Continuous and Discontinuous Ribosome Scanning on the Cauliflower Mosaic Virus 35 S RNA Leader Is Controlled by Short Open Reading Frames*

Lyubov A. Ryabova, Mikhail M. Pooggin‡, Diana Ines Dominguez§, and Thomas Hohn¶

From the Friedrich-Miescher-Institute, P.O. Box 2543, CH–4002 Basel, Switzerland

The pathways of scanning ribosome migration controlled by the cauliflower mosaic virus 35 S RNA leader were investigated in vitro and in vivo. This long (600 nucleotides) leader contains several short open reading frames (sORFs) and folds into an extended hairpin structure with three main stable stem sections. Translation initiation downstream of the leader is cap-dependent and occurs via ribosomal shunt under the control of two cis elements, a short open reading frame A (sORF A) followed by stem section 1. Here we show that a second similar configuration comprising sORF B followed by stem section 2 also allows shunting. The efficiency of the secondary shunt was greatly increased when stem section 1 was destabilized. In addition, we present evidence that a significant fraction of reinitiation-competent ribosomes that escape both shunt events migrate linearly via the structured central region but are intercepted by internal AUG start codons. Thus, expression downstream of the 35 S RNA leader is largely controlled by its multiple sORFs.

Most translation initiation events in eukaryotic cells involve mRNAs that are capped, have short unstructured leaders (5′ untranslated regions), and are monocistronic, because initiation is limited to the 5′ AUG codon. To account for unusual situations such as viral mRNAs with multiple AUG start codons, Kozak (1, 2) proposed a modified or “leaky” scanning model, in which the first AUG codon could be bypassed if it is in a suboptimal nucleotide context for initiation. In the latter case, secondary structure within a certain distance from the AUG can increase the efficiency of recognition (3).

Reinitiation in eukaryotes appears to be inefficient and one or two small upstream ORFs (uORFs) can reduce protein yields considerably, whereas long ORFs will usually abolish it (2, 4, 5). The ability of ribosomes to reinitiate translation at downstream AUGs after scanning correlates negatively with the length of the upstream ORF (5, 6). Longer intergenic distances favor reinitiation (4, 6, 7). It is thought that initiation factors dissociate from the ribosome upon each initiation event, and the limiting parameter for subsequent reinitiation is the time needed to reacquire these factors (8).

In the case of multiple short uORFs, ribosomes can bypass (or scan through) other uORFs after translation of the first one and become competent to translate a main ORF located further downstream. The best known example is the regulated translation of the GCN4 mRNA in yeast. In this case, ribosomes must translate the 5′ proximal uORF, uORF 1, then resume scanning to reinitiate at either uORF 4 or at the main ORF depending on the availability of the eIF-2/GTP/Met-tRNA{Met} ternary complex (9).

An alternative strategy used by a number of cellular mRNAs and many viruses involves direct ribosome binding at the mRNA start codon (or upstream of it) mediated by an internal ribosome entry site (10, 11). A second alternative to the linear scanning route is a ribosome shunt, as shown for the RNAs of Sendai virus (12, 13), cauliflower mosaic virus (CaMV) (14, 15), rice tungro bacilliform virus (16), adenovirus (17, 18), and papillomavirus (19). The shunt process allows ribosomes to bypass RNA regions that may include AUG codons and secondary structures inhibiting linear migration (for review, see Ref. 20).

The CaMV 35 S RNA leader contains seven to eight sORFs and folds into an elongated hairpin structure comprising three main stem sections (21). It does not function as an internal ribosome entry site when placed between two cistrons (22). Expression downstream of the 35 S RNA leader occurs via ribosome shunt, which requires three cis elements within the leader, a capped 5′ end (14, 23), a 5′ proximal sORF (sORF A; 4 codons in length), and, following it after 6 nt, a stable stem (stem section 1) (22, 24, 25). The translation event at the sORF is needed for efficient shunting (26, 27). The elongated hairpin structure promotes shunting by bringing the shunt “take off” and “landing” sites into close spatial proximity (15, 22). Shunting does not require viral or specific host factors, because it functions in plant protoplasts, wheat germ extract, and reticulocyte lysate (14, 23, 26), but it can be enhanced by a CaMV-encoded transactivator protein (27).

