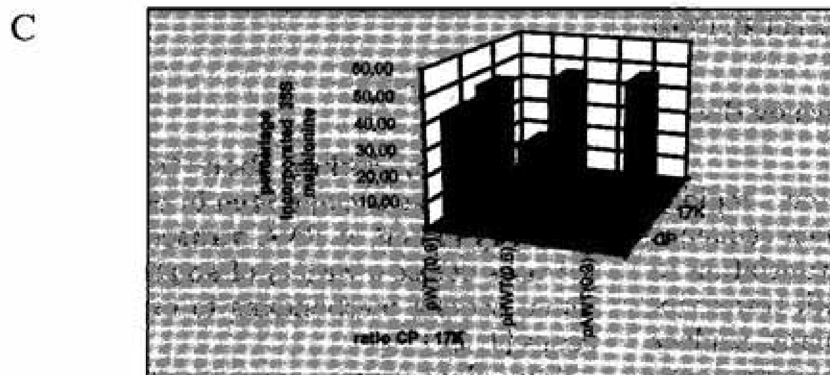

Figure 5. The effect of leader sequence on the efficiency translation of sgRNA.

A. Schematic representation of sgRNA and constructs used to study influence of LS. The sgRNA starts at position 3376 nucleotide and is 2506 nucleotides long. The shaded boxes represent open reading frames. The position of AUG and UAG codons are designated. The black box represents the **T7** promoter and **L** represents the leader sequence. P1, P2, P3, P4 and P5 represent primers used to amplify cDNA fragments (see Table 1). B. Translation products of tWT (lane 1), tHWT (lane 2) and tAWT (lane 3). CP and 17K are coat protein and M_r 17000 protein, respectively. C. Diagram representing the ratio of CP:17K as the percentage of [^{35}S]methionine incorporated into proteins.

in which initiation occurs at two nearby out-of-frame AUGs.

The aim of this work was to elucidate the translational mechanisms of three ORFs located on sgRNA. Two models of translation of the initiation codon in Eukaryota have been proposed: the scanning model and the internal initiation model [9]. According to the scanning model the 40S ribosomal subunit complex, including initiator $tRNA^{Met}$ and several initiation factors, binds at the 5′ end of the mRNA and scans until it reaches the first AUG, at which the 60S subunit binds to form the 80S ribosome and protein synthesis begins. The alternative to this distal entry of part of the translational machinery and its linear migration is the internal entry of ribosomes guided by the "internal ribosome entry site" (IRES) [10]. In the case of the scanning model of translation the introduction of the strong secondary structure with a high free energy upstream of initiation codon AUG can completely stop the scanning 40S subunits. The experiments with the construct pPWT containing the palindrome before AUG codon indicated that sgRNA is translated according

