The pregenomic 35 S RNA of cauliflower mosaic virus (CaMV) belongs to the growing number of mRNAs known to have a complex leader sequence. The 612-nucleotide leader contains several short open reading frames (sORFs) and forms an extended hairpin structure. Downstream translation of 35 S RNA is nevertheless possible due to the ribosome shunt mechanism, by which ribosomes are directly transferred from a take-off site near the capped 5′ end of the leader to a landing site near its 3′ end. There they resume scanning and reach the first long open reading frame. We investigated in detail how the multiple sORFs influence ribosome migration either via shunting or linear scanning along the CaMV leader. The sORFs together constituted a major barrier for the linear ribosome migration, whereas the most 5′-proximal sORF, sORF A, in combination with sORFs B and C, played a positive role in translation downstream of the leader by diverting scanning ribosomes to the shunt route. A simplified, shunt-competent leader was constructed with the most part of the hairpin including all the sORFs except sORF A replaced by a scanning-inhibiting structure. In this leader as well as in the wild type leader, proper translation and termination of sORF A was required for efficient shunt and also for the level of shunt enhancement by a CaMV-encoded translation transactivator. sORF A could be replaced by heterologous sORFs, but a one-codon (start/stop) sORF was not functional. The results implicate that in CaMV, shunt-mediated translation requires reinitiation. The efficiency of the shunt process is influenced by translational properties of the sORF.

At least three distinct steps involving 40 S ribosomal subunits and the mRNA 5′-untranslated region (5′-UTR) precede the formation of translation competent 80 S ribosomes on most mRNAs in eukaryotic cells. First, the 40 S subunit binds to the capped 5′ end of the mRNA; second, ribosomes translocate to the start codon by linear scanning; and third, the start codon is recognized (1–3). A number of ribosome- or RNA-associated protein factors are involved in cap recognition, removal of RNA secondary structure, and initiation complex formation (4). The helix-destabilizing capability of the scanning ribosome appears limited. Double-stranded regions with a free energy of less than −50 kcal/mol block the scanning process (5). Besides secondary structure, alternative translation start sites could interfere with scanning. Since the composition of the scanning complex is altered upon translation initiation, most notably by “consumption” of the bound eIF2-GTP-initiator methionyl tRNA complex, it was long assumed that eukaryotic ribosomes can initiate only once on an mRNA. However, particularly after translation of a short ORF (sORF), at least a fraction of ribosomes can reinitiate translation on the same mRNA. How ribosomes regain initiation capacity, how they reach further downstream ORFs, and how all this is influenced by the nature of the first ORF is still unclear. In the yeast GCN4 system (6) and in in vitro systems (7), reinitiation is distance-dependent, suggesting that a certain time is required to reestablish initiation competence. In GCN4, this time (translated to a scanning distance) was inversely correlated to the concentration of active eIF2.

An increasing number of eukaryotic mRNAs have been detected with strong secondary structures and/or alternative start sites in their 5′-UTR, particularly those of transcription factors or protooncogenes (8). Alternative start sites may create short ORFs or N-terminally extended variants of the major ORF. Alternative start sites are not necessarily easily predictable, because “non-AUG” codons, i.e. codons deviating from AUG in one position, may act as start codons (e.g. Ref. 9), particularly in conjunction with downstream secondary structures that may pause scanning ribosomes (10).