In this study we applied in vitro and in vivo translation systems to study the mechanisms by which the complex leader of the CaMV 35 S RNA regulates polycistronic translation from this RNA. Inspection of the CaMV leader sequence revealed that the combination of an sORF followed by a stem section is repeated (sORF B/stem section 2; see Fig. 1B). In this paper we show that this second configuration also supports shunting. We also demonstrate that ribosome migration controlled by the CaMV 35 S RNA leader is characterized by both continuous and discontinuous scanning.
Upper case letters indicate the original CaMV leader sequence. Bold letters indicate mutated nucleotide sequence introduced with double-stranded oligo. Lower case letters show nucleotide changes. Dashes, as well as Δ, indicate deleted nucleotides.

**TABLE I**

| Construct | Description of cloning | Sequence of original and mutated regions |
|----------|------------------------|----------------------------------------|
| Lm       | XhoI/NheI sequence     | TCAGAAATAAATGTTGAGATTTGCTCTGGGATAAGGAGGATTGCTTCTATAGGGTTTCGG |
| LeA:B     | Clai/IcoI sequence     | TCAGAAATATGTTGAGATGCTCCGAGGATAAGGAGATTGCTTCTATAGGGTTTCGG |
| L/AUG569  | XhoI/IcoI oligo        | CAGTTAAAGAAAATgTCGGGTGAGGCCGCTTAAATATTGTACGAGC |
| LeA′B     | XhoI/IcoI oligo        | TCAGAAATATGTTGAGATGCTCCGAGGATAAGGAGATTGCTTCTATAGGGTTTCGG |
| LeA: B    |                         | TCAGAAATATGTTGAGATGCTCCGAGGATAAGGAGATTGCTTCTATAGGGTTTCGG |
| LeA′B/st2a | Spel/BglII oligo      | CTGTTATATATctaaATCTCTAAATCCAAAATATCAGATATTAAAACCCCTTCAGATGTTATGTT |
| LeA′B/st2c | BglII/ClaI sequence    | CTGATTATATTcaataATCTCTAAATCCAAAATATCAGATATTAAAACCCCTTCAGATGTTATGTT |
| LeA′B/F−:C | BglII/ClaI PCR fr     | A(T)505(T)ACGCTAAGGGAtTaCTTTGTATTTACCCTATATACCCTATAGGCCCCTTTAT |
| LCsaA′B  | PCR mutagenesis        | ATGCTGAGGAG(TAACTGTTATCGATGTTATGTT |

**MATERIALS AND METHODS**

**Constructs—**Plasmid LmF-CAT (Lm) containing the T7 promoter, the whole CaMV S strain (25) leader carrying a deletion of Δ289 (numbering is from the 5′ end of the 35 S RNA), and the CAT reporter gene fused to the AUG of ORF VII were described previously (22). A series of derivatives lacking parts of the leader or having mutated leader sequences are listed in Table I. The insertion of a double-stranded oligonucleotide between the Clai–IcoI sites in Lm yielded L/AUG569 resulting in insertion of an AUG codon just downstream of the shunt landing site. Mutation of the sORF A stop codon was achieved by the insertion of oligonucleotides of the desired sequence between the XhoI–IcoI sites in Lm resulting in an A:B fusion ORF, (LsA::B). In LsA::B, the IcoI–StuI fragment was exchanged using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Gels were also analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**In Vitro Translation—**Wheat germ extract was prepared according to Roberts and Paterson (31). Rabbit reticulocyte lysate (gel-filtered) was from Roche Molecular Biochemicals. 0.1 pmol/μl of each transcript was used for in vitro translation. The capped mRNA has been shown to be relatively stable during 40 min of incubation in vitro (26). In vitro translation reactions with wheat germ extract (WGE) or rabbit reticulocyte lysate (RRL) were performed as described in (26).

3 μl of translation reaction mixtures were resolved on high cross-linking (16.5% T (total percentage concentration of acrylamide and bisacrylamide) and 6% C (percentage concentration of the cross-linker)) Tricine-SDS polyacrylamide gels as described by Schagger and von Jagow (32). Gels were fixed in the presence of 10% glycerol, dried at 50 °C overnight, and exposed to x-ray film (Fuji). Gels were also analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Protoplast Transfections and Reporter Gene Assays—**The transient expression experiments in Oriyahphragmus violaceus protoplasts were performed as described (27). CAT-expressing plasmids (30 μg) were always cotransfected with 2.5 μg of β-glucuronidase-expressing plasmid (pV594) to serve as an internal standard of transfection efficiency. For transactivation, 5 μg of plasmid pHELPP7 (33) expressing the TAV protein was also added. For each CAT construct, transfections were repeated three times (± TAV) in independent batches of protoplasts. CAT expression was always calculated relative to the β-glucuronidase activity of internal controls. Protein levels and β-glucuronidase activities were measured by a CAT ELISA assay (Rocke Molecular Biochemicals) and a 4-methylumbelliferyl β-glucuronide fluorometric assay, respectively.