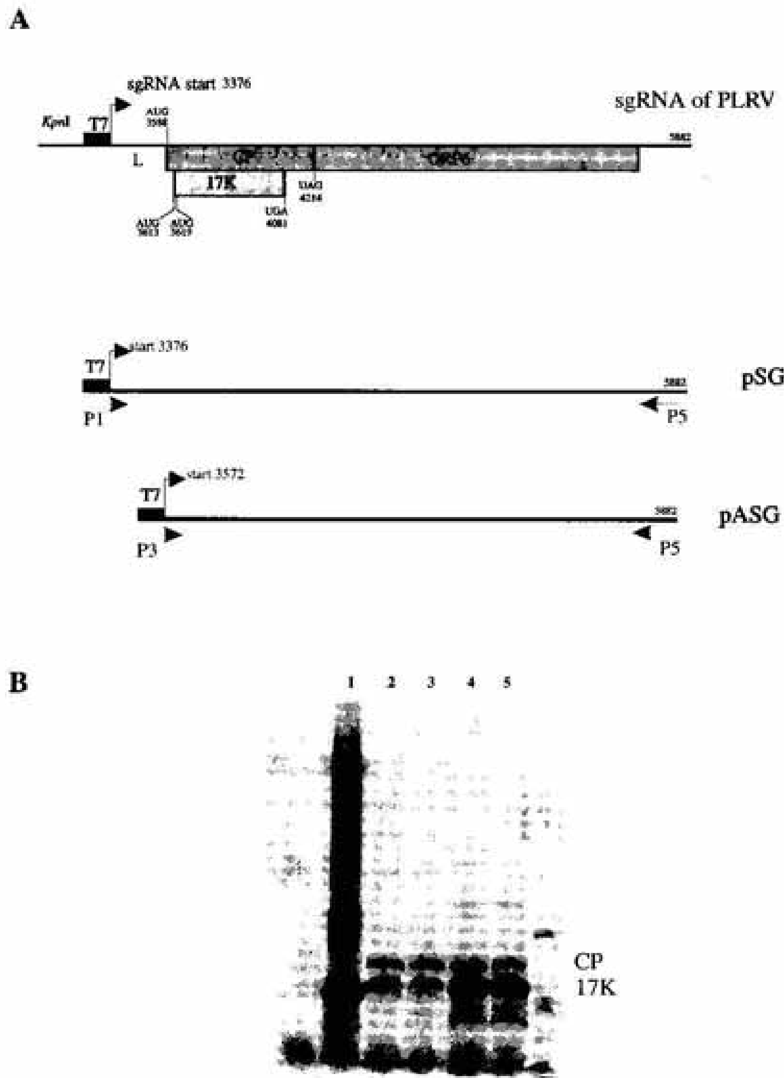

Figure 6. The effect of the 3′ untranslated region (UTR) on efficiency of translation of sgRNA.

A. Schematic representation of sgRNA and constructs used to check the effect of the 3′UTR. The description of Fig. 6A is like on Fig. 5A. B. Translation products of tWT (see Fig. 5A) and tSG represent lanes 2 and 3, respectively. Translational products of tAWT (see Fig. 5A) and tASG represent lanes 4 and 5. As control of translation brome mosaic virus RNA was

to scanning model (Fig. 3). The insertion palindrome prevented the translation of tPWT (Fig. 3).

The stop codon readthrough is used as regulatory strategy by a large number of animal and bacterial viruses. Many viruses, including luteoviruses suppress a stop codon by causing ribosomes to continue translating through a stop codon that separates two in frame ORFs [18]. The peptide chain is released from most ribosomes when they reach the termination codon, but a fraction of the ribosomes suppress this stop codon and continue translation until the next stop codon is reached. Stop codons are read as sense codons and certain amino acids are incorporated in the nascent peptide. Like frameshifting, this mechanism facilitates control of synthesis of relatively low amounts of a carboxy-terminal extended viral protein and relatively high amounts of an amino-terminal portion. In a few cases the signals that permit or promote readthrough have been identified but the mechanisms by which they act are not understood. Interestingly, it has been shown that termination is profoundly affected by the bases following the stop codon.

Recently, it has been shown that besides bases following the stop codon of the coat protein gene of barley yellow dwarf virus (PAV serotype) a second more distal element, normally located nearly 700 bases behind the stop codon, is required for its readthrough [19]. The results presented in this paper (Fig. 3) indicate that ORF5 is expressed by the readthrough mechanism since addition of suppressor tRNAs to the translation mixture of tSG resulted in the synthesis of a third of M_r 79000 protein which is a fusion of the coat protein and the product of ORF5, whereas

without suppressor tRNA, only 17K and CP were synthesised.

There are a number of examples in which the LS regulates the efficiency of translation. The 68 base length leader from tobacco mosaic virus (TMV) genomic RNA and the 36 base length leader from alfalfa mosaic virus (AMV) RNA 4 were the first leaders examined for their ability to enhance translation *in vivo* and *in vitro* [20, 21]. Both viral RNAs are naturally capped. The mechanism of translation enhancement by plant viral RNA 5′ leaders has been extensively studied. One of the most plausible hypotheses proposes that enhancement is due to a low secondary structure of viral 5′ leader sequences that makes translation less dependent on cap-binding initiator factors and facilitates mRNA interaction with 40S ribosomal subunit according to the scanning model [9].

Although other naturally capped viral leaders, e.g. from brome mosaic virus RNA 3 [22] and potato virus X [23], also enhance translation, not all LS have a stimulatory effect. The genomic leader from turnip yellow mosaic virus did not improve the *in vitro* or *in vivo* translation of chimeric constructs [24]. The results presented in this paper (Fig. 5B) indicated that LS of sgRNA has the same inhibitory effect on translation of genes located downstream and it affects the ratio of translated genes. The inhibitory effect of LS was observed independently whether truncated transcripts (tWT, tHWT and tAWT) or the full length of sgRNA (tSG and tASG) were translated in RRL (Fig. 6).

This observation seems to be very interesting since so far most studied LS of viral genomes show either a translational enhancer effect or they did not play any role. The results obtained with tSG and tASG indicated that the presence of 3′ untranslated region has no effect on the translation of sgRNA.

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