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Positive and negative control of translation by the leader sequence of cauliflower mosaic virus pregenomic 35S RNA

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We have studied the influence of the 600 nt long leader sequence of cauliflower mosaic virus 35S RNA on downstream translation. Plant protoplasts were transfected with plasmids expressing a CAT reporter gene from a mRNA, containing wild-type or mutant forms of the 35S RNA leader. Deletion analysis revealed the presence of three separate stimulatory sequence regions, S1, S2 and S3. The latter two interact with each other to enhance downstream translation 5- to 10-fold. This enhancement was not observed in protoplasts from a non-host plant. In the absence of either S2 or S3, the region I2, located in between, exerts an inhibitory effect on downstream translation, probably due to the presence of short open reading frames. Expression of a reporter gene inserted into I2 increases 2-fold upon deletion of either S2 or S3. We propose that mRNA regions S2 and S3 form a complex with cellular factors that allows scanning ribosomes to bypass region I2.

Key words: caulimovirus/pararetrovirus/plant protoplast/ribosome scanning/transient expression/translational control

Introduction

The 35S RNA is the major cauliflower mosaic virus (CaMV) RNA species in infected plants (Guilley *et al.*, 1982). This RNA, which covers the complete viral genome and carries direct terminal repeats, functions as a template for reverse transcription during the viral replication cycle. It also contains all of the viral open reading frames (ORFs) in a tight arrangement and is the only viral RNA encompassing ORFs IV – VII that has been reproducibly detected (Figure 1; for review see Gronenborn, 1987; Bonneville *et al.*, 1988). The polarity of mutants in the non-essential ORFs II and VII has led to the suggestion that at least ORFs I and III are translated from a polycistronic mRNA by reinitiation after translation of the respective upstream ORFs VII and II (Sieg and Gronenborn, 1982; Dixon and Hohn, 1984). Such a mechanism is unusual for eukaryotic cells (Kozak, 1989a). Recently, we and others have shown that expression of a downstream coding region on the 35S RNA is possible in plant protoplasts, but requires expression of the viral ORF VI product as a transactivator (Hohn *et al.*, 1989; Bonneville *et al.*, 1989; Gowda *et al.*, 1989). These findings suggest that 35S RNA indeed acts as a polycistronic mRNA. The use of 35S RNA as mRNA is further complicated by the presence of a 600 nt leader sequence preceding the first longer ORF, ORF VII (Figure 2A). [We define here as leader the sequences preceding the principal coding region of a messenger RNA. We hesitate to use the otherwise common term UTR (untranslated region) since leaders can (and in the case of CaMV do) contain small ORFs (sORFs) which might be translated.] This leader sequence can theoretically be folded into a large stem-and-loop structure and contains several small open reading frames (sORFs), some of which can be expressed when fused to reporter genes (Fütterer *et al.*, 1988).

The generally accepted model of translation initiation in eukaryotes, i.e. the modified scanning hypothesis (Kozak, 1989a), proposes that the 40S ribosomal subunit together with a variety of other factors binds to messenger RNA at or near the cap structure and then scans the RNA until an AUG start codon is encountered. The 80S ribosome is assembled there and translation is initiated. In mammalian cells the frequency of translation initiation for any given AUG depends on its immediate sequence context (Kozak, 1986a, 1987a). An AUG in an unfavourable context will be used inefficiently and will allow continuation of scanning and translation initiation at a start codon situated further downstream (Kozak, 1986a and 1987b). Such a mechanism ensures, for instance, the expression of the correct molar ratio of two SV40 capsid proteins (Sedman and Mertz, 1988) as well as the translation of two proteins from the Sendai virus P/C mRNA (Curran and Kolakofsky, 1988a). In plants and in yeast the sequence requirements for translation initiation do not seem to be equally stringent and only minor variations in the efficiency of start codons within different sequence contexts have been reported (Lütcke *et al.*, 1987; Baim and Sherman, 1988).

After termination of translation, 60S ribosomal subunits are thought to dissociate from the RNA. The fate of the 40S subunits has still to be elucidated definitely, but cases have been observed in which they resume scanning and reinitiation occurs at an AUG further downstream (e.g. Peabody and Berg, 1986; Peabody *et al.*, 1986). The efficiency of reinitiation is usually low (Kozak, 1984; Liu *et al.*, 1984). Parameters by which this efficiency is determined are not well understood; to date there is just one report correlating an increase of intercistronic distance with increase in downstream translation for a synthetic, dicistronic preproinsulin RNA (Kozak, 1987b).

The scanning model therefore offers an explanation for how upstream AUGs interfere with the expression of a downstream coding region. Of the mRNAs characterized so far, only a small percentage contain upstream sORFs (for a compilation of vertebrate mRNAs see Kozak, 1987c). The function of these sORFs is best understood in the case of yeast GCN4 (for a review see Hinnebusch, 1988) and CPA1 mRNAs (Werner *et al.*, 1987), where upstream sORFs modulate expression of the downstream coding region in response to metabolic conditions. Also, some viruses produce mRNAs in which a major ORF is preceded by one or more

Fig. 1. Map of the CaMV genome. Long and short ORFs are indicated by shaded arrows in the outer circles. The two major viral RNAs are shown as arrows along the respective coding regions and labelled at the transcription start sites.

sORFs. In at least some cases, expression products of such sORFs have been detected (Nomura *et al.*, 1983; Hackett *et al.*, 1986; Khalili *et al.*, 1987) or indirect evidence for their translation has been reported (Fütterer *et al.*, 1988; Peterson *et al.*, 1989). Still, apart from modulating expression of the downstream ORF (Sedman *et al.*, 1988; Geballe and Mocarski, 1988), the only known function of such a sORF is for the SV40 agnoprotein (Hay and Aloni, 1985). In the case of picornavirus RNAs, a long leader sequence with several sORFs does not inhibit expression of the downstream ORF in infected cells. Ribosomes apparently associate with these RNAs in a cap-independent manner at an internal entry site (Pelletier and Sonenberg, 1988; Jang *et al.*, 1988).

Gene expression can also be influenced by the secondary structure of the mRNA leader sequence: stable hairpins have been shown to inhibit either cap-dependent binding (Chevrier *et al.*, 1988; Parkin *et al.*, 1988; Kozak, 1989b) or the scanning of ribosomes (Baim and Sherman, 1988; Kozak, 1989b). Such structures can mediate expression control in response to changes of environmental parameters such as osmotic pressure (Kozak, 1988).

With its potential secondary structure and its sORFs, the CaMV 35S RNA leader sequence thus contains two features of interest for studying translational control in plant cells. We and others have recently shown that this leader sequence indeed reduces expression of a downstream reporter gene in plant protoplasts and in *in vitro* translation systems (Baughman and Howell, 1988; Fütterer *et al.*, 1989). However, in protoplasts prepared from CaMV host plants, constructs encoding a complete leader sequence still gave rise to about one-third of the reporter gene activity induced by a leaderless version (Fütterer *et al.*, 1989). Interpreted on the basis of the scanning model, this appears to be a rather low degree of inhibition; therefore, questions are raised about