**RESULTS**

**Destablization or Removal of Stem Section 1 Reveals a Second Shunting Site—**Our basic test construct (Lm; Fig. 1, A and B) contains the CaMV leader with a frameshift mutation in sORF F bringing it in frame with ORF VII, which it normally overlaps (LCwt; Fig. 1A). Thus, the sORF F start codon, the internal F′ AUG, and several functional non-AUG codons (26) are in-frame with the start codon of ORF VII (see Fig. 1, A and B). In this study the CAT reporter gene was placed downstream of the CaMV leader at the position of the original ORF VII. In a WGE translation system, a battery of CAT-related bands corresponding to initiation events at these start codons is revealed (Fig. 1D, lane 1). Some of the following are produced by shunting via stem section 1: N′:CAT, N′:CAT, N′:CAT, and CAT; these start codons are located downstream of the shunt landing site (26). In contrast, because the F′ and F′ AUGs are located upstream of the main shunt landing site, they must be reached either by linearly migrating ribosomes or by a secondary shunt event.

Previous results have shown that integrity of stem section 1 is critical for an efficient ribosome shunt (22, 26). Indeed,
disruption of stem section 1 by destabilizing mutations (Lst1\textsuperscript{2}) or by ribosomes translating an elongated A::B fusion ORF, LsA::B, results in a substantial decrease in synthesis of CAT, N::CAT, N\textsuperscript{9}::CAT, and N\textsuperscript{0}::CAT proteins (Fig. 1\textsuperscript{D}, lanes 2 and 3). In both cases, the major initiation event now takes place at the AUG of F\textsuperscript{9}. Given that the upstream start codon of F is in a better initiation context (ggaAUGc) than that of F\textsuperscript{9} (cuaAUGa, lacking a purine at position -3 and a guanosine at position +4, with respect to the A of the start codon (2)), it is unlikely that F\textsuperscript{9}:CAT ORF is translated only by linearly migrating ribosomes.

An extra AUG codon inserted just downstream of the shunt landing site at the position of the first non-AUG codon (L/AUG\textsuperscript{569}) was efficiently recognized by shunting ribosomes, whereas expression from the downstream CAT ORF was inhibited (Fig. 1\textsuperscript{D}, lane 4). Recognition of AUG\textsuperscript{569} was impaired by mutations destabilizing stem section 1 (Lst1\textsuperscript{2}/AUG\textsuperscript{569}; Fig. 1\textsuperscript{D}, lane 5) proving that shunting ribosomes rather than scanning ribosomes utilize this AUG. Again, there was a concomitant increase in translation from the F\textsuperscript{9} AUG.

Thus, the start codon of sORF F\textsuperscript{9} is preferentially recognized in mutants with a destabilized stem section 1 despite the presence of the remaining structured regions of the leader and multiple internal sORFs. One explanation could be that the F\textsuperscript{9}AUG is recognized via an alternative shunt event at the base of stem section 2 initiated by ribosomes that have translated sORFs B or A::B.

To test this hypothesis, a stem section 1 deletion mutant was constructed in which the 5\textsuperscript{9} arm of stem section 1 was removed. This deletion removes the region between sORFs A and B resulting in a novel sORF, sORF A*B (LsA*B). The predicted secondary structure of this construct is shown in Fig. 1C. Our
model places a putative second shunt landing site between the F and F′ AUGs. The main polypeptide translated from this mRNA comigrated with F′:CAT polypeptides translated from Lst1′ mRNA (Fig. 1E, cf. lanes 1 and 2). To ensure that the start codon of sORF F′ is the real start site of the observed fusion polypeptide, this AUG was mutated to GUA. As a result, the F′:CAT band disappeared, and there was a concomitant increase in CAT ORF expression (Fig. 1E, lane 3). We assume that ribosomes that would have otherwise recognized the F′ AUG now scan further downstream and initiate efficiently at the CAT AUG.

