2854–2860 *Nucleic Acids Research, 1997, Vol. 25, No. 14*

Plant ribosome shunting *in vitro*

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Received March 25, 1997; Revised and Accepted May 30, 1997

ABSTRACT

It has been proposed that cauliflower mosaic virus 35S RNA with its 600 nt long leader uses an unusual translation process (the translational shunt). A wheat germ *in vitro* translation assay was used to improve the study of this mechanism. Deletions, the introduction of stable stem–loop structures, and the inhibitory effect of antisense oligonucleotides on gene expression were used to determine the roles of various parts of the leader. It was found that the 5′- and 3′-ends of the leader are absolutely required for translation whereas the middle part is apparently dispensable. These results confirm the data already reported from transient expression experiments with protoplasts. However, the *in vitro* data suggest in contrast to protoplast experiments that only two relatively short regions at both ends, ~100 nt each, are required. The *in vitro* system provides tools for further studying the shunt model at the molecular level and for examining the involvement of proteins in this mechanism. Shunting was also found to occur with the rice tungro bacilliform virus leader. As wheat is neither a host plant of cauliflower mosaic virus nor rice tungro bacilliform virus, the shunt seems to be host independent, a finding that deviates from earlier studies in protoplasts.

INTRODUCTION

Caulimo- and badnaviruses contain a double-stranded DNA genome and their pregenomic RNAs are used not only for reverse transcription but also for translation of their open reading frames (ORFs) (1–3). The 600–700 nt long leaders of these RNAs fold into extended secondary structures and contain several small ORFs (sORFs), that could potentially interfere with translation (4). Despite these features, reporter constructs containing cauliflower mosaic virus (CaMV) and rice tungro bacilliform virus (RTBV) leaders are translated in several types of transfected protoplasts with an efficiency of ~30% compared with their leader-less counterparts (5–8). This translation is inhibited by addition of a strong stem–loop structure to the 5′-end of the leader, suggesting that an initiation complex is formed at the cap as usual (9) and not at an internal entry site (10). In contrast, strong stem–loop structures and long ORFs in the centre of the leader are not inhibitory, suggesting that ribosomes do not scan linearly along the whole leader. These results were explained by the ‘ribosome shunt’ model, according to which the scanning complex can bypass (shunt) inhibitory regions. The shunt model was strongly supported by the construction of a ‘trans-shunt’ system, in which the two halves of the leader, transcribed from separate plasmids, allowed translation of a reporter ORF (7). The shunt in CaMV is enhanced by, but does not depend on, the presence of the viral translation transactivator (7), a protein that is known as the inclusion body protein. It is encoded by gene VI and translated from its own monocistronic messenger RNA. Shunting also occurs in Sendai virus, where it was described as ‘scanning-independent cap-dependent initiation’ (11,12), and in late adenovirus translation, where it is directed by a group of secondary structures in the tripartite leader (13).

In plants the shunt model has only been tested so far in transient expression experiments using protoplasts transfected with plasmid DNA. This approach has the disadvantage that expression depends on all steps of information transfer from DNA to protein, and leader sequences of retro- or pararetroviruses affect not only translation but also transcription (14–16), RNA processing (17–21) and RNA export (22). These problems should not arise in *in vitro* translation, where mature RNA is the starting material.

Shunting in transient expression experiments was highly dependent on the source of protoplasts used. Reporter genes attached to the CaMV leader were not translated in protoplasts from *Daucus carota* (23), and were poorly translated in those derived from several *Solanaceae*, and efficiently in protoplasts derived from *Cruciferae* (5). This suggests the involvement of host-specific factors. The probability that a translation system originating from either reticulocytes or wheat germ would allow shunting was thus considered low. However, we have developed a wheat germ *in vitro* system which efficiently translates a reporter gene attached to the CaMV or the RTBV leader. This system allows the study of the ribosome shunt mechanism at the molecular level; with antisense inhibition experiments the influence of parts of the leader on shunting can be determined and the important regions can be looked at in greater detail.