In general, complex 5′-UTRs have negative effects on translation efficiency and it has been argued that their function could be to down-regulate expression. However, such features may also be used for more subtle post-transcriptional control of gene expression, although only a few examples have been studied in any detail (6, 11–16). How the eukaryotic translation machinery negotiates complex 5′-UTRs is still far from being understood, even though a number of alternatives to the scanning process have been described. Direct binding to internal ribosome entry sites (IRES) was observed in many viruses and some cellular mRNAs (17). Entry points can be directly at the start codon or upstream of it, thus requiring a translocation step to the start codon. The other alternative to continuous scanning is the ribosome shunt, which leads to the bypass of scanning-inhibitory regions by direct transfer of ribosomal subunits between two RNA regions. Ribosome shunt has so far been described mainly for viral RNAs: cauliflower mosaic virus (CaMV) (18, 19), rice tungro bacilliform virus (RTBV) (20), Sendai virus (21, 22), adenovirus (23), human papillomavirus (24), and budgerigar fledgling disease virus (25). In most of...
these cases, viral factors are not required, even though they may enhance the process, suggesting that ribosome shunt is a normal, cellular mechanism and might also occur on cellular RNAs. Ribosome shunt combines the efficient binding of ribosomes through the mRNA cap and the versatility of internal ribosome entry. The mechanisms that allow 40 S ribosomes to bypass RNA regions are not understood. Here we describe analysis of some of the requirements for ribosome shunt on the leader of the CaMV 35 S RNA. This polycistronic RNA begins with a long leader of 612 nucleotides preceding ORF VII. The leader is inhibitory for the scanning process, because it contains up to nine AUGs creating sORFs, 2–35 codons in length (26), and forms an extended central hairpin (27). Downstream translation is nevertheless possible via the ribosome shunt (18, 19). Shunting functions in various translation systems including plant protoplasts (19, 28), wheat germ extract (29), rabbit reticulocyte lysate (30), and transgenic plants (31). It requires the formation of a stable stem section at the base of the central hairpin (stem section 1) (32, 33) and the presence of an sORF (sORF A) in front of this or a similar stable structure (19, 32, 34). The importance of sORF A has also been underscored by revertants obtained after infection of plants with CaMV mutants (26).

In this report, we investigated (i) combinatorial and individual effects of the sORFs in the CaMV leader on ribosome shunt and linear scanning along the leader and (ii) the effect of the CaMV-encoded transactivator of polycistronic translation, TAV (35, 36), on shunt-mediated expression. The group of sORFs constituted a major inhibitory barrier for linear scanning, whereas the most 5′-proximal sORF A, in combination with sORFs B and C, alleviated this inhibition by diverting scanning ribosomes to the shunt route. Parameters of sORF A were further investigated in a simplified shunt-competent leader. In this leader mimicking the wild type CaMV leader, proper elongation and termination of sORF A were required for efficient ribosome shunt and also for the level of shunt enhancement by TAV. sORF A could be functionally replaced by heterologous, naturally occurring sORFs; however, a start/stop codon sequence was not functional. These results support a reinitiation model of the ribosome shunt.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids for Transient Transfections**—The “wild type” plasmid used in this study as a base-line control has been previously described as pLC20 (18). Its expression unit consists of (i) the fragment of the CaMV strain CM4–184 genome comprising the 35 S RNA promoter and the complete leader, (ii) a CAT reporter gene fused to the ORF VII AUG, and (iii) the CaMV terminator region. All point mutations were introduced into the leader sequence by the polymerase chain reaction ligation method (37). The polymerase chain reaction primers and conditions used as well as the detailed description of the mutations removing all the sORFs have been published elsewhere (26).

The sequences of various point mutations in sORF A are depicted in the corresponding figures. The mutant versions of the leader were subcloned from plasmid pV322 (26) into pLC20 using unique sites EcoRV and Clal naturally occurring in the promoter and near the 3′ end of the leader, respectively. Two large deletions in the leader were made taking advantage of (i) new restriction sites Xhol and HindIII created by the point mutations of sORFs B and F, respectively, yielding delB and its derivatives delMgX, delMgXTG, delMgXtaTG, and A:F, which carry additional point mutations in sORF A; or (ii) the sites Xhol and Clal, yielding delA, and its derivative delMgXG lacking sORF A. In both cases, plasmid MALL-A, which contains sORF A in an otherwise AUG-free leader or its derivatives with the mutated sORF A (26), was cut with the corresponding endonucleases, and the ends filled in with Klenow, and ligated.