Initiation of translation in LsA*B occurred also at F AUG with an efficiency of ~20% (Fig. 1E, lane 2). Most likely, translation-competent ribosomes could reach this AUG by a scanning-reinitiation process in contrast to initiation via shunting at the internal F′ AUG. Insertion of a strong hairpin into the BglII site (see Fig. 1C) in the center of the LsA*B and Lst1′ constructs (LsA*B(ks) and Lst1′(ks)) abolished F′:CAT translation, indicating that the AUG of F is indeed recognized by ribosomes linearly migrating through stem section 3 (Fig. 1E, lanes 4 and 5). In contrast, translation of F′:CAT was only slightly affected by the scanning-inhibiting stable stem, thus supporting our hypothesis that F′:CAT is translated via an alternative ribosomal shunt.

sORF B and Stem Section 2 Control Efficient Alternative Shunt—sORF A′B in LsA*B contains two in-frame AUGs, one at the start position and one at the original start codon of sORF B (now internal). These were mutated to stop codons in different combinations (Fig. 2A). Removal of the first AUG (LsA−B) revealed that the 9-nt-long sORF B alone can promote shunting more efficiently than the longer 18-nt sORF A′B fusion (Fig. 2B, lanes 1 and 2). CAT, but not F′:CAT, expression was also increased in this mutant, perhaps because of leaky scanning through the AUG of F′. Removal of both AUGs together (LsA−B′−B′), or elongation of sORF A′B until the stop codon of sORF C (LsA−B:C), had a strong inhibitory effect on F′:CAT production (Fig. 2B, lanes 3 and 4), confirming the requirement of a sORF terminating in front of stem section 2 for efficient second shunt.

We subdivided stem section 2 into the following 3 regions as indicated in Fig. 1C; 2a (upstream of sORF C), 2b (sORF C), and 2c (downstream of sORF C; st2a, st2b, and st2c). The base paired regions, 2a or 2c, were destroyed separately by introduction of point mutations into the left arm of st 2 (LsA*B/st2a′ and LsA*B/st2c′; Fig. 2C). Disruption of st2a in the LsA*B construct caused an increase in F′:CAT production and a significant reduction of F′:CAT synthesis (Fig. 2D, lane 2), suggesting that it is a key element for the alternative shunt. Disruption of st2c was less inhibitory (Fig. 2D, lane 3). Stabilization of st 2c (mutant LsA*B/st2c′−) caused a roughly 2-fold increase in F′:CAT protein production (Fig. 2D, lane 4), with concomitant reduction of F′:CAT protein production.

Thus, destabilization of stem section 2 led to a higher level of F′:CAT ORF translation and a lower level of F′:CAT ORF translation, whereas stabilization had the opposite effect, indicating that linear migration of ribosomes through stem section 2, as well as the second shunt, was affected. In summary, there is strong evidence that the combination of sORF B and stem section 2 promotes an alternative shunt event in the structured region of the CaMV 35 S RNA leader.

Evidence for the Second Shunt in Plant Protoplasts—To address whether the second shunt operates in vivo, CaMV leader-CAT transcription units were introduced into expression vectors under the control of the 35 S RNA promoter and terminator (see “Materials and Methods”). To visualize the second shunt-mediated expression, we took advantage of the natural configuration of sORF F′ that overlaps, and therefore down-regulates, the first ORF following the leader (ORF VII in the viral context or the CAT ORF in this study). According to the in vitro results (Fig. 1E, cf. lanes 2 and 3), the sORF F′ AUG is first recognized by ribosomes arriving by the second shunt. These ribosomes translate sORF F′ and would therefore not initiate at the AUG of the CAT ORF. In the absence of the F′ AUG, translation would initiate at the CAT AUG. Because the in vitro expression system measures only translation of CAT protein, comparison of CAT expression levels in the presence and absence of the sORF F′ AUG indirectly measures the efficiency of the second shunt.