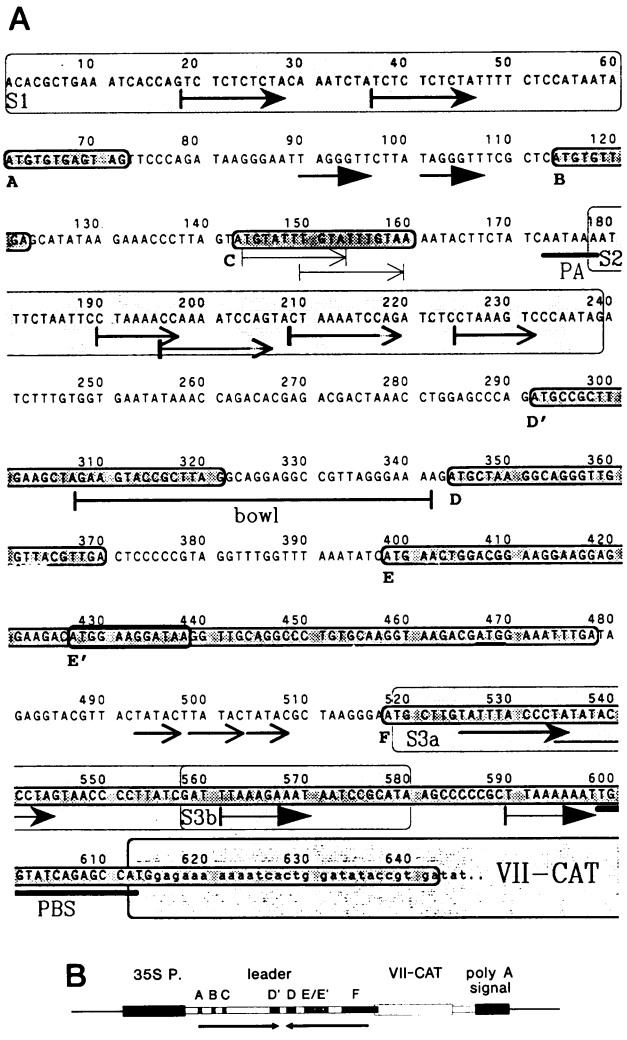

Fig. 2. (**A**) Sequence of the CaMV 35S RNA leader. The DNA sequence encoding the 35S RNA leader of CaMV strain CM4-184 (Dixon *et al.*, 1986), starting from the presumptive cap-site, is shown in upper case letters, together with the beginning of the CAT ORF, fused to the ATG codon of ORF VII, in lower case letters (wide, darkly shaded box). The sORFs in the leader are shown in narrow, darkly shaded boxes and designated A–F below the ATG codon. Stimulatory regions as defined in the text are marked by wide, lightly shaded boxes. Several repetitive elements are underlined by arrows and sequence elements with known functions in the viral life cycle are underlined: PA, conserved sequence within the polyadenylation signal; PBS, primer binding site for reverse transcription. The location of a sequence highly conserved between different caulimoviruses is indicated as bowl (Fütterer *et al.*, 1988). (**B**) Schematic representation of the relevant portion of plasmid LC20 (not to scale). The CaMV 35S transcription control regions are derived from plasmid pDH51 (Pietrzak *et al.*, 1986) and are shown as hatched boxes. Thin lines represent vector sequences. Transcribed regions between the transcription control signals contain the leader sequence (black boxes denote the sORFs; sORF E′ within sORF E is shown as shaded box), the CAT ORF and a 3′ untranslated region derived predominantly from the source of the CAT fragment. Arrows underline leader regions that are involved in formation of the proposed secondary structure (Fütterer *et al.*, 1988).

the efficiency of sORFs in inhibiting translation in plants or on mechanisms counteracting such inhibitory effects.

The results described here indicate that a counteracting mechanism is active in translation regulation by the CaMV 35S RNA leader sequence.

Table I. Plasmids used

Mutant	Construct		Sequence around mutation
	Parent	Description of cloning	
LC20	Fütterer *et al.* (1989)		see figure 1A
-a1	LC20	on.d.m.	CATA-580.....608-AGCC
-a2	LC20	on.d.m.	TTAT-556.....608-AGCC
-a3	LC20	on.d.m.	GCTT-523.....580-AAGC
-b1	LC20	*Cla*I/T4 Pol.	AACC-550.....557-CGAT
-b2	LC-f1	*Hin*dIII/*Cla*I/fi	aGCT-522..... 557-CGAT
-b3	LC20	*Cla*I/Bal31	GTAA-462.....557-CGAT
-b4	LC20	*Cla*I/Bal31	CCCC-377.....557-CGAT
-b5	LC20	*Cla*I/Bal31	CCGC-317.....557-CGAT
-b6	LC20	*Bgl*II/*Cla*I/fi	GATC-222.....557-CGAT
-b7	LC-x	*Sac*I/*Cla*I/T4 Pol.	TGTG- 67.....557-CGAT
-b8	LC-d1	*Xho*I/*Cla*I/fi	TCTC- 53..ga..557-CGAT
-b9	LC20	*Cla*I/fi/*Sal*I→TZDH:*Sma*I/*Sal*I	aggg 557-CGAT
-c1	LC-x	*Sac*I/*Sal*I/T4 Pol.→TZDH:*Sma*I	aggg 133-CATG
-c2	LC-d1	*Xho*I/fi/*Sal*I→TZDH:*Sma*I/*Sal*I	aggg ...tc..... 121-GAGC
-c3	LC20	*Bgl*II/fi/*Sal*I→TZDH:*Sma*I/*Sal*I	aggg 239-GATC
-c4	LC20	*Hin*dII/*Sal*I→TZDH:*Sma*I/*Sal*I	aggg 369-GACT
-c5	LC20	*Eco*O109/*Sal*I→TZDH:*Sma*I/*Sal*I	aggg 447-GCCC
-d1	LC20	on.d.m.	TCTC- 53..... 121-GAGC
-d2	LC-d1	*Xho*I/Bal31 + *Xho*I-linker	TCTC- 53.gagg. 159-AAAA
-d3	LC-d1	*Xho*I/Bal31 + *Xho*I-linker	TCTC- 53.gagg. 178-AATT
-d4	LC-d1	*Xho*I/Bal31 + *Xho*I-linker	TCTC- 53.gagg. 206-GTAC
-d5	LC-d1	*Xho*I/*Bgl*II/fi	TCTC- 53..g...238-AGAT
-d6	LC20	on.d.m.	TTCC-190..cc.. 238-AGAT
-d7	LC20	*Bgl*II/Bal31	ATCC-204..g...239-GATC
-f1	LC20	on.d.m.	GGAA-518...a..520-GCTT
-f2	LC20	*Cla*I/fi	ATCG-558..cg..559-ATTT
-g1	LC-f1	*Eco*O109/fi→TZDH:*Sma*I	see -c5 and -f1
-g2	LC-f2	*Eco*O109/fi→TZDH:*Sma*I	see -c5 and -f2
-g3	LC-b2	*Eco*O109/fi→TZDH:*Sma*I	see -c5 and -b2
-g4	LC-a2	*Eco*O109/fi→TZDH:*Sma*I	see -c5 and -a2
-h1	LC20	on.d.m.	ATTT-476.GgTg.581-GAGG
-h2	LC20	*Cla*I/Bal31	AGGT-460.....557-CGAT
-j1	LC-a2	*Bgl*II/fi/*Eco*RI→LC-x:*Sac*I/T4 Pol/*Eco*RI	see LC-a2 and -j2
-j2	LC-x	*Sac*I/*Bgl*II/T4 Pol	TGTG- 67.....239-GATC
-k1	= LC421, Fütterer *et al.* (1988)		GATC-222cccgctcgagc369-GA
-k2	LC20	*Bhl*II/Bal31	GATC-222...c..413-GGAA
-x	LC20	on.d.m.	TGAG- 69.....111-CTCT

A simplified description of the construction of the plasmids used is given in the second and third columns. Abbreviations used: on.d.m., oligonucleotide-directed mutagenesis; fi, filled in by Klenow DNA polymerase; T4 Pol, T4 DNA polymerase treatment. (→) denotes isolation of a DNA fragment created by digestion of the parent plasmid and cloning into another plasmid as indicated. Plasmid TZDH has been described previously (Fütterer *et al.*, 1989). To obtain unidirectional deletion mutants in the case of Bal31 digestion, fragments of the original digestion products have been recloned. Plasmid LC-x, the parent of several of our constructs, is shown in the last line. The sequence around the site of mutation is shown in the last column. Numbers designate the positions of bases bordering the respective deletions. Upper case letters: original CaMV sequence; lower case letters: mutations or insertions.

Results

Experimental strategy

Measurement of reporter gene expression in transfected cells is a standard method for the analysis of the transcriptional properties of DNA sequence elements. The method has also been used to study translational control, e.g. in the case of the yeast GCN4 mRNA (e.g. Miller and Hinnebusch, 1989); we describe here a similar analysis of the CaMV 35S RNA leader sequence.

Plasmid pLC20, containing the complete CaMV 35S promoter, the 600 bp long leader region, the CAT reporter coding region fused to the first codon of ORF VII and the CaMV polyadenylation signal (Fütterer *et al.*, 1988, 1989), was used as a reference construct (Figure 2B). A series of derivatives lacking part of the leader region was produced (Table I and Figures 3, 5 and 6). Protoplasts derived from cell cultures of the host plant *Orychopragmus violaceus* and the non-host plant *Hyoscyamus muticus* were transfected with circular forms of the resulting plasmids, and the effect of mutations within the leader on expression was studied by assaying CAT activity in both systems. CAT activity was found to increase linearly for at least 20 h and the ratio of CAT activities for different mutants remained constant during and after this time (tested with LC20, -a2, -b6 and -d1; data not shown).