MATERIALS AND METHODS

Construction of clones

Plasmid pSPLC20 contains the SP6 promoter, the entire leader of CaMV (strain S), the chloramphenicol acetyl transferase (CAT) reporter gene fused to the AUG of ORF VII and the CaMV polyadenylation site. pTBLC4 contains a deletion of the first 242 nt of the leader [numbering of the sequence is according to (6)]. To obtain this clone, a *Bgl*II/*Sal*I fragment of pSPLC20 was subcloned in the vector pT3T7 (Boehringer Mannheim). pT3sLC2 contains at

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its 5′-end a stable stem–loop structure. This stem–loop region plus the first 222 nt up to the first *Bgl*II site was taken from clone pS1LGC.St1 (7). The rest of the leader with the CAT reporter gene was used from pSPLC20 and cloned in a three-fragment ligation reaction into plasmid pT3T7. To introduce the stem–loop structure into the center of the leader, the palindromic oligonucleotide GATCGGGCGCGTGGTGGCGGCGCAGCCGCCACCACGCGCCCGATC (2,7) was introduced between the two *Bgl*II sites of pSPLC20. This manipulation led to clone pSPLCs108. Relevant regions of all plasmids were verified by sequencing. Plasmids used for transient expression experiments were as published (pLC20, see ref. 6) or were obtained from the *in vitro* translation expression plasmids by exchanging the bacterial promoters with the CaMV 35S promoter.

The clones containing RTBV leader sequences (24) were derived by subcloning from those used in transient expression assays (25). In all four cases, a *Sma*I/*Acc*65I fragment was subcloned into vector pT3T7 cut with *Hin*dIII (blunted) and *Acc*65I. The *Sma*I site is positioned just upstream of the RTBV leader sequence and the *Acc*65I site downstream of CAT.

Oligonucleotides

The synthesised oligodeoxyribonucleotides (Fig. 4a) were lyophilised, ethanol-precipitated and purified on a 12% denaturing polyacrylamide gel.

Gel-shift assay

The ability of the oligonucleotides to anneal to the transcript was monitored in a gel-shift experiment. Each oligonucleotide (1 pM) was labelled with [γ-^{32}P]ATP (Amersham, 3000 Ci/mM) using T4 polynucleotide kinase (Biofinex). The labeling was checked on a 12% denaturing TBE–polyacrylamide gel. In the annealing reaction, 1.5 pM leader RNA from pSLC20 was mixed with a 10-fold molar excess of unlabelled oligonucleotide and 10 000 c.p.m. of the corresponding labelled oligonucleotide dissolved in a buffer containing 100 mM KAc, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.6. The mixture was heated to 67°C for 5 min and slowly cooled to room temperature before loading onto a 6% native TBE–polyacrylamide gel. Annealing of oligonucleotides to leader RNA was visualised by a shifted signal on the autoradiogram.

In vitro transcription and translation

RNAs were prepared by *in vitro* transcription of linearised plasmids, using SP6 (prefix: ‘SP’; Boehringer Mannheim)-, T3 (prefix: ‘T3’; Boehringer Mannheim)- or T7 (prefix: ‘T’; Biofinex)-RNA polymerase according to the manufacturer’s instructions. For preparation of capped transcripts, the initial concentration of GTP was reduced from 500 to 50 µM and the cap analogue ^{7}mGpppG was added to a final concentration of 0.25 mM. For antisense experiments, template DNA was digested with DNase RQ1 (Promega) at 37°C for 10 min. The integrity of the synthesised transcripts was evaluated on a 6% denaturing polyacrylamide gel. Transcripts were translated *in vitro* in a wheat germ extract (Promega) provided with ^{35}S-met. In standard reactions, KAc was added to a final concentration of 100 mM. The translation reaction was carried out in a final volume of 25 µl at 25°C. After 1 h incubation, 1.5 µl of the reaction mixture was analysed by SDS–PAGE. Gels were fixed for 30 min in 50% MeOH, 12% HAc, and then dried and exposed to X-ray films. Quantification was carried out using a PhosphorImager (Molecular Dynamics). For translation in the presence of antisense and sense oligonucleotides, 1 µg of RNA was mixed with a 10-fold molar excess of oligonucleotides in annealing buffer prior to the translation step. This mixture was heated to 67°C for 5 min and cooled slowly to room temperature. In experiments without annealing, the heating step was omitted.