The simplified shunt-competent construct delMgX-KS was generated by replacing the Xhol-HindIII fragment of MALL-A with the desired oligonucleotides, such that the HindIII site was maintained. In the latter construct, new Xhol and AflII sites in front of sORF A and the KS sequence, respectively, were introduced by oligonucleotide-directed mutagenesis (CAT54Ag and CG119a), yielding KSALA. Disruption of stem section 1 in the latter construct or modifications in the sORF A region were performed by replacing the Xhol-AflII fragment with the corresponding oligonucleotides. Restoration of stem section 1 was carried out by replacing the HindIII-AflII fragment with an oligonucleotide carrying the desired compensatory mutations. The Kozak stem in the sense strand of these mutants was created by the full-length leader and a fragment between two BglII sites (positions 218 and 238) with the self-complementary oligonucleotide 5′-GATCggggcgcgtggtggcggctgcagccgccaccacg-cgcccc-3′. The inhibitory effects of some of the individual sORFs was tested by reintroduction of their start codons. sORF D’ alone was not inhibitory, sORF E’ was only slightly inhibitory, and sORF F that overlaps ORF VII::CAT was stronger than the others (Fig. 2, lines 4, 5, and 6, respectively). Inhibition by sORF combinations was not additive and not predictable. Combination of sORFs E’ and F (line 7) was more inhibitory than E’ alone but less than F alone; addition of D’ to E’ and F (line 8)
created a greater inhibition, although D alone had had no effect at all. Addition of sORFs D, E, and E’ to the latter construct only slightly reduced expression (Fig. 2B, line 9). These data could be explained by analogy to the yeast GCN4 mRNA with distance-dependent reinitiation (39); a fraction of ribosomes that resume scanning and reinitiate after translation of E’ would bypass the start codon of F because of the short intervening distance and thus the negative effect of the latter sORF is partially alleviated. In a similar way, after translation of D, ribosomes can efficiently reinitiate at the CAT ORF, unless they are intercepted by the intervening sORFs. In the later cases, a potential second round of reinitiation (after sORFs D, E, E’, or E’) is probably inefficient.

While individual readdition of the sORFs to the 3’ half of an otherwise AUG-free leader revealed their potential for translation inhibition, their individual removal from the wild type, sORF-containing leader had no effect on expression (data not shown), indicating that, in this context, the individual sORFs have no major influence on the expression level. This contrasts with the behavior of sORFs A, B, and C; removal of any of these sORFs individually or in combination reduced expression (constructs in Fig. 2B, compared with wild type), suggesting that they alleviate the negative effect of the rest of the leader. sORF A removal had the most striking effect, decreasing expression 5-fold (line 12). The positive function of these sORFs was only detectable in the context of the wild type leader. In combination with other sORF mutations, individual sORFs A, B, and C could also display negative effects on overall expression (e.g. Fig. 2, pairs 16/2 or 15/9 for A, 11/10 for B, and 10/9 for C).

**Fig. 1.** Overall inhibitory effect of sORFs and secondary structure elements on translation controlled by the CaMV leader in plant protoplasts. Primary and secondary structures (predicted with the GCG MFold program) of the wild type leader and its mutated variants are schematically depicted. sORFs A to F are indicated by boxes, and their AUG mutations by crosses. Borders of large deletions removing structural elements are shown with vertical lines; the corresponding restriction sites used are also indicated on the leader structure. Relative expression levels of the downstream CAT ORF are given.

**Fig. 2.** Individual and combinatorial effects of sORFs on shunt- and scanning-dependent expression. Relative CAT expression downstream of the AUG-free leader (A and C) or the wild type leader (B), or their derivatives containing the scanning-inhibitory Kozak stem (indicated by a thick arrow), in the absence (−TAV) or the presence (+TAV) of the CaMV TAV protein, is given. sORFs are indicated by boxes; their AUG mutations are shown by crosses; the resulting mutant triplets are shown for sORFs A, B, and C, with mutated nucleotides in lowercase. The deduced mechanisms (scanning or/and shunt) of downstream translation are indicated with + or − in the Interpretation column; +/− stands for inefficient shunt (when the sORF A AUG is mutated to a non-AUG start codon UUG, or when the sORF B and C AUGs are missing).
lacking the sORFs A, B, and C greatly reduced expression (Fig. 2, lines 2 and 9 versus 1), i.e., in the absence of sORFs A, B, and C, most or all downstream translation depends on continuous, linear scanning and only a small proportion (if any) of the ribosomes perform a shunt.

The presence of sORF A in the otherwise AUG-free leader allowed some ribosomes to overcome the KS barrier (Fig. 2C, line 16 versus line 2), and the presence of sORFs A, B, and C completely restored shunting (line 17 versus line 1, with KS). In the absence of KS, translation from these mRNAs occurs by shunting and scanning. Comparison of mutants in one or several of the respective sORFs with and without KS revealed that removal of any of the first three sORFs increased the contribution of scanning to overall expression and reduced the proportion of shunting (Fig. 2B). In the presence of the KS, the knock out mutation of sORF A (line 12) had the most drastic inhibitory effect on shunt-mediated translation (~13-fold), while the individual mutations of sORFs B and C (lines 13 and 14) were less inhibitory (~1.5- and 4-fold, respectively).