A mosaic of stimulatory and inhibitory sequences in the CaMV leader

Analysis of the CAT reporter gene activities induced by pLC20 and a series of leader mutations in transfected

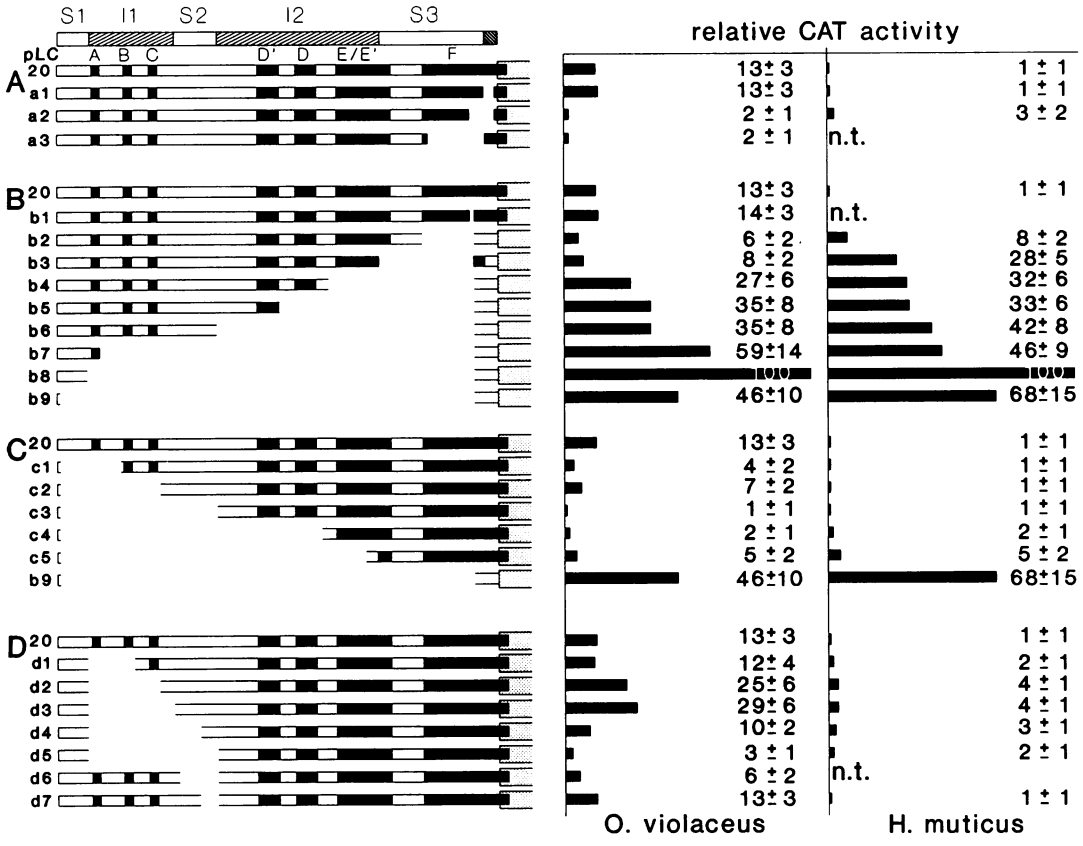

Fig. 3. General deletion analysis of the leader sequence. The 35S RNA leader and deletion mutants are represented schematically as in Figure 2(B) (sORF E′ is omitted for clarity). Gaps designate the extent of the individual deletions. The location of the stimulatory (S) and inhibitory (I) regions is shown in the first line. The average induced CAT activity in *O. violaceus* or *H. muticus* protoplasts is shown in the right panels. Values are normalized with respect to plasmid LC-b8, whose expression was set to 100%. (n.t.) not tested.

O. violaceus protoplasts showed that the leader consists of elements which either inhibit or stimulate downstream gene expression. Deletion series B (Figure 3) reveals the effect on gene expression of successively larger deletions from the 3′ end of the leader. Although most sequential deletions in this series, and notably those which remove a further sORF, lead to an increase in reporter gene activity, some (Figure 3, lanes b2 versus b1 and b9 versus b8) have the opposite effect. Together with the results of deletion series A (Figure 3, lanes a2 and a3 versus a1), this shows that sequences at the 5′ and 3′ ends of the leader also have stimulatory effects on reporter gene activity.

In contrast, two long stretches in the internal region covering sORFs A to C (Figure 3, b7 and b8 versus b6 in series B and d2 and d3 versus 20 in series D) and sORFs D′ to E (e.g. b3 versus b6) have inhibitory effects. The gradual increase in reporter gene activity upon successive deletion of these sORFs suggests that the inhibitory effect is caused by these sORFs and that each of them contributes slightly (at most an ~3-fold reduction) to the overall effect. The deletions in series C (especially c3 versus c2 in Figure 3) disclose a third region whose deletion leads to reduced activity. This stimulatory region lies between the two major inhibitory regions. The three stimulatory regions have been named S1, S2 and S3, and the inhibitory regions I1 and I2 (Figure 3).

The stimulatory region S1 is operationally defined as the first 60 nucleotides (Figure 3, lanes b8 versus b9 or c2 versus d2); its detailed analysis is not covered by this paper.

S2 is operationally defined as nucleotides +178 to +239 (Figure 3, c2 versus c3 or d3 versus d5). Region S2, however, may extend into I1, since the strongest reduction of downstream gene expression was observed upon deletion of not only the core S2 region but also I1 (Figure 3, d5 versus d6), in spite of the clear inhibitory effect of I1 alone. Certain truncated forms of the S2 region (Figure 3, d4 and d7) still elicited a partial stimulation. This might be explained by the presence of several redundant sequence motifs that are active in the stimulatory mechanism. Such a sequence motif might be CPyAAAPuPyC, which occurs four times in S2 (Figure 2a). Deletion of either the first two or the last two copies of this motif (Figure 3, d4 and d7) is less detrimental to reporter gene expression than deletion of all four of them (Figure 3, d5 and d6).

Region S3 is subdivided into two sections, S3a and S3b, because two non-overlapping deletions in this region cause a reduction of downstream gene expression: the location of S3a is defined by mutant pLC-b2 (Figure 3) as +519 to +555 and that of region S3b by the deletion end points of mutants pLC-a1 and pLC-a2 as +557 to +580. Individual deletion of each of the regions resulted in a reduction of gene expression, with a more dramatic effect of deletion of S3b (Figure 3, a2 versus 20 in series A and b2 versus 20 in series B). A combined deletion had a similar effect as the individual deletion of S3b alone (Figure 3, a2 versus a3). These findings could indicate that the actual signal spans the *Cla*I site at +555. This is, however, unlikely since a small deletion from +550 to +556 did not change the CAT

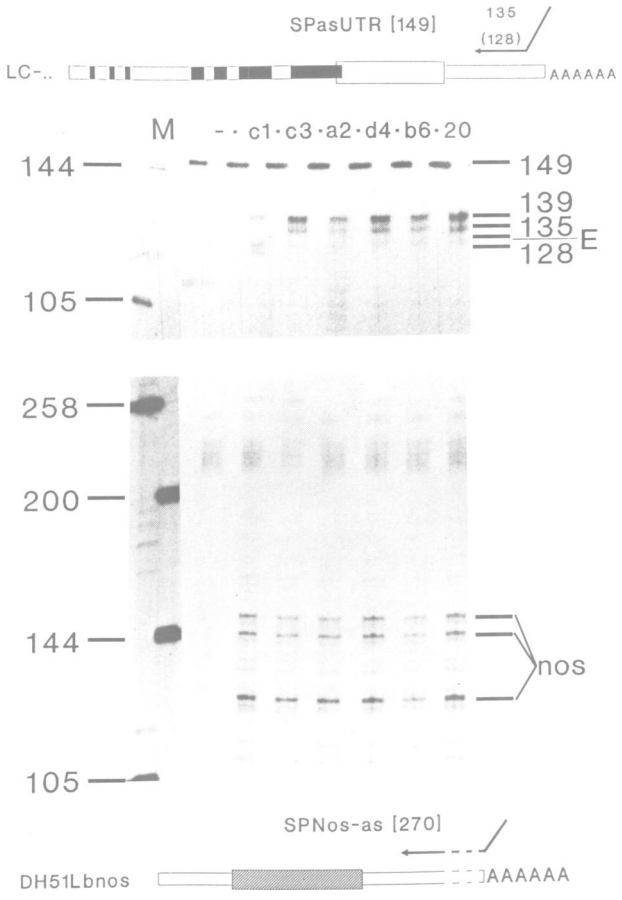

Fig. 4. RNA quantification. Total RNA from transfected *O. violaceus* protoplasts was analysed by RNase A/T1 mapping as described in Materials and methods. A representative set of pLC-plasmids was cotransfected with the internal standard plasmid pDH51Lbnos. The induced RNA species were hybridized to two different antisense RNAs, each specific for parts of the 3′ untranslated regions, to compare the relative amounts of both RNA species. The structure of the expected mRNAs and their interaction with the respective antisense probes is shown above and below the autoradiographs. RNase resistant fragments were separated and detected as described in Materials and methods. The length of the unprocessed probes is given in square brackets with the names of the antisense RNA generating plasmids, and the expected size of protected fragments is indicated. Lanes designated M: size markers; the fragment size is shown at the left. Lanes designated (.): unprocessed probes. Lanes designated -, c1, c3, a2, d4, b6 and 20: RNA induced by calf thymus DNA or the respective LC plasmids plus DH51Lbnos. **Upper panel**: analysis with the SPasUTR probe. Fragment sizes are indicated on the right. pLC-c1 mRNA protects a 128 nt fragment (in contrast to 135 nt in the case of the other LC plasmids), because a *Sal*I site within the probed region was filled-in during cloning. The 139 nt fragment is probably caused by an accidental, imperfect base-pairing between a 5′ part of the probe and a RNA region close downstream of the perfect homology. E: non-specific band. **Lower panel**: analysis with the SPNos-as probe. Due to heterogeneity in the 3′ end formation of mRNA at the nos terminator signal, SPNos-as protects three fragments (Bevan *et al.*, 1982) which are marked.