RNA analysis

RNAs were obtained by *in vitro* transcription as described above, but in the presence of [α-^{33}P]UTP (Amersham), in a ratio of labelled to cold UTP of 1:2500. Transcripts were incubated in the wheat germ extract under standard conditions. At 10, 20 and 60 min an aliquot of the translation mix was taken and treated with 13.6 µg of proteinase K for 20 min at 37°C. RNA was then purified by phenol–chloroform extraction and ethanol precipitation, and analysed by 4% denaturing PAGE. Quantification was carried out using a PhosphorImager.

RESULTS

In vitro translation of RNAs containing CaMV leader sequences

The effect of the leader on translation in the *in vitro* system was tested by comparing the leader-containing RNA derived from plasmid pSPLC20 with the leader-less derivative from plasmid pSPC3 (Fig. 1). We first optimised *in vitro* translation in a commercial wheat germ lysate using several capped mRNAs obtained by *in vitro* transcription. All experiments were performed in the presence of 2 mM $MgAc_2$ (as provided with the extract). Addition of more Mg^{2+} strongly decreased the translation efficiency of all tested RNAs. The optimal KAc concentrations were also distinct and the ranges of ionic strength applicable for translation differed for the individual test RNAs. Routinely, we used 100 mM KAc and performed translation experiments either with or without *in vitro* transcribed U1A RNA (26) as an internal control. The lysate was not saturated with 0.4 pmol for both the test and the control RNA.

Under these conditions, the amount of CAT protein translated from the leader-containing RNA (pSPLC20) was on average 17% of that translated from the leader-less RNA derived from pSPC3 (Fig. 1). In transient expression experiments using *Orychophragmus violaceus* protoplasts with the corresponding ^{35}S promoter-driven constructs (7), the leader-containing construct (pLC20) gave rise to CAT activity levels of 30% of those obtained with the leader-less construct (pC3). Thus, in both systems, the leader is not a complete hindrance to translation. Transient expression experiments previously revealed that sequences within the 5′- and the 3′-ends of the leader are required for shunting, while the middle portion can be deleted or loaded with insertions without affecting shunting (7). The results of our *in vitro* experiments confirm these findings, since removal of the first 220 nt of the leader sequence from the 5′-end (pTBLC4), or insertion of a strong stem (pT3sLC2) (2,9) near the 5′-end, almost abolished translation (Fig. 1); insertion of the stem into the centre of the leader (pSPLCs108) had no effect.

Our results could reflect different RNA stabilities in the translation mixture rather than differences in translatability. Therefore, we routinely analysed the test RNAs by gel electrophoresis. In all cases, 30–60% of the input RNA could still be recovered after a 60 min