Because of difficulties with quantification of the very low levels of reporter RNA obtained from transfected protoplasts (data not shown), we could not exclude that some mutations might affect RNA stability. However, the fact that the same combination of the sORF point mutations could have either positive or negative effects on expression, depending on the leader context and consequently on the mode of ribosome migration (Fig. 2, compare lines 1 and 2, in the absence or presence of KS), suggests that mainly the rate and/or the mechanism of translation initiation are affected. Previously, it has been shown (29) that in a shunt-competent wheat germ extract the CAT transcripts containing the wild type CaMV leader without or with the Kozak stem (see Fig. 2, line 1) were as stable as the leader-less CAT transcript. This also supports that neither the CaMV sORFs nor the Kozak stem sequence represent instability determinants.

These results show that sORFs can fundamentally alter the translation properties of an mRNA not just by regulating the initiation capacity of ribosomes but also by changing the mode how ribosomes access the internal mRNA regions. sORF A was confirmed as a major determinant of efficient ribosome shunting, but its effects are influenced by the more internal sORFs B and C.

Effects of the Transactivator—The CaMV transactivator TAV acts by enhancing the reinitiation potential of ribosomes that have already translated one ORF (36). Here, TAV was found to enhance expression downstream of the 35 S RNA leader by a factor of 2–3 in all cases involving significant ribosome shunting (Fig. 2, see Interpretation column), allowing expression levels approaching 30% of the maximal translation potential; the latter can be deduced from the reference construct lacking all inhibitory elements (Fig. 1, line 4). Scanning-mediated expression was influenced not at all or much less. When an RNA is translated by both mechanisms, the shunting-dependent fraction is activated by TAV whereas the scanning-dependent fraction is hardly influenced (Fig. 2C, line 17). Shunt requires sORF A and thus all constructs showing a strong effect of TAV contain this sORF. When the start codon was mutated to UAG (Fig. 2B, line 12), downstream translation occurred only by scanning and TAV had at most a small effect; however, with the non-AUG codon UUG, a small amount of shunting is apparent in the presence of TAV (Fig. 2B, line 11, +KS), suggesting that the UUG is active as a start codon in this context.

TAV may function in restoring, at least partially, the initiation capacity of shunting ribosomes that have translated sORF A. For scanning-dependent reinitiation after sORF A translation, this function might not be required because the distance in the leader is long enough to acquire reinitiation capacity during the scanning process (e.g. Fig. 2, line 16).

Translation of sORF A—The data presented so far provide strong evidence that sORF A is translated. To investigate how efficiently ribosomes recognize the sORF A start codon, we fused sORF A in the short leader containing only stem section 1 to the AUG-less remnants of sORF F, which overlap the ORFVII:CAT for 35 nucleotides (Fig. 3, line 2). The mutation drastically reduced expression, suggesting that only a small proportion of scanning ribosomes skips the sORF A AUG start codon. As a control, additional mutation of the latter AUG to UAG fully derepressed translation (line 3).

Construction of a Simplified, Shunt-Competent, and TAV-responsive Leader—To study the specific role of sORF A and TAV in shunting, the complexity of translation events on the reporter RNA was reduced by replacement of the upper part of the leader hairpin (including all other sORFs) with the much shorter but energy-rich Kozak stem (Fig. 4A). In this simpler expression unit, CAT translation was almost completely dependent on the presence of sORF A. Expression levels, the effects of sORF A start codon mutations to UAG or UUG (see Fig. 5A, line 8), and the response to TAV were similar to those observed with the wild type leader (Fig. 4A), suggesting that in both cases shunting occurs. This similarity was maintained only when the KS was present and stem section 1 intact. In the absence of KS, sORF A exhibited a 2.5-fold inhibition (Fig. 4B), indicating that the respective RNA is translated mainly by scanning. In the presence of KS, disruption of stem section 1 by multiple point mutations (Fig. 4C) completely abolished expression as expected for a strictly scanning-dependent mRNA with a KS element. Restoration of stem section 1 by compensatory mutations on the descending arm restored expression, albeit not fully, and effective response to TAV (Fig. 4C), which is consistent with our previous results for the full-length CaMV leader (32, 33). These data suggest that all minimal cis-elements required for shunting are contained in the simplified construct and confirm the key role of the combination of sORF A and stem structure at a proper distance in the shunt mechanism, as already inferred from our previous in vitro analysis (32, 34).