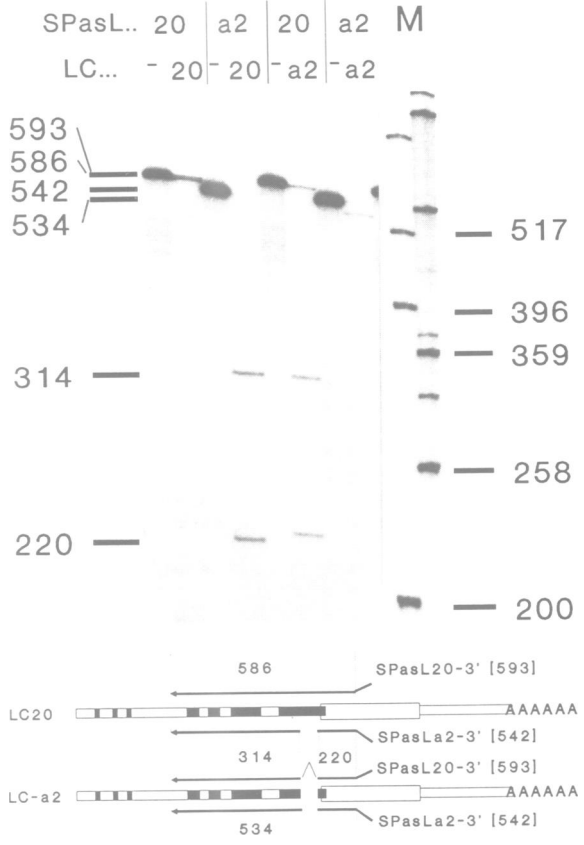

Fig. 5. Analysis of the LC-RNA leader region. RNA was prepared from protoplasts transfected with pLC20 or pLC-a2. Both RNA species were probed with antisense RNA from SPasLC20-3′ and SPasLCa2-3′ as indicated in the first two lines. The structures of the respective RNA and the expected interaction with the different probes are shown schematically, as in Figure 4. The fragment sizes are indicated at the left. Lanes designated (−): undigested probe; M: size markers. Sizes are shown at the right. The slight difference in size of the 220 nt fragment is probably caused by the different accessibility of the nucleotides at the site of discontinuity in the two mRNA−probe interactions.

expression level (Figure 3, b1 versus 20). So it seems that the stimulatory effect of region S3 is made up of contributions by two non-contiguous sequences, whose influences are interdependent but not equally strong.

RNA analysis

To investigate whether the stimulatory effects observed were due to an increase in RNA amount or due to an enhancement of translation we analysed the RNA. Total RNA was prepared from *O. violaceus* protoplasts transfected with a representative selection of mutants and CAT mRNA levels were determined by RNase A mapping and compared with an unrelated RNA species, transcribed from a co-transfected plasmid (Figure 4). CAT RNA levels were similar for all constructs tested, whereas the CAT activities varied by a factor of 30 between the lowest (pLC-c3) and the highest (pLC-b6) in the series (Figure 3).

The observed slight variations in RNA levels could have been caused by different RNA stabilities, however, variations from experiment to experiment were in the same order of magnitude. The significance of these differences remains uncertain at present.

Spliced CaMV RNAs have not been detected in plants infected with wild-type strains of CaMV and the 35S RNA leader does not contain a dicotyledonous plant mRNA intron according to the rules of Goodall and Filipowicz (1989). However, abnormal types of splicing or other types of processing might occur; for this reason, we analysed the upstream region of the CAT transcripts by RNase A/T1 mapping (for a representative example see Figure 5). The antisense probe transcribed from SPasL20-3′ covers 216 nt

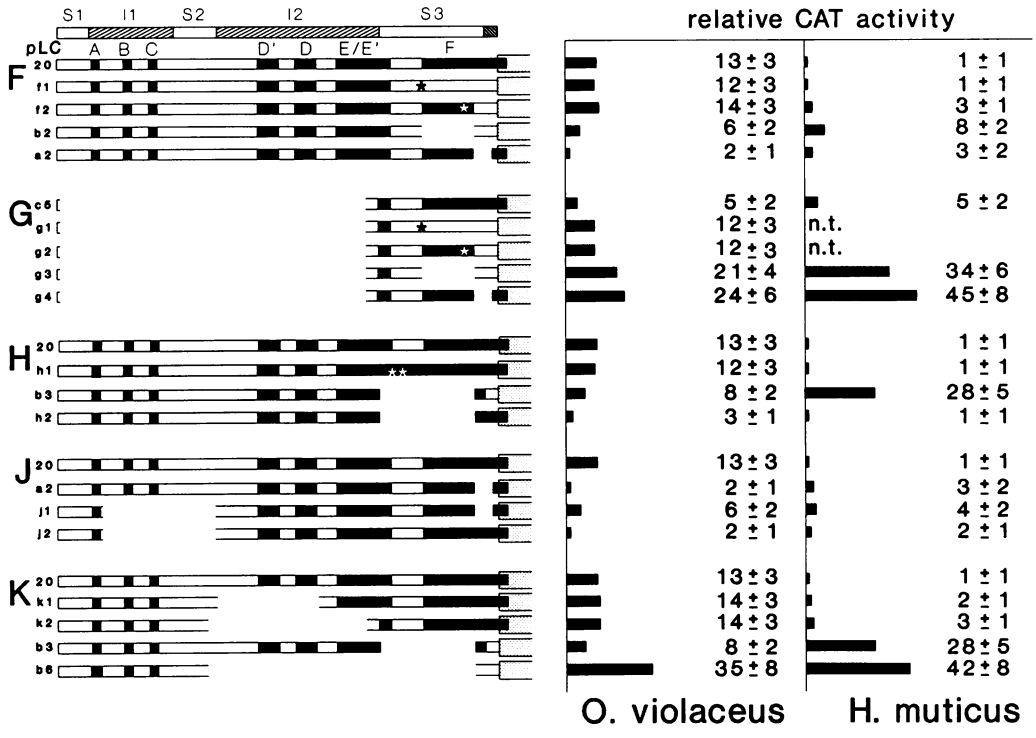

Fig. 6. Context dependent effects of leader mutants. Mutants are shown as in Figure 3. (★) denotes point-mutations. The average induced CAT activity is shown relative to that of pLC-b8. (n.t.) not tested.

of the CAT ORF and 370 nt of the leader (Figure 5); a spliced, functional mRNA would therefore produce a protected fragment of less than full length but longer than 216 nt. No such fragment was obtained when the pLC20-derived mRNA was probed with the SPasL20-3′ antisense RNA (Figure 5, second lane). A processed mRNA would have been detected, if present, as is shown by reconstitution experiments; here a discontinuity was deliberately introduced between the mRNA and the antisense probe by deletion in either the mRNA (transfection with pLC-a2 and probing with SPLas-20-3′ RNA) or in the antisense probe (SPasLa2-3′ antisense RNA used as probe for pLC20 mRNA). In all these cases, and also in probing pLC-a2 RNA with the homologous SPasLa2-3′ antisense RNA, exclusively those protected RNA fragments were observed that are expected for unprocessed mRNAs (Figure 5). Also, no indications of RNA processing were obtained when the 5′ half of the leader was used as antisense probe (not shown).

Low abundance mRNA species may remain undetected in this type of assay. However, interpretation of the data presented in Figure 7 (see below) on the grounds of splicing would postulate processing of ~50% of the RNA molecules.

Since no detectable RNA processing takes place, and since the different CAT mRNAs are present in equivalent amounts, we conclude that the large differences observed in CAT expression reflect different translation efficiencies of the respective mRNA species. Minor variation (e.g. between pLC-c1 and pLC20), however, could still be caused by changes in transcription or mRNA stability.