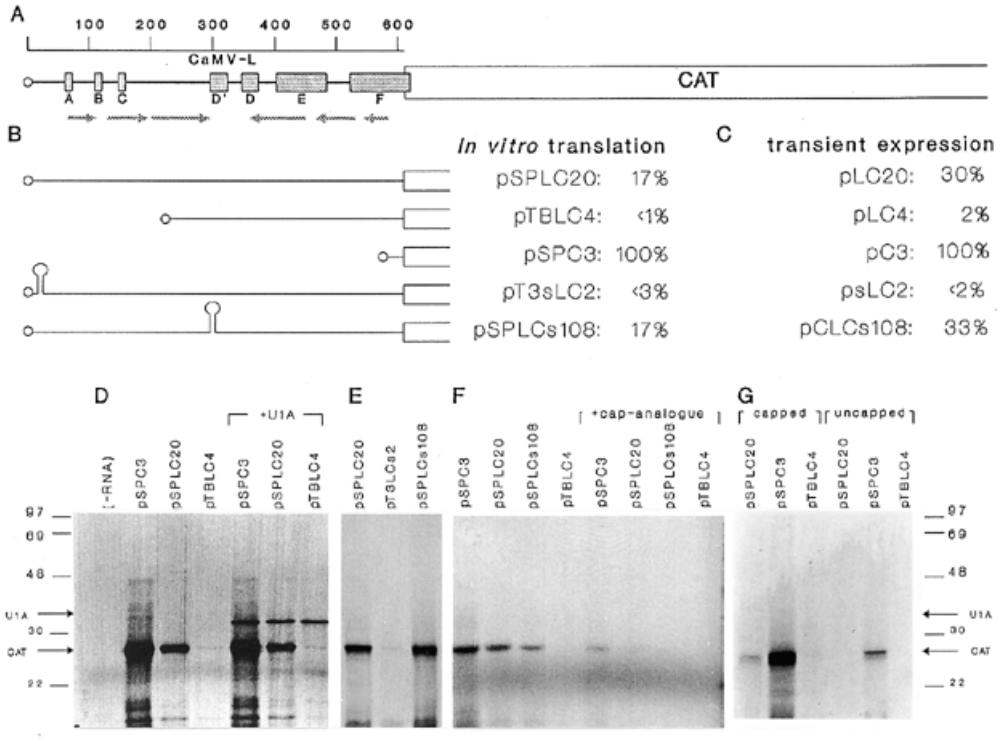

Figure 1. *In vitro* translation of CaMV-leader-containing RNAs. (**A**) Map of the leader indicating the small ORFs (boxes A–F) and the main stem structures (arrows). (**B**) Summary of *in vitro* translation data. The transcripts of the plasmids used containing the full-length leader and truncated versions of it are symbolised as well as variants carrying an energy-rich hairpin (see Materials and Methods). Plasmids were provided with bacteriophage promoters for *in vitro* transcription. The values are the means from at least three independent experiments of the PhosphorImager scanning data of CAT bands separated on SDS–PAGE. The amount of CAT protein obtained by translation of pSPLC20 RNA was set to 100%. These data were compared with (**C**) the values gained from transient expression experiments in protoplasts. (**D**) *In vitro* translation in the absence and the presence of an internal standard (U1A RNA). (**E**) *In vitro* translation from RNAs carrying a strong stem–loop structure at different positions. (**F**) *In vitro* translation in the absence and the presence of the cap analogue ^{7}mGpppG at a final concentration of 0.2 mM. (**G**) Translation of capped and uncapped RNAs. The plasmids were transcribed in the presence or the absence of the cap analogue. Molecular weight standards and the positions of the CAT and U1A proteins are indicated.

incubation (shown for the CaMV derivatives in Fig. 2). Although there was some variation in the efficiency of RNA recovery and the non-translated RNAs were degraded faster, low or negligible CAT expression cannot be explained by RNA degradation, showing that our results indeed reflect translation efficiencies rather than RNA stabilities. The results reported here showed that the *in vitro* translation system can be used to investigate the shunt further, as it confirmed the data obtained *in vivo*.

Addition of the cap-analogue m^{7}GTP (final concentration 0.25 mM) to the translation mixture drastically reduced the translation level in all cases (Fig. 1F), showing that the reaction was cap dependent. Residual translation most probably still depended on recognition of the 5′-end, since introduction of a strong stem at the 5′-end abolished expression completely. Furthermore, a comparison between *in vitro* transcribed capped and uncapped RNAs showed that the translation efficiency of uncapped RNA of pSPC3 was only 15% of its capped counterpart (Fig. 1G). The leader-containing construct (pSPLC20) gave a similar result, whereas the RNA with a deletion at the 5′-end (pTBLC4) was not translated, regardless of the presence or absence of the cap.

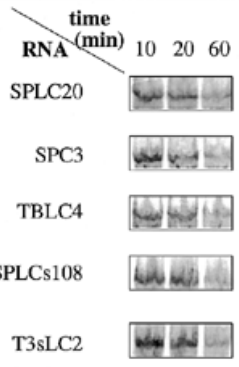

Figure 2. Stabilities of test RNAs during the *in vitro* translation reaction. (**A**) RNA labelled with [α-^{33}P]UTP was extracted at the indicated times from the wheat germ translation mix as described in Materials and Methods and analysed on a 4% denaturing PAGE.

Translation of RTBV RNA

To investigate whether shunting could also be observed *in vitro* with RTBV leader-containing constructs, reporter plasmids containing this leader or parts of it were constructed for *in vitro* transcription and translation. The RTBV leader allowed levels of *in vitro* translation similar to those of the CaMV leader. A transcript containing the complete RTBV leader (Fig. 3, lane 2) was translated with an efficiency of 15% relative to a transcript from which most of the leader including all sORFs was deleted