Effects of Alterations of sORF A on Shunting and Transactivation—For a closer analysis of the sORF A role in shunting, we generated a number of additional mutants with alterations of the translation start or stop site of sORF A or its coding content and evaluated their effects on shunt and its enhancement by TAV in the simplified, shunt-competent construct (Fig. 5, A and B). A simple alteration of the stop codon (UAG to UAA) did not have any significant effect (Fig. 5A, line 2). In contrast,
shortening or elongating sORF A by one triplet significantly reduced the basal level of shunt-mediated expression (1.7- and 2.7-fold, respectively). For both mutants the response to TAV was drastically reduced (lines 3 and 4). The sORF elongation introduced a new triplet UAC (coding for Tyr), which might be also responsible for the drastic reduction. To clarify this point, this triplet was mutated to GAG (coding for Glu), the codon preceding the stop codon of the original sORF A. With this mutation, the reduced basal level of expression was not significantly changed, whereas the response to TAV was restored (line 5). Reciprocally, mutation of the GAG codon of the original sORF A to UAC only slightly affected the basal level of shunting but significantly reduced the response to TAV (line 6), thus implying involvement of the sORF A coding potential in shunt enhancement by TAV.

Mutation of the second codon UGU to GCU (Cys to Ala) had only a slight negative effect both in the absence and presence of TAV (Fig. 5A, line 7). With this mutation the sequence context of the start codon was optimized (U1-4 to G). Weakening the sORF A AUG context (A1-3 to U) also did not affect expression significantly (Fig. 5B, line 11). The insufficiency of the sORF A start codon context for shunt-mediated expression is another indication that the start codon in this particular location (see “Discussion”) is recognized very efficiently and allows only a small amount of leaky scanning (see also Fig. 3). This efficiency allowed that even an sORF opened by the non-AUG start codon UUG could promote some shunting in the presence of TAV (Fig. 5A, line 8 and Fig. 2B, line 11).

In a control experiment, replacement of the sORF A sequence with a polyCU stretch of the same length that contains no non-AUG start codon drastically reduced expression both in absence and presence of TAV (Fig. 5B, line 12). A small residual level of expression in this case, as well as in the case of the UAG mutant (line 9), is most likely due to a small fraction of scanning ribosomes that do penetrate through the Kozak stem barrier (see also Fig. 2A, line 2, +KS).

Replacement of sORF A by Heterologous sORFs—Our results show that shunt-mediated expression does not depend on a certain amino acid sequence of the sORF, although the coding potential may modulate shunt efficiency, especially in the presence of TAV (Fig. 5A). We then tested a few natural sORFs derived from other plant pararetroviruses or from the yeast GCN4 mRNA for their effects on shunting.

In the RTBV RNA that is also translated via ribosome shunt (20), the most 5′-proximal sORF terminates 7 nucleotides upstream of the extended hairpin. A similar configuration occurs in the pregenomic RNA leaders of CaMV and other plant pararetroviruses (40). Replacement of the sORF A region preceding stem section 1 by the respective, sORF-containing sequence from RTBV did not affect shunt-mediated expression (Fig. 5C, line 13). This suggests that the RTBV sORF 1 can functionally substitute sORF A in ribosome shunt, despite the differences in length and coding potential. In contrast, a start/stop sORF occurring in soybean chlorotic mottle virus (40) did not support efficient shunting when incorporated in place of sORF A (Fig. 5C, line 16). This was also seen with another, independently
generated one-codon sORF (Fig. 5C, line 17), excluding that the effect of the ShbMV sORF is due to context changes, e.g. in the sequence following the stop codon.

Finally, sORF A was replaced by two functionally distinct sORFs involved in translation regulation of the yeast GCN4 mRNA, sORF 1 that allows efficient reinitiation and sORF 4 that inhibits reinitiation (6). Again, both sORFs were introduced together with their flanking sequences to preserve natural initiation and termination context; the stop codon was 7 nucleotides upstream of the base of stem section 1. The GCN4 sORF 1 sequence slightly increased shunt-mediated expression, whereas the sORF 4 sequence reduced the expression significantly (2.5-fold). In both cases, effective response to TAV was observed (Fig. 5C, lines 14 and 15).