Interdependence of inhibitory and stimulatory effects

While the effect of region S1 was detectable regardless of whether the rest of the leader was present or not (Figure 3, b8 versus b9 or c2 versus d2), a stimulatory effect of regions S2 or S3 was observed only when both regions were present. This can be inferred from mutant series B (Figure 3, b6 versus b7) and C (Figure 3, c5 versus b9) but is most clearly shown in series J (Figure 6): deletion of either S2 or S3 caused similar reduction of CAT activity (a2 versus j2). By combining both deletions (j1), this activity level was not reduced further but increased slightly.

Likewise, the inhibitory effect of region I2 was only observed in the absence of either S2 or S3 (Figure 3, series C and B, respectively). Its deletion then led to an ~5-fold increase of CAT activity (b3 versus b6 and c3 versus c5) while its deletion in the presence of S2 and S3 had no effect (Figure 6, series K).

These data clearly indicate that, in the context of the complete leader, the inhibitory effect of region I2 is offset by other leader sequences.

Simultaneous assay of translation from sORF D and ORF VII

Since the results described above indicated that the S3 region may influence expression from within I2, we constructed a plasmid series to analyse simultaneous expression of sORF D within region I2 and of ORF VII. A β-glucuronidase (GUS) reporter gene (Jefferson, 1987) was fused to the second codon of sORF D. Downstream of the GUS ORF we added leader regions I2 and S3 followed by the ORF VII CAT fusion, yielding plasmid LC20-DG (Figure 7). The same deletions of S2 and S3, respectively, as in pLC-a2 and pLC-d5 were introduced into pLC20-DG to analyse the effect of those regions (Figure 7, a2-DG and d5-DG). In these constructs region I2 is elongated by ~2 kb and contains 34 additional AUG codons. Although the absolute level of CAT expression from pLC20-DG is only in the range 25–50% of that from pLC20, CAT expression from ORF VII shows the same qualitative dependence on regions S2 and S3

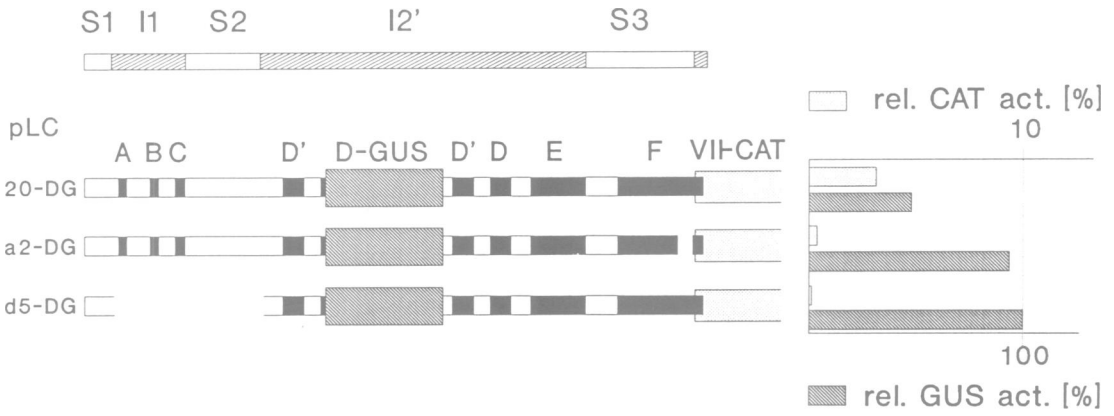

Fig. 7. Simultaneous assay of translation from sORF D and ORF VII. The relevant part of the plasmids is shown as in Figures 3 and 6. A GUS ORF (hatched box, not to scale) is fused to sORF D. The region around sORF D' is duplicated in these plasmids. The induced enzyme activities in transfected *O. violaceus* protoplasts were determined as described in Materials and methods. Average activities are represented in the left panel. Shaded bars denote CAT activity relative to construct pLC-b8, hatched bars denote GUS activity relative to pLC-d5-DG.

(Figure 7). The reduction of CAT expression relative to pLC20 could be caused by interference of the inserted sequence with mRNA accumulation or with translation of the second ORF. Interestingly, GUS expression from sORF D is 2-fold lower in the presence of S2 upstream and S3 downstream than in the absence of either one of these regions. We conclude that the mechanism mediated by regions S2 and S3 simultaneously allows translation from ORF VII and reduces it from the sORFs in region I2.

Expression of the ORF VII/CAT fusion is not inhibited by an overlapping upstream sORF in the presence of S2 and S3

The last sORF within the leader sequence (sORF F) overlaps ORF VII. Reading frame overlap has been shown to be particularly detrimental to expression of a downstream ORF when an upstream one is expressed (Kozak, 1986a). We have analysed the influence of sORF F by mutating its start codon (Figure 6, f1) or by introducing a frameshift within its coding region (Figure 6, f2) by insertion of two bases. In the context of the complete leader sequence, both mutations had no effect on downstream CAT expression (Figure 6, series F). However, in deletion mutants lacking, among others, the S2 region, both mutations led to an ~2-fold increase of CAT activity (Figure 6, c5 versus g1 and g2). By fusing sORFs E and F we created an overlapping sORF that started with an AUG codon that had been shown earlier to function as an initiation codon (Fütterer *et al.*, 1988). The overlap was only inhibitory in the absence of S3 (Figure 6, series H; compare mutants pLC-b3 and -h2, differing by only two bases) but had no effect when S2 and S3 were present (Figure 6, h1 versus 20). This is another example of translation inhibition caused by an upstream sORF being alleviated by the presence of S2 and S3 in the CaMV 35S RNA leader.

Region S3 is inhibitory in the absence of other leader sequences

A comparison of mutants pLC-c5 and -b9 (Figure 3, series B) shows that the 3′ end of the leader exerts a strong inhibitory effect. As shown above, this is caused to some degree by the overlap of sORF F and ORF VII. An internal AUG of sORF E, which is in an optimal sequence context may also contribute to this effect. Still, mutant series G and J revealed inhibitory effects in region S3 that seems to be independent of translation of any sORF (Figure 6, g3 versus g1 and g4 versus c5; j1 versus j2). Since the respective RNA segment is also devoid of any obvious secondary structure, inhibition of translation could best be explained by tightly bound cellular factor(s) as for ferritin mRNA (Leibold and Munro, 1988; Rouault *et al.*, 1988). The same factor(s) may be involved in the stimulatory mechanism active in the presence of S2 and S3, since deletion of the presumptive binding region interfered with the stimulation (Figure 3, b2 and a2).

Stimulation by regions S2 and S3 is inefficient in H.muticus protoplasts

We have investigated the influence of the leader on downstream gene expression in protoplasts derived from plant species that do not support virus infection.

A comparison of the expression of mutant series A–D (Figure 3) in *H. muticus* (non-host) and in *O. violaceus* (host) protoplasts showed that all constructs lacking one of the two stimulatory regions S2 and S3 induce similar levels of CAT activity in the two systems (e.g. b3 to b9 and c3 to c5 in Figure 3). The construct containing both the S2 and S3 regions, however, expressed CAT much less efficiently in *H. muticus* protoplasts. In fact, in certain constructs region S3 exhibited a particularly strong inhibitory effect in these protoplasts [compare b2 and 20 (Figure 3) or c5 and g3 and g4 (Figure 6) for the two protoplast species]. Most protoplast batches obtained from *Nicotiana plumbaginifolia* leaves or from *N. tabacum* cell suspensions (non-hosts) reacted like *H. muticus* protoplasts in yielding only very low levels of CAT activity after transfection with leader-containing constructs. However, some batches responded like *O. violaceus* protoplasts or showed an intermediate response (Fütterer *et al.*, 1989). Since the behaviour of these types of protoplasts was unpredictable, no systematic study was performed. The results obtained suggest that the variability described here is caused by the variation of host factors participating in the stimulatory effect of regions S2 and S3.

In contrast to regions S2 and S3, regions I1, I2 and S1 are similarly active in all types of protoplasts (e.g. Figure 3 series B for *H. muticus*).