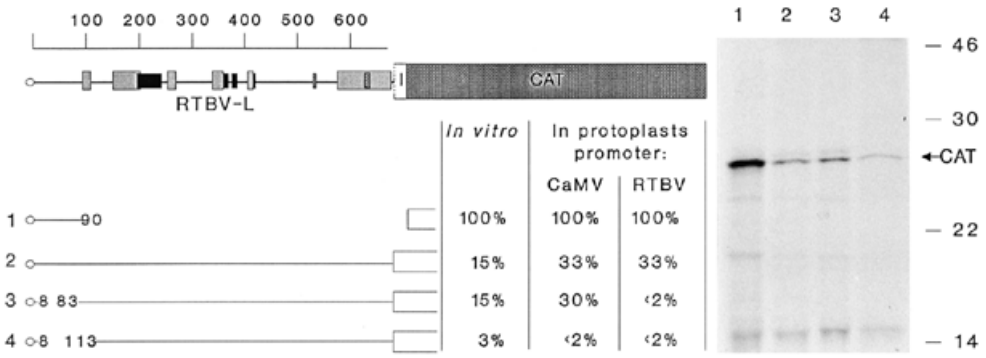

Figure 3. Translation of RTBV-leader-containing RNAs. Top, schematic representation of the RTBV leader with its sORFs indicated as boxes. Middle, plasmid constructs used for *in vitro* transcription (the numbers indicate the positions of the deletions in the leader) and the *in vitro* translation from capped mRNAs. Comparison with the data obtained from transient expression experiments with plasmids carrying either the CaMV ^{35}S or the RTBV promoter is shown.

(lane 1). These results coincide well with those obtained by Chen *et al*. (25) in transient expression experiments (Fig. 3).

Protoplast transfection studies revealed that the RTBV leader contains sequences that appear to affect either transcription or translation (14,25). This is exemplified by transient expression experiments using leader-deletion mutants transcribed from either the RTBV or the CaMV promoter (Fig. 3). The RTBV leader region between nt +8 and +83 was important for expression in the context of the RTBV but not of CaMV 35S promoter (lane 3), while the region between nt +83 and +113 was important for expression with either promoter (lane 4). From these observations, it was suggested that the region +8 to +83 includes promoter/enhancer elements important for transcription in the context of RTBV upstream promoter sequences (14), while region +83 to +113 is involved in translational regulation. As an illustration of how the *in vitro* system allows the dissection of sequences important for translation independent of transcriptional effects, a construct containing a leader deletion from +8 to +83 was translated as well as one containing the complete leader (compare lanes 2 and 3), confirming the above model.

Inhibition of translation by antisense oligonucleotides

A series of antisense oligodeoxynucleotides was tested for effects on *in vitro* translation of the CaMV leader-containing construct (pSPLC20; Fig. 4a). Translation inhibition experiments were performed under two conditions: in the first case ('preannealing'), target RNA and antisense probe were mixed at 25°C, heated to 67°C for 5 min and cooled slowly to 25°C. In the second case, translation reactions were started immediately after target RNA and antisense oligonucleotide were mixed at 25°C. Using the preannealing regime and the same salt conditions as in the translation experiments, the labelled oligonucleotides were tested by gel-shift experiments in a native polyacrylamide gel for their ability to bind to the CaMV leader sequence. The mobilities of all antisense oligonucleotides except $A_{172–134}$ were shifted to lower values, indicating their binding to the leader sequence. Why $A_{172–134}$ did not bind, is unknown; however, it is relatively poor in G- and C-residues and the corresponding target sequence is resistant to chemical modification, indicating its possible involvement in a stable higher order structure (27). Because of this abnormality, antisense inhibition results with $A_{172–134}$ were not considered further. However, as it only inhibits translation by 8%, it served as a control to demonstrate that the addition of antisense oligonucleotides *per se* did not inhibit translation of the leader-containing RNA. Furthermore, little inhibition was observed in a control experiment using a sense oligonucleotide ($S_{123–161}$; Fig. 4).

Using antisense oligonucleotides, we distinguished three different effects. Some oligonucleotides inhibited translation strongly, regardless of whether target and probe were preannealed or not. This class included the antisense oligonucleotides covering sequences close to the 5′- and 3′-ends of the leader ($A_{77–39}$, $A_{600–562}$ and A_{ATG}; Fig. 4). We assume in these cases, that the target is relatively unstructured, or becomes available during ribosome scanning and translation initiation. A second class of antisense oligonucleotides inhibited translation only strongly ($A_{105–66}$ and $A_{546–509}$) or moderately ($A_{580–543}$) if the preannealing step was performed. We assume, in these cases, that strong secondary structures have to be melted before the antisense oligonucleotide can be annealed. The remaining class of antisense oligonucleotides, which were designed to interact with the more central regions of the leader, inhibited more weakly after preannealing and very weakly or not at all without preannealing. We assume that these sequences are neither scanned by ribosomes involved in shunting nor are they required as shunting structures.