**Effect of sORF A Variants on Scanning-dependent Reinitiation**—To evaluate whether sORFs are boxed; their amino acid sequences are shown below the boxes. C, replacement of the sORF A region by heterologously, naturally occurring sORFs with the flanking sequences (shown in italics). Constructs of series B and C contain additional restriction sites XhoI (underlined) and Apal (preceding the Kozak stem sequence).

**FIG. 5. Parameters of sORF A translation important for shunt-mediated, TAV-responsive expression versus scanning-dependent expression.** A and B, effect of alterations in the start site or the stop site or the coding content of sORF A on shunt-mediated expression from the simplified construct (depicted schematically on the top) in the presence and absence of TAV (two penultimate columns), or on scanning-dependent expression from the construct with a full-length AUG-free leader (last column; see Fig. 2C, line 16). Sequence preceding the base of stem section 1 is shown. Numbering is from the mRNA 5’ end. Mutated nucleotides are in lowercase. sORFs are boxed; their amino acid sequences are shown below the boxes. C, replacement of the sORF A region by heterologically, naturally occurring sORFs with the flanking sequences (shown in italic). Constructs of series B and C contain additional restriction sites XhoI (underlined) and Apal (preceding the Kozak stem sequence).

**DISCUSSION**

sORF A Is a Positive Regulator of Downstream Translation—Short ORFs in 5’-UTRs of eukaryotic mRNAs are usually regarded as elements that influence downstream translation negatively. The few exceptions described so far, including Rous sarcoma virus (15, 41) and the yeast GCN4 system (6, 39), are always associated with the presence of additional start codons between the first sORF and the regulated, long ORF. For instance, the first of four sORFs in the GCN4 mRNA is required to alleviate the negative effects of the other sORFs in the leader sequence under conditions of amino acid starvation. Ribosomes that have translated sORF 1 conditionally fail to regain reinitiation capacity until they have scanned past the other sORFs and thus can reach the GCN4 ORF. This illustrates that the effect of an sORF is not only dependent on its translation but also on poorly characterized properties that can influence the post-termination fate of the ribosomes. Reinitiation frequency is influenced by the intercistronic distance (6, 7), by the sequences surrounding the termination codon (6, 42), by the length of the sORF (43), and by the coding potential of the sORF (e.g. Ref. 11). The additional influence of metabolic conditions (6, 13, 16) makes detection of such effects dependent on the choice of the assay system.

The first sORF (sORF A) in the leader of the CaMV 35 S RNA is required for translation downstream of this leader. In this respect, it is similar to the sORF 1 of the GCN4 mRNA. Here we demonstrated that sORF A alleviates the strong negative effect caused by the other sORFs present in the CaMV leader by diverting ribosomes to the shunt route (Fig. 2). We showed indirectly that sORF A is translated (Fig. 3), and this translation event correlates with efficient ribosome shunt (Fig. 4).
Model for CaMV Shunt—The results presented here and in parallel work (30, 32, 34) are consistent with the model for ribosome shunt depicted in Fig. 6. Scanning ribosomes initiate translation at the sORF A start codon. Even in a suboptimal context, recognition of this start codon is efficient (Figs. 3 and 5B), probably because stem section 1 is located 14 nucleotides downstream of it. Secondary structure at this distance has been shown to enhance initiation at AUG codons in weak context and even at non-AUG codons, probably due to pausing of scanning ribosomes (10). Upon translation termination, the first 8 base pairs of stem section 1 should be disrupted by the translating 80 S ribosomes (see legend to Fig. 6). This exposes the region that has been previously mapped as the shunt landing site at the 3′ end of the 5′-UTR (19), allowing binding by a new ribosome, entering internally, or by a ribosome from the 5′ part of the leader. The internal binding model appears unlikely, since no deletion variant of the leader was ever found which would have supported such binding. Therefore, simple masking of the acceptor region by upstream leader regions can not be a reason for inactivation of an otherwise functional IRES. We favor the possibilities shown in Fig. 6B, particularly the model involving transfer of the same ribosome that has translated sORF A. In this case, downstream translation involves reinitiation explaining the positive effect of TAV, which enhances translation reinitiation (36). However, at this stage, alternatives for TAV action as indicated in Fig. 6 cannot be excluded.