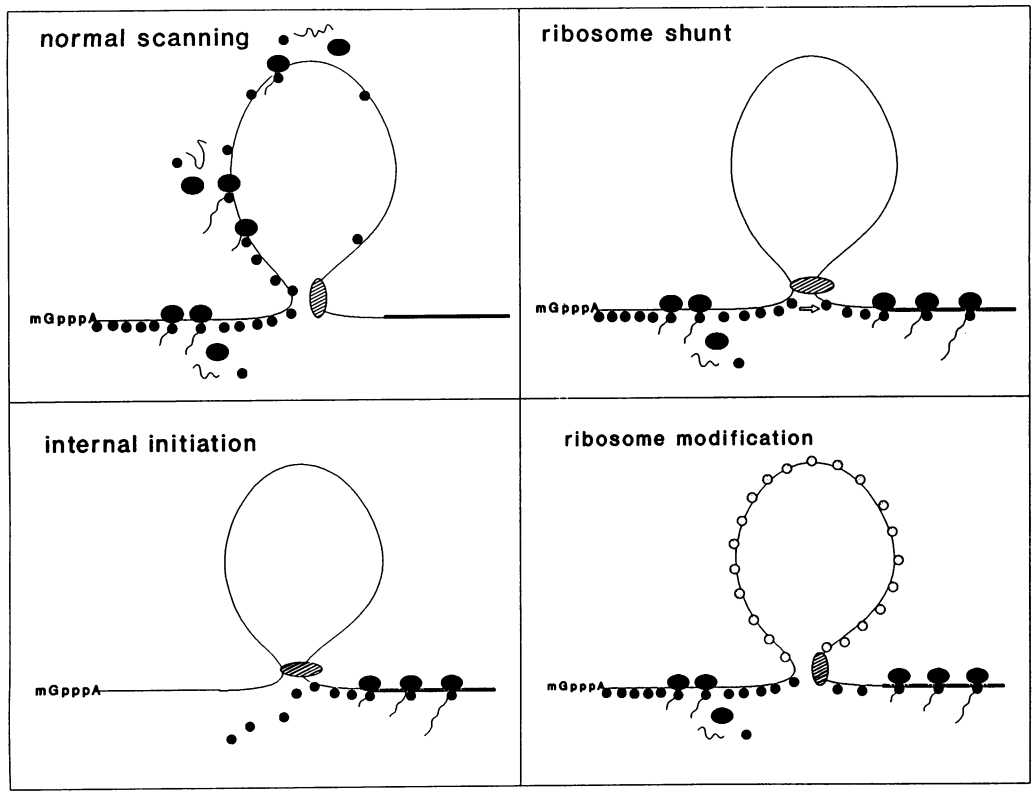

Fig. 8. Models for translation initiation downstream of the 35S RNA leader. Schematic representation of potential translation events within and downstream of the leader sequence. A thin line represents the leader sequence and the hatched bar the first longer ORF downstream of the leader. The sORFs in the leader are only shown indirectly as sites, where 40S (●) and 60S (⬬) ribosomal subunits and peptides (∿) are produced. Small open circles (○) in the ribosome modification model denote modified 40S ribosomal subunits, no longer inhibited in scanning by the sORFs in the leader. (▨): cellular factor(s) bound to region S3. The models are explained in the text.

Discussion

We found that the 600 nt long CaMV 35S RNA leader sequence consists of a mosaic of inhibitory and stimulatory sequences (S1−I1−S2−I2−S3), which influence the expression level of a downstream reporter gene in plant cells. Stimulator region S1, located directly downstream of the cap-site, stimulates expression ~2-fold, regardless of the type of plant cells used and regardless of the properties of the rest of the leader. We assume that S1 is either important in mRNA production or provides an efficient ribosome binding site, e.g. leader sequences of some plant virus mRNA species (Jobling and Gehrke, 1987; Gallie *et al.*, 1987). A duplicated $(CT)_n$ sequence motif (Figure 2a) that is conserved between different caulimoviruses might be involved in the stimulation process (our unpublished observations).

In conclusion, from RNA analysis we assume that the leader region following S1 exerts its influence on gene expression at the level of translation. This region has the potential to inhibit downstream translation 50- to 100-fold; such a degree of inhibition is observed in the absence of either region S2 or S3 or the relevant host factors. Inhibition in this case is in accordance with the scanning model for translation initiation (Kozak, 1989a) and apparently mainly reflects the dissociation of ribosomes from the RNA after translation of sORFs (Figure 8). Every sORF, including sORF F, which overlaps ORF VII, has only a small inhibitory effect (1.5- to 3-fold). Apart from sORFs, sequences within S3 that may tightly bind a cellular factor also contribute to the inhibition of scanning.

In protoplasts derived from the CaMV host plant *O. violaceus*, repression of downstream translation by the leader sequence is alleviated. This requires the simultaneous presence of regions S2 and S3 and results in a 5- to 10-fold increase of downstream gene expression. When all the components of the derepression process are present, region I2 does not significantly influence downstream translation. Insertion of a large additional reporter gene into I2 is tolerated and comparison of the reporter gene activities within I2 and downstream of the leader showed that translational activity in I2 is reduced by ~50%, when downstream expression is derepressed. The requirement for two translation stimulating regions upstream and downstream of a presumptive obstacle for scanning ribosomes is particularly difficult to reconcile with the modified scanning model for translation initiation in eukaryotes (Kozak, 1989a). It is unlikely that these stimulatory sequences simply act as spacers to enhance the ability of ribosomes to reinitiate by increasing the distance between a terminator codon and the next AUG codon, as was observed in other systems (Kozak, 1987b; Miller and Hinnebusch, 1989), since the requirement for S2 is observed in the absence of any preceding sORF (d3 versus d4, Figure 3) and since translation of ORF VII/CAT is clearly independent of the configuration of translation start and stop codons in the context of the complete leader (Figure 6, mutants f1, f2 and h1). Furthermore, a deletion of 17 nucleotides of S3b (including

nucleotides +559 to +575) had a similar negative effect on translation as complete deletion of S3, although it changes the spacing less than, for example, the neutral deletions in mutant a1 (our unpublished observations).

Our findings suggest that ribosomes initiating translation at the ORF VII AUG either do not scan region I2 at all, or possess altered properties while passing this region. We see three possible ways that this could be achieved (Figure 8).

(i) Internal initiation. Regions S1 and S3 would form a structural element that allows direct binding of ribosomes. Internal initiation occurs in prokaryotic operons but is unusual in eukaryotic systems. It probably occurs on the uncapped picornavirus RNAs (Pelletier and Sonenberg, 1988; Jang *et al.*, 1988) and has been proposed for a variety of capped viral mRNAs (for review, see Herman, 1989). However, direct internal binding of ribosomes should not be negatively influenced by sequence elements upstream of the binding site. Data from mutant series D demonstrate that there is an inhibitory effect of leader region I1 on downstream expression, which would suggest that ribosomes pass this region before they initiate translation at ORF VII.

(ii) Ribosome modification. Scanning ribosomes, while passing region S2, would become modified to lose competence for translation initiation but retain competence for scanning until region S3 is reached, where the modification would be reversed. This would correspond to an enhancement of leaky scanning. Since we did not observe any inhibitory effect of region S2 on expression from the sORF D-GUS or CAT fusion in the absence of S3 (our unpublished observations), presence of S2 alone cannot be sufficient for modification to initiation incompetence. A mechanism that involves both regions S2 and S3 in such a modification process is theoretically possible, but we regard it as highly improbable.

An alternative to enhancement of leaky scanning could be a modification that increases the reinitiation efficiency, e.g. by inhibiting post-translational ribosome disengagement from the mRNA. However, the decrease in I2 translation and the requirement for sequence regions that are far apart from each other argue against such a model.

(iii) Ribosome shunt. Regions S2 and S3, in cooperation with host factors, would form a bypass complex, i.e a structural element allowing direct translocation of 40S ribosomal subunits from a region near the 5′ end of the leader to a position directly upstream of the AUG of ORF VII, thereby skipping the inhibitory region I2. A related model based on RNA secondary structure and ribosome binding studies has been proposed for translation of reading frames downstream of avian retrovirus leader sequences (Darlix *et al.*, 1982). Hull (1984) suggested a similar model that involves RNA folding and discontinuous ribosome translocation for expression of CaMV ORFs I–V; however, no experimental data in support of this model have been published so far.

In effect, modification of the translation initiation capabilities of scanning ribosomes and ribosome shunt could result in very similar expression patterns. On the other hand, ribosome shunt and direct internal ribosome binding may be related. In the shunt process, transfer of ribosomal subunits from S2 to S3 would involve loosening of the ribosome–mRNA interaction around region S2 and novel binding to region S3. Ribosome binding at the cap and subsequent scanning to region S2 may serve to increase the concentration of ribosomes in the vicinity of S3 so that binding would become more efficient. This may be necessary since the CaMV 35S RNA, in contrast to picornaviral RNAs, has to be translated in competition with capped cellular mRNAs. It seems possible that in (some) picornaviruses a similar shunt could enhance the frequency of ribosome-binding to the internal 'landing-pad'. Data from deletion analysis of the leader of encephalomyocarditis virus would be consistent with both mechanisms (Howell *et al.*, 1990). So far, the ribosome shunt model fits our data best. Since at present the differences in reporter gene expression that might distinguish between the models are only small, additional experiments are required for a final discrimination between the different possibilities. Such experiments are in progress.