DISCUSSION

A wheat germ *in vitro* translation system was established which allowed translation of a reporter ORF positioned downstream of either the CaMV or the RTBV leader. In contrast to earlier attempts (see ref. 28), this system yielded results resembling those obtained in transient expression experiments. The efficiency of the system was highly dependent on the source of the wheat germ and on ionic strength and we did not observe truncated proteins. Interestingly the leader-containing RNA was translated over a much narrower ionic strength range than other RNAs. The reason might lie in the importance of secondary structure for translation efficiency of RNAs with large leaders (27). Translation was largely cap dependent and, accordingly, inhibited by the addition of a cap analogue. Furthermore, uncapped RNAs were only weakly translated compared with their capped counterparts. This shows that translation starts at the cap site and that internal initiation plays no significant role as a mechanism to overcome the inhibitory leader. Since the lysate was free of nuclei, only

a

oligo	%GC	length	oligo	%GC	length
A_{77-39}	37	38	$A_{510-473}$	32	37
A_{105-66}	44	39	$A_{546-509}$	38	37
$A_{137-100}$	43	37	$A_{580-543}$	32	37
$A_{172-134}$	26	38	$A_{600-562}$	37	38
$A_{275-238}$	43	37	A_{ATG}	37	41
$A_{342-306}$	53	36	$S_{123-161}$	26	38
$A_{434-399}$	54	35			

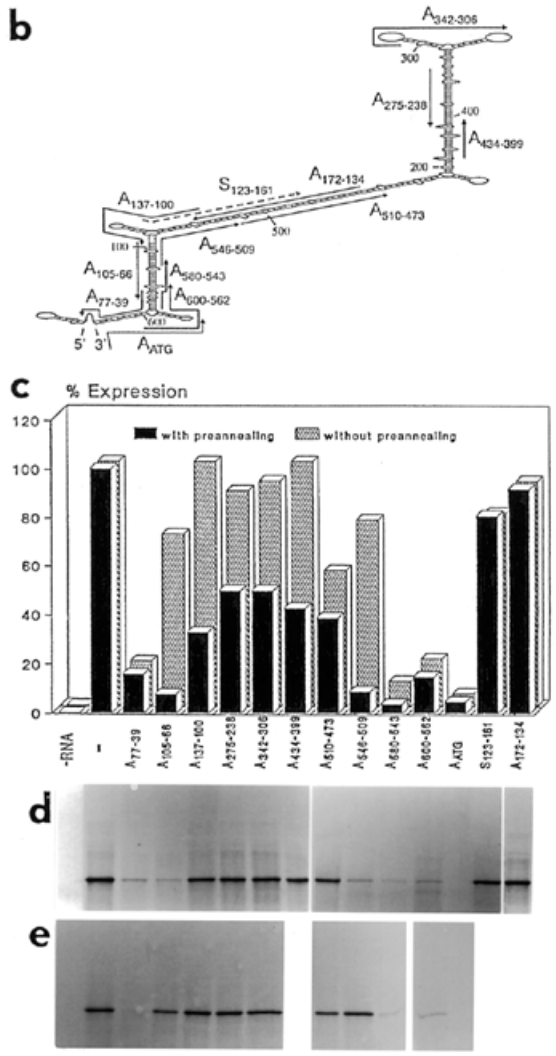

Figure 4. Translation inhibition experiments using antisense oligodeoxynucleotides. (**a**) The series of antisense oligonucleotides used for the inhibition experiments. The numbers following the A indicate the complementary region in the leader. A_{ATG} is complementary to the region around the initiation codon of the CAT reporter gene. The lengths and GC contents of each oligonucleotide are shown. (**b**) Secondary structure of the CaMV leader as predicted by the 'fold' computer program. The regions complementary to the antisense oligonucleotides are indicated. (**c**) Yield from *in vitro* translation of pSPLC20-derived RNA in presence of a 10-fold molar excess of antisense oligonucleotides performed with and without a preannealing step. Efficiencies were determined by PhosphorImager analysis from the labelled CAT translation product separated on SDS–PAGE. The efficiency in absence of oligonucleotides was set to 100%. Numbers given are the means of at least three independent experiments. (**d** and **e**) Typical SDS gels of labelled CAT obtained by *in vitro* translation from pSPLC20-derived RNA in the absence and the presence of preannealed (d) and not preannealed (e) antisense oligonucleotides.