The reinitiation model is also supported by our observation that the replacement of sORF A by the GCN4 sORF 4, which inhibits reinitiation in yeast, significantly reduced shunt-mediated expression (Fig. 5C). The reduction in our case was observed even though only 7 of 10 natural nucleotides downstream of the sORF 4 stop codon, thought to be required for the inhibiting effect in yeast (42), were preserved in our construct due to the distance constraint. In contrast, the GCN4 sORF 1 that allows efficient reinitiation in yeast, could substitute sORF A without any effect on shunt-mediated expression. Together with the recent observation that an sORF encoding the peptide MAGDIS (13), which causes ribosome stalling in mammals, also inhibited shunt-mediated translation in place of sORF A (30), these findings suggest that translational interactions between sORFs and ribosomes may be quite conserved among eukaryotes.

The minimal shunt-promoting structure consists of sORF A and stem section 1 (Refs. 32 and 34 and Fig. 4). However, in the context of the wild type 35 S RNA leader, shunt was stimulated by further internal sequences in the sORF B and, especially, C regions (Fig. 2B). One possible explanation is that in the absence of sORFs B and C, the increased flow of scanning ribosomes through the leader (as seen from Fig. 2C) should melt secondary structure reducing the probability of reformation of stem section 1. Alternatively, the mutations in these sORFs might themselves modify the leader structure (26). As structural elements these sequences may again ensure the proper formation of stem section 1 in the complete leader, a function that could also be performed by the KS element in the simplified, shunt-supporting construct (Fig. 4A). It is noteworthy that some deletion and point mutations, described previously as interfering with ribosome shunt (19), in fact disturb the proper alignment of the same structural region. In contrast, improvement of the base pairing in this region stimulates shunt (32).

Important Parameters of sORF A Translation—Our previous in planta analysis revealed that sORF A start codon mutations reverted by restoring an in-frame AUG at various positions, while the stop codon mutation was only restored at its wild type location (26). This finding complements our in vitro translation analysis (32), indicating the importance of the positioning of sORF A with respect to stem section 1. Here we confirmed that shifting the stop codon even for only one triplet upstream or downstream could significantly reduce shunt efficiency (Fig.
Role of a Short ORF in Ribosome Shunt

5A). The configuration of an sORF terminating 5–10 nucleotides upstream of a stable hairpin is conserved in the leaders of most plant pararetroviruses (40). The coding content of the respective sORFs varies, but the most frequent amino acids are Ser or Cys in the penultimate and Gln or Gly in the last position (40). Our mutagenesis experiments revealed that variants of sORF A are also functional, but they could modulate translation efficiency (Fig. 5). The sORF could affect the number of ribosomes emerging from sORF translation in a mode that allows reinitiation, or it could directly influence the choice of ribosomes for scanning or shunting. Reinitiation frequency was reduced at most 2-fold (Fig. 5, line 17) but, in most cases, less (line 5) or not at all. Therefore, some effects (Fig. 5, lines 3, 4, and 17) are clearly shunt-specific and may directly affect the post-termination ribosome translational process.

None of the sORF variants tested resulted in a significantly higher expression, suggesting that the natural sORF A with sequence MCE is functionally optimal. Furthermore, sORF A could be replaced by the corresponding 5′-proximal sORF from RTBV (MAQVSE) without notable effect on shunting (Fig. 5C). Our preliminary results indicated that mutation of this sORF in the RTBV leader abolished shunt-mediated expression.2 This supports the conservation of the shunt mechanism in the family of plant pararetroviruses.

An interesting observation reported here is that the start/stop sORFs could not functionally replace sORF A in shunting (Fig. 5C). Most likely, the initiation or the termination process in this one-codon sORF without peptide-elongation phase is detrimental to further ribosome activity. Consistent with this, we have previously shown that the mutation in CaMV sORF A creating the one-codon sORF9 (Fig. 5C, line 17) drastically reduced infectivity of the virus and resulted in second site revertants in planta that eventually restored the wild type sORF A (26). This findings also suggest that SbCMV indeed represents an exceptional case in plant pararetroviruses and might not use the shunting strategy; mapping of the transcripts is not complete for this virus, and a predicted leader sequence is shorter and contains fewer AUGs than in other plant pararetroviruses (40).