None of the three models excludes the possibility that a fraction of ribosomes continue to scan into I2. We cannot calculate this fraction for the original leader, but in the case of plasmid LC20-DG, about half of the ribosomes follow the bypass-route, while the remaining ones resume normal scanning. All three models imply a structural element containing regions S2 and S3.

Sequences required for optimal stimulation are distributed in S2 and S3 over a range of ~60–80 nucleotides each. In both cases partial deletions within the respective region still allow partial stimulation, indicating the presence of multiple active sequence elements. One such element could be CPyAAAPuPyC, which is repeated four times in region S2 (Figure 2a). Direct base pairing between regions S2 and S3 cannot be predicted from the sequence. Instead, an RNA complex might be formed with a cellular factor(s), the influence of which has been documented by our host-range experiments. Involvement of a cellular factor in internal ribosome binding was recently proposed for poliovirus (Meerovitch *et al.*, 1989; del Angel *et al.*, 1989).

Although we have not performed a systematic analysis of the influence of the leader secondary structure on ORF VII expression, our data suggest that this structure is not important for the operation of the bypass mechanism. In the central portion of the predicted hairpin (Fütterer *et al.*, 1988) deletions (Figure 6, k1 and k2) as well as insertions (Figure 7, 20-DG) are tolerated. Furthermore, sequences in the 5′ part of the leader involved in formation of the base of the hairpin structure are dispensible (Figure 3, d3). Interestingly, the bypass process described here occurs in plant cells in the absence of virus infection and involves specific cellular factors. Similar mechanisms may therefore be active in the normal expression of certain plant genes and perhaps in other organisms as well. The potentially increased competitiveness by using the cap-dependent ribosome binding seems to favour a bypass mechanism over direct internal ribosome binding for capped mRNAs. The cap-dependence but scanning-independence of translation of the Sendai virus X protein from a downstream ORF on the P/C mRNA (Curran and Kolakofski, 1988b) could be explained by the ribosome shunt model. A more detailed analysis of the leader sequences of some of the complex mRNAs, e.g. many oncogene mRNAs (Kozak, 1987c; Marth *et al.*, 1988;

Rao *et al.*, 1988; Muller and Witte, 1989), may reveal the presence of similar, cell factor-dependent stimulatory sequences.

For CaMV, a bypass mechanism as described above might allow the translation of ORFs even further downstream on the 35S RNA. Since such a process could be regulatable, the respective ORFs could then be expressed differentially. In addition, AUG start codons within an ORF could be used to produce N-terminally truncated proteins. Preliminary data from an analysis of requirements in *cis* for expression of further downstream ORFs on the 35S RNA support such a model. This mode of gene expression could exist together with the termination−reinitiation mechanism that was suggested previously for ORFs I and III (Sieg and Gronenborn, 1982; Dixon and Hohn, 1984), but it is also possible that our findings could lead to reinterpretation of the genetic data on which the proposal of the reinitiation model was based.

In spite of the bypass process, the overall effect of the leader sequence in the experiments described is a reduction of downstream translation. This inhibition can be alleviated by coexpression of the virus encoded transactivator (J.F. and T.H., in preparation) that is also absolutely required for translation of further downstream CaMV ORFs (Bonneville *et al.*, 1989; Gowda *et al.*, 1989). The leader sequence may therefore regulate the use of the 35S RNA as mRNA or as template for reverse transcription depending on the availability of the transactivator.

Materials and methods

Plasmids for transient expression

ORF VII/CAT fusions. The construct pLC20, containing a CAT reporter gene fused to the AUG of CaMV ORF VII downstream of a complete 35S RNA leader sequence and under control of CaMV transcription signals, was described previously (Fütterer *et al.*, 1988, 1989). A variety of deletions was introduced into the leader sequence with the use of naturally occurring restriction sites or of restriction sites previously introduced into the leader by oligonucleotide-directed mutagenesis (Zoller and Smith, 1983). Most members of mutant series B and D were generated by Bal-31 digestion starting at the *Cla*I-site (B) of pLC20 or the *Xho*I-site of mutant pLC-d1 (D). Mutants pLC-c1, -j1 and -j2 are derivatives of a deletion mutant in which sORFs A and B have been fused by oligonucleotide-directed mutagenesis under creation of a *Sac*I-site (pLC-X; Table I). Mutants of series C and D and pLC-b9 were constructed by insertion of appropriate restriction fragments into the vector pDHTZ described previously (Fütterer *et al.*, 1989). Plasmid pLC-b9 is the same as pC20 of Fütterer *et al.* (1989) and plasmid pLC-k1 is the same as pLC421 of Fütterer *et al.* (1988). For a short description of the mutants see Table I. All steps in the cloning procedures were performed as recommended by the suppliers of the respective enzymes or as described (Maniatis *et al.*, 1982).

The 35S RNA leader sequence used in this study was derived from CaMV strain CM4-184 (Figure 2a; Dixon *et al.*, 1986). The numbering refers to the transcription start site as +1, which is located at genome position 7014 [in analogy to the transcription start site of the Cabb-S strain (Guilley *et al.*, 1982)].

Double-reporter gene constructs. Plasmid LC15 (Fütterer *et al.*, 1988) was opened at its *Xho*I site, treated with Klenow DNA polymerase, digested with *Eco*RI and the large fragment isolated. Into this a GUS reading frame was cloned as a *Nco*I(filled in)−*Bam*HI fragment derived from plasmid RAJ275 (Jefferson, 1987), together with the small *Bgl*II−*Eco*RI fragment of plasmids LC20 or LC-a2, yielding plasmids LC20-DG or LC-a2-DG, respectively. To create pLC-d4-DG, the small *Bgl*II(filled in)−*Eco*RI fragment of pLC20-DG was cloned into the large *Xho*I(filled in)−*Eco*RI fragment of pLC-d4.

Suspension cultures and protoplast preparation

Cell suspension cultures of *O. violaceus* and of *H. muticus* were gifts of C.Matsui and P.King, respectively. Culture conditions and protoplast preparation have been described previously (Fütterer *et al.*, 1989).

Transient expression assays

Routinely, 2×10^6 protoplasts were transfected by electroporation (Fromm *et al.*, 1985) with 10−15 μg of circular plasmid DNA as described (Fütterer *et al.*, 1988, 1989). After overnight incubation (12−24 h) a protein extract was prepared and the CAT or GUS activity was determined as described previously (Gorman *et al.*, 1982; Jefferson *et al.*, 1986; Bonneville *et al.*, 1989). For CAT quantification, assay conditions were chosen such that not more than 20% of the radioactive chloramphenicol was converted to the acetylated forms.

The response of plant protoplasts to transfection with a given construct varied by about ±20% of the respective induced reporter enzyme activity levels. To compare slightly different expression levels reliably, most constructs were tested >10 times and none less than four times, using at least two different DNA preparations. For all plasmids that induced very low levels of CAT activity, two independent clones were tested. Since the absolute expression levels varied between the batches of protoplasts used, while the relative levels remained constant (within the range mentioned), we present the CAT expression data relative to the reference plasmid pLC-b8 and GUS expression data relative to plasmid LC-d5-DGus.

RNA analysis

Quantitative and qualitative RNase A/T1 mapping was performed on total RNA, isolated 6−8 h post-transfection as described previously (Bonneville *et al.*, 1989). As an internal standard for quantification, plasmid DH51Lbnos [a derivative of pDH51Lb (Wiebauer *et al.*, 1988)], containing part of a leghaemoglobin gene under control of the CaMV 35S promoter and the nos polyadenylation signal, was cotransfected with the leader mutants. The nos polyadenylation signal was derived as a *Pst*I−*Hin*dIII fragment from plasmid NosCAT (Fromm *et al.*, 1985) and inserted between the same restriction sites of pDH51Lb. The same fragment was cloned between the respective restriction sites of plasmid Gem-1 (Promega), yielding pSPNos-as. pSPNos-as was linearized with *Pst*I and transcribed with SP6 polymerase (Melton *et al.*, 1984) to produce radioactively labelled RNA antisense to the 3′-untranslated region of the RNA transcribed from pDH51Lbnos. For generation of labelled RNA antisense to the 3′-untranslated region of the pLC-series, which was used as the probe in the quantification experiment, the small *Pst*I−*Bgl*II fragment of plasmid LC20 was cloned into the *Pst*I−*Bam*HI opened plasmid Gem-1 (Promega) (pSPasUTR). Prior to transcription with SP6 RNA polymerase the resulting plasmid was cut with *Eco*O109.