post-transcriptional effects are investigated, and since in all cases a considerable fraction of imput RNA could be recovered even 60 min after incubation with the wheat germ extract, the effects are mainly translational. Hence, this *in vitro* translation system is an appropriate model system to study the shunt mechanism at a molecular level.

Our findings that a deletion of the first 220 nt of the CaMV ^{35}S RNA leader or the insertion of a strong stem–loop structure at its 5′-end inhibited translation almost completely, confirm the conclusion reached from transient expression experiments that this part of the leader is required for translation. The middle part of the CaMV leader with its inhibitory sORFs, on the other hand, seemed to be bypassed by the translational shunt mechanism, since insertion of a strong stem structure into this region did not inhibit translation.

In vitro translation allows the testing of the effect of antisense oligodeoxyribonucleotides on the mRNA in a more defined way than previously, and also allows interpretation of the results as translational effects. *In vivo*, the effect of antisense oligonucleotides on translation is far more difficult to assess because additional steps of expression, such as intracellular transport and the distribution of RNA, may also be affected. For example, antisense RNA might act in the nucleus by inhibiting RNA export. Several mechanisms for the inhibition of gene expression by oligodeoxyribonucleotides have been proposed, including inhibition of the formation of the translation complex, inhibition of scanning and digestion of the hybrid by RNAseH (11,29). Annealing of an oligonucleotide might also influence translation by inhibiting the binding of proteins or by destroying an important secondary or tertiary structure of the RNA. Analysis of the RNA isolated from the translation reaction showed us that RnaseH was present in the extract. Therefore, RNAseH digestion likely contributed to our results. The differential and reproducible effects of the various antisense oligonucleotides that bound to the test RNA with similar efficiency can, however, best be interpreted as interference with processes connected to translation, e.g. scanning and shunting. Despite a high excess of antisense oligonucleotides, residual translation of the test RNA was found in all cases. This residual activity is unlikely to result from translation of truncated test RNAs due to RNAseH activity, since the truncated RNAs would be uncapped and we showed that our system translates uncapped RNA only very poorly. Furthermore, oligonucleotides like $A_{275–238}$ inhibited translation from the test RNA by 50%; in this case a truncated RNA would resemble the RNA obtained from clone pTBLC4, which is not translated even when capped. On the other hand, truncation of the CaMV leader annealed with $A_{546–509}$, $A_{580–543}$ and $A_{600–562}$ by RNAseH activity would result in a monocistronic mRNA without upstream sORFs like pSPC3. Uncapped RNAs of this species would be translated about as efficiently as leader-containing RNAs (Fig. 1G). Addition of these oligonucleotides, however, drastically inhibited translation by $>80\%$. Antisense oligonucleotides against the centre of the leader had a moderate effect on translation. This correlates with results obtained *in vivo* (Fig. 5) (7), showing that the central portion of the leader can be deleted or interrupted by insertions without affecting translation in protoplasts. Translation would result both from the exclusion of this region from scanning if the RNA stays intact, and from the prevalence of the assumed shunt structure, even when the central portion of the leader is interrupted due to RNAseH digestion, which would correspond to a trans-shunt mechanism (7). Antisense oligonucleotides against