Mechanistic Considerations—What could be the function of sORF A? In its absence, no shunting could be detected and downstream structural elements are simply inhibitory for scanning ribosomes. In the presence of the sORF but with a disturbed shunt-supporting structure, ribosomes can apparently re-enter a scanning mode with high efficiency and in this state are equally susceptible to inhibition by secondary structure (Figs. 2C and 4C). It appears that, immediately after translation termination, ribosomes are in a state that allows either shunting or reestablishment of scanning properties. During the initiation event, a number of translation factors dissociate from the ribosome. For reinitiation, some of them, e.g., eIF2, have to be reacquired. Possibly, factors necessary for scanning are also lost and, as long as they are lacking, a slower and less directed mode of ribosome migration might occur. The missing factor may be the helicase eIF4A (44). In the case of the adenovirus major late mRNAs, shunting does not require an sORF, but it becomes the predominant mechanism under conditions where the activity of RNA cap-binding complex that includes eIF4A is reduced (23). Lack of a proper set of initiation factors could also be responsible for initiation at non-AUG start codons, since, e.g., eIF5 has been implicated in proofreading of the initiation step (45); eIFs 1 and 1A also seem to destabilize preinitiation complexes (46). Non-AUG start codons can be recognized at shunt landing sites in CaMV (30), RTBV (20), and Sendai virus (22); such codons are usually not significantly recognized by scanning ribosomes unless scanning is slowed down due to secondary structure (10).

Reinitiation after Shunting and Effect of TAV—Since the start codon of sORF A is recognized efficiently, downstream translation requires reinitiation. In the GCN4 mRNA, this switch to reinitiation seems to be the only function of sORF 1, allowing the regulation of expression in response to the concentration of active eIF2. For CaMV, the scanning distance between sORF A and ORF VII is long enough to allow efficient reinitiation (Fig. 2C, line 16; Fig. 5A). Notably, in the latter case, almost no response to the CaMV TAV protein could be observed. However, in the wild-type leader, reinitiation most likely occurs already at some of the other sORFs, which are evenly spaced throughout the leader, unlike the GCN4 mRNA, where the distance between the regulatory sORF 4 and the main ORF is quite long. After shunting, the scanning distance becomes much shorter and therefore recovery of initiation capacity is probably the step that is enhanced by TAV. The crucial step in acquiring reinitiation capacity is probably the recycling of the eIF2-GDP complex and its association with tRNA. After dissociation from the initiating 40 S ribosome, the eIF2-GDP complex may, at least transiently, associate with the 60 S subunit (47), i.e. remain in close proximity to the 40 S subunit, and the large ribosomal subunit seems to play a role in recycling and translation reinitiation (48). A mechanism that would make this complex available for rebind to the 40 S subunit after translation of an sORF, allowing it to recycle while bound to one of the ribosomal subunits, could allow distance-independent translation reinitiation. It is conceivable that such a mechanism could be influenced by nascent peptides. We observed that two sORF A variants ending in Tyr and an sORF encoding the peptide MC had a reduced response to TAV in shunting but did not impair reinitiation after linear scanning (Fig. 5A). The scanning-dependent and the TAV-dependent mechanisms by which ribosomes can regain initiation capacity may therefore be different. It remains to be investigated whether translated peptides or RNA sequence features influence TAV activity, or whether TAV may enhance not just reinitiation but also the shunt process itself.

Implications for Other Complex mRNAs—The translational events on RNAs with complex leader sequence are difficult to predict by the simple model of linear ribosome scanning. sORFs and secondary structure not only suppress the scanning process. They can make internal RNA regions inaccessible for translation initiation even under conditions where overall translation is cap-dependent. Ribosomes emerging from translation of an sORF may also, at least transiently, possess a different set of initiation factors allowing initiation at start codons not normally recognized. It is noteworthy that most examples of naturally active non-AUG start codons are found on RNAs with complex leader sequences or on RNAs that attract ribosomes by means other than a cap structure (8). From the features described here, we would predict that a ribosome shunt could function on any mRNA containing a proper sORF ending close to a stable secondary structure element; such an sORF may also start with a non-AUG start codon. Although it appears that other cis-acting features can also induce shunting (22, 23, 49), these have not been characterized to any detail. At the present state of knowledge, it appears possible that some cases of IRESs found in capped mRNAs could be examples of ribosome shunting. Indeed, recent work on the c-myc protooncogene has challenged a previous IRES hypothesis for that case, suggesting a shunt mechanism instead (50).

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2 M. Poggin, unpublished results.
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