To detect potential spliced RNA species, antisense RNA probes were prepared by SP6 transcription of *Bgl*II linearized plasmids SPasL20-3′ or SPasLa2-3′ containing the small *Eco*RI−*Eco*RV fragment of pLC20 or pLC-a2, respectively, in pGem-2 (*Eco*RI−*Sma*I).

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References

de Angel,R.M., Papavassiliou,A.G., Fernandez-Tomas,C., Silverstein,S.J. and Racaniello,V.R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8299−8303.
Baim,S. and Sherman,F. (1988) *Mol. Cell. Biol.*, **8**, 1591−1601.
Baughman,G. and Howell,S.H. (1988) *Virology*, **167**, 125−135.
Bevan,M., Barnes,W. and Chilton,M.D. (1982) *Nucleic Acids Res.*, **11**, 369−385.
Bonneville,J.M., Hohn,T. and Pfeiffer,P. (1988) In Domingo,E., Holland,J.J. and Ahlquist,P. (eds), *RNA Genetics*. CRC Press, Boca Raton, FL, Vol. 2, pp. 23−42.
Bonneville,J.M., Sanfaçon,H., Fütterer,J. and Hohn,T. (1989) *Cell*, **59**, 1135−1143.
Chevrier,D., Vézina,C., Bastille,J., Linhard,C., Sonenberg,N. and Boileau,G. (1988) *J. Biol. Chem.*, **263**, 902−910.
Curran,J. and Kolakofsky,D. (1988a) *EMBO J.*, **7**, 245−251.
Curran,J. and Kolakofski,D. (1988b) *EMBO J.*, **7**, 2869−2874.
Darlix,J.-L., Zuker,M. and Spahr,P.-F. (1982) *Nucleic Acids Res.*, **10**, 5183−5196.
Dixon,L. and Hohn,T. (1984) *EMBO J.*, **3**, 2731−2736.

Dixon,L., Nyffenegger,T., Delley,D., Martinez-Izquierdo,J.A. and Hohn,T. (1986) *Virology*, **150**, 463–468.
Fromm,M.E., Taylor,L.P. and Walbot,V. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5824–5828.
Fütterer,J., Gordon,K., Bonneville,J.M., Sanfaçon,H., Pisan,B., Penswick,J. and Hohn,T. (1988) *Nucleic Acids Res.*, **16**, 3877–8390.
Fütterer,J., Gordon,K., Pfeiffer,P., Sanfaçon,H., Pisan,B., Bonneville, J.M. and Hohn,T. (1989) *Virus Genes*, **3**, 45–55.
Gallie,D.R., Sleat,D.E., Watts,J.W., Turner,P.C. and Wilson,T.M. (1987) *Nucleic Acids Res.*, **15**, 3257–3273.
Geballe,A.P. and Mocarski,E.S. (1988) *J. Virol.*, **62**, 3334–3340.
Goodall,G.J. and Filipowicz,W. (1989) *Cell*, **58**, 473–483.
Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
Gowda,S., Wu,F.S., Scholthof,H.B. and Shepherd,R.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9203–9207.
Gronenborn,B. (1987) In Hohn,T. and Schell,J. (eds), *Plant DNA Infectious Agents*. Springer Verlag, Berlin, pp. 1–29.
Guilley,H., Dudley,R.K., Jonard,G., Balazs,E. and Richards,K.E. (1982) *Cell*, **30**, 763–773.
Hackett,P.B., Petersen,R.B., Hensel,C.H., Albericio,S.I., Gunderson,A.C., Palmenberg,A.C. and Barany,G. (1986) *J. Mol. Biol.*, **190**, 45–57.
Hay,N. and Aloni,Y. (1985) *Mol. Cell. Biol.*, **5**, 1327–1334.
Herman,R.C. (1989) *Trends Biochem.*, **14**, 219–222.
Hinnebusch,A.G. (1988) *Trends Genet.*, **4**, 169–174.
Hohn,T., Bonneville,J.M., Fütterer,J., Gordon,K., Pisan,B., Sanfaçon,H., Schultze,M. and Jiricny,J. (1989) In Staskawicz,B., Ahlquist,P. and Yoder,O. (eds) *UCLA Symposium on Plant–Microbe Interactions*. Alan R.Liss, Inc. New York pp. 153–165.
Howell,M.T., Kaminski,A. and Jackson,R.T. (1990) In Brinton,M. and Heinz,F.X. (eds), *Positive Strand RNA Viruses*. ASM Publications, in press.
Hull,R. (1984) *Plant Mol. Biol.*, **3**, 121–125.
Jang,S.K., Kräusslich,H.-G., Nicklin,M.J., Duke,G.M., Palmenberg,A.C. and Wimmer,E. (1988) *J. Virol.*, **62**, 2636–2643.
Jefferson,R.A. (1987) *Plant Mol. Biol. Rep.*, **5**, 387–405.
Jobling,S.A. and Gehrke,L. (1987) *Nature*, **325**, 622–625.
Khalili,K., Brady,J. and Khoury,G. (1987) *Cell*, **48**, 639–645.
Kozak,M. (1984) *Nucleic Acids Res.*, **12**, 3873–3893.
Kozak,M. (1986a) *Cell*, **44**, 283–292.
Kozak,M. (1986b) *Proc. Natl. Acad. Sci. USA*, **83**, 2850–2854.
Kozak,M. (1987a) *J. Mol. Biol.*, **196**, 947–950.
Kozak,M. (1987b) *Mol. Cell. Biol.*, **7**, 3438–3445.
Kozak,M. (1987c) *Nucleic Acids Res.*, **15**, 8125–8148.
Kozak,M. (1988) *Mol. Cell. Biol.*, **8**, 2737–2744.
Kozak,M. (1989a) *J. Cell. Biol.*, **108**, 229–241.
Kozak,M. (1989b) *Mol. Cell. Biol.*, **9**, 5134–5142.
Leibold,E. and Munro,H.N. (1989) *Proc. Natl. Acad. Sci. USA*, **85**, 2171–2175.
Liu,C.-C., Simonsen,C.C. and Levinson,A.D. (1984) *Nature*, **309**, 82–85.
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.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marth,J.D., Overell,R.W., Meier,K.E., Krebs,E.G. and Perlmutter,R.M. (1988) *Nature*, **332**, 171–173.
Meerovitch,K., Pelletier,J. and Sonenberg,N. (1989) *Genes Dev.*, **3**, 1026–1034.
Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Theor. Appl. Genet.*, **59**, 191–195.
Miller,P.F. and Hinnebusch,A.G. (1989) *Genes Dev.*, **3**, 1217–1225.
Muller,A.J. and Witte,O.N. (1989) *Mol. Cell. Biol.*, **9**, 5234–5238.
Nomura,S., Khoury,G. and Jay,G. (1983) *J. Virol.*, **45**, 428–433.
Parkin,N.T., Cohen,E.A., Darveau,A., Rosen,C., Haseltine,W. and Sonenberg,N. (1988) *EMBO J.*, **7**, 2831–2837.
Peabody,D.S. and Berg,P. (1986) *Mol. Cell. Biol.*, **6**, 2695–2703.
Peabody,D.S., Subramani,S. and Berg,P. (1986) *Mol. Cell. Biol.*, **6**, 2704–2711.
Pelletier,J. and Sonenberg,N. (1988) *Nature*, **334**, 320–325.
Petersen,R.B., Moustakas,A. and Hackett,P.B. (1989) *J. Virol.*, **63**, 4787–4796.
Pietrzak,M., Shillito,M., Hohn,T. and Potrykus,I. (1986) *Nucleic Acids Res.*, **14**, 5857–5868.
Rao,C.D., Pech,M., Robbins,K.C. and Aaronson,S.A. (1988) *Mol. Cell. Biol.*, **8**, 284–292.
Rouault,T.A., Hentze,M.W., Caughman,S.W., Harford,J.B. and Klausner,R.D. (1989) *Science*, **241**, 1207–1210.
Sedman,S.A. and Mertz,J. (1988) *J. Virol.*, **62**, 954–961.
Sedman,S.A., Good,P.J. and Mertz,J.E. (1989) *J. Virol.*, **63**, 3884–3893.
Sieg,K. and Gronenborn,B. (1982) *Abstracts of the NATO Advanced Studies Institute. Advanced Course*. p. 154 (abstract).
Werner,M., Feller,A., Messenguy,F. and Pierard,A. (1987) *Cell*, **49**, 805–813.
Wiebauer,K., Herrero,J.-J. and Filipowicz,W. (1988) *Mol. Cell. Biol.*, **8**, 2042–2051.
Zoller,M.J. and Smith,M. (1983) *Methods Enzymol.*, **100**, 468–500.

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