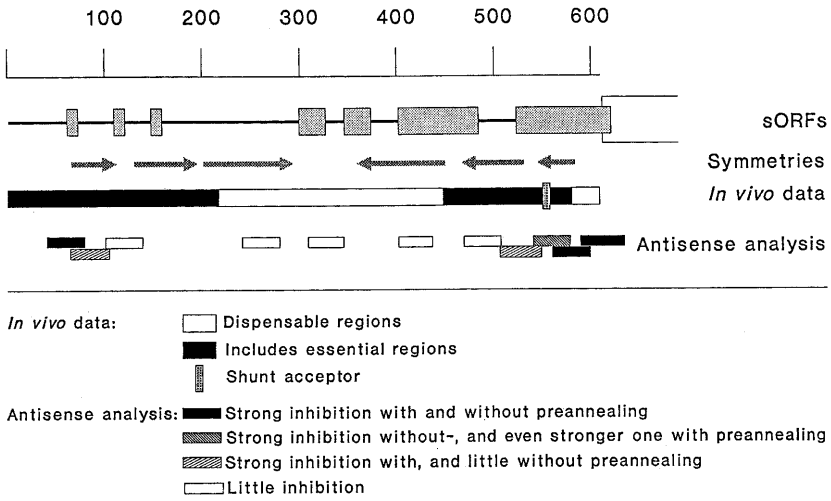

Figure 5. Translation of a reporter gene attached to the CaMV leader. Graphical representation of the results obtained from leader deletion analysis in transfected protoplasts (*in vivo*) (6,7) and inhibition experiments with antisense oligonucleotides *in vitro* (this publication). The CaMV leader with its sORFs is shown; arrows indicate the main symmetric regions (stems). The regions essential or dispensable for translation of a reporter gene *in vivo* are compared with the location and the effect on translation *in vitro* of the antisense oligonucleotides.

the termini of the leader, on the other hand, strongly inhibited translation, whereby both outermost regions were inhibited either with or without preannealing, and penultimate regions mainly after preannealing; thus these regions are critical for translation. We assume that RNA regions affected independently of preannealing are relatively unstructured and, therefore, easily accessible to the oligonucleotides. These regions are thought to be scanned by ribosomes before and after shunting and consequently their annealing to oligonucleotides would interfere with translation. Another possibility would be that these regions are melted during scanning and, therefore, become available for hybridisation. Those that are not affected without preannealing, however, are probably highly structured. This is in accordance with computer prediction (30) and enzymatic and chemical modification analysis (27). The latter shows these regions to be part of the base of the long leader stem structure (Fig. 4b), which is thought to be melted poorly during scanning and thus might cause pausing of scanning and thereby favor shunting. The strongest inhibition in fact was achieved with the antisense oligonucleotide covering the region previously identified as the shunt acceptor ($A_{580–543}$; Fig. 5) (7). We speculate that this region is not scanned but is involved in the shunting process. Oligonucleotide AATG covers the initiation codon and inhibited translation by up to 95%. A similar degree of inhibition was also observed in a rabbit reticulocyte lysate system using antisense RNAs covering the initiation codon of mammalian genes (31,32).

Thus, *in vitro* translation- and protoplasts transfection experiments lead to the same conclusion, namely that both ends of the leader are required for shunting, whereas the central region is dispensable. In protoplasts the first ~200 and the last ~150 nt have been shown to be required for the shunt. The *in vitro* inhibition experiments allowed a more detailed mapping and has shown that only the first and the last 100 nt are needed. These two regions can form a quite extended and stable stem structure. The *in vitro* translation system now allows us to investigate further the minimal sequences and/or structures needed for shunting.

Another deviation from experiments using protoplasts concerns the host specificity. The efficiency of the shunt involving the CaMV leader in transfected protoplasts depended on the source of protoplasts used (5,6), e.g. protoplasts from *Cruciferae* species allowed much more efficient expression than those derived from several *Solanaceae* (5) and carrots (23). Because *Cruciferae* are hosts of CaMV, it was tempting to assign this effect to host specificity. However, the effect could also reflect deficiencies in certain translation factors or induction of inhibitors during protoplasting. Our observations that shunting occurs in the germ of wheat, a non-host plant, and that this system functions equally well with leaders from CaMV and RTBV, viruses which affect very different host plants, suggest that shunting is a more general mechanism independent of specific virus-host adaptations.

The *in vitro* translation system will allow the testing of possible protein factors required for shunting, which could be done by providing additional factors, such as the leader-binding proteins described by Dominguez *et al.* (33) and the viral transactivator protein, or by depleting conventional translation factors when antibodies become available.

ACKNOWLEDGEMENTS

We gratefully acknowledge the active interest and help of our colleagues, especially G. Chen, J. Fütterer, H. Rothnie and L. Ryabova, and the critical reading of the manuscript by P. King. We thank G. Simpson for providing the U1A clone and H. Moser and D. Hüsken, CIBA, and P. Müller, FMI, for the provision of oligonucleotides. This work was partially funded by an EMBO fellowship to WS-P.

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