CAT expression obtained with the wild type CaMV leader construct (LCwt; see Fig. 1A) was set to 100% (Table II). Translation of CAT downstream of the wild type CaMV leader is only slightly increased in the absence of the F′ AUG (cf. pLCwt and
pLC/F\(^{-}\)) showing that the first shunt landing site downstream of the F\(^{+}\) AUG is most efficient and giving an estimated efficiency for the second shunt of about 10% of that of the first. In contrast, if stem section 1 is deleted, CAT expression is 4-fold higher in the absence of the F\(^{+}\) AUG (pLCsA/B/F\(^{-}\)) than in its presence (pLCsA/B) in accordance with our second shunt predictions. In the absence of the F\(^{+}\) AUG, the level of second shunt-mediated expression is slightly higher than that mediated by the main shunt on the full-length construct (cf. pLC/F\(^{-}\) and pLCsA/B/F\(^{-}\)). This correlates well with the in vitro results (cf. Fig. 1D, lane 1 and Fig. 1E, lane 2).

In this in vivo system we could also measure the effect on shunting of the CaMV-derived translational transactivator, TAV, which promotes reinitiation at internal ORFs on polycistrionic mRNA (34). (TAV does not function in our in vitro system.) TAV stimulates the main shunt event about 2–3-fold (27). We observed that TAV also promoted about a 2-fold increase in the initial level of ribosomal shunting via stem section 2 (Table II). Thus, in vivo expression of the CAT ORF by shunting ribosomes via stem section 2 can also be inferred to require translation of sORF A\(^{A'}\)B, and, as in the case of the main shunting event, TAV stimulates reinitiation.

Ribosomal Progression into Central Internal Regions of the CaMV Leader—The F::CAT ORF is most likely translated by ribosomes that migrate linearly through the central region of the leader (Fig. 1B). Indeed, destabilization of stem section 1 significantly increased F::CAT ORF expression (Fig. 1D, cf. lanes 1 and 3 and 4 and 5). We have shown recently that sORF A is recognized by the majority of the scanning ribosomes (26, 27), and the translational event at sORF A is a prerequisite for translation of the F::CAT, F::CAT, and CAT ORFs (26). Thus, recognition of the central ORFs is not a primary initiation event but requires reinitiation.

The central structured region of the CaMV leader possesses two long sORFs (D (27 nt) and E (42 nt); see Fig. 3A) with the AUGs of sORFs D and E in a moderate initiation context and an internal AUG 9 nt from the end of sORF E (E\(^{+}\)) in a strong context. The longest sORF, sORF E, was mutated alone or in combination with sORF E\(^{+}\) and sORF D (Fig. 3B). Removal of the sORF E start codon did not significantly increase the efficiency of initiation at sORF F in the full-size leader (Lm) but increased it about 3-fold in the shortened version (LsA\(^{A'}\)B; Fig. 3C, cf. lanes 1 and 2 and 5 and 6). Removal of sORF D, in addition to sORF E, increased translation of sORF F in the full-sized leader more than 3-fold (cf. Lm\(^{E'}\) and Lm\(^{D'}\)). However, it was not significantly affected in LsA\(^{A'}\)B/D\(^{E'}\) (Fig. 3C, cf. lanes 2 and 3 and 6 and 7). Thus, it appears that sORF D is better recognized by ribosomes in Lm RNA than in its shorter derivative, LsA\(^{B}\)E\(^{B}\). This favors the following distance-dependent model of reinitiation (7); ribosomes emerging from translation of sORF A are most competent for reinitiation at sORF D (at a distance of 253 nt), ribosomes having translated A\(^{B}\) have only traveled 201 nt before they encounter sORF D, and initiate much more efficiently at sORF E after scanning another 54 nt. In both cases, a distance of about 250 nt is scanned before efficient reinitiation can occur (see Fig. 3A).

**TABLE II**

| Constructs | Relative CAT expression | -TAV | +TAV | +TAV/TAV |
|-----------|-------------------------|------|------|----------|
| pLCwt     | 100                     | 241 ± 25 | 2.4 |
| pLC/F\(^{-}\) | 112 ± 13                | 246 ± 30 | 2.2 |
| pLCsA/B   | 31 ± 3                  | 96 ± 16 | 3.1 |
| pLCsA/B/F\(^{-}\) | 127 ± 17               | 241 ± 29 | 1.9 |

In vivo, the second shunt is slightly higher than that mediated by the main shunt on the full-length construct (cf. pLC/F\(^{-}\) and pLCsA/B/F\(^{-}\)). This correlates well with the in vitro results (cf. Fig. 1D, lane 1 and Fig. 1E, lane 2).

In this in vivo system, we could also measure the effect on shunting of the CaMV-derived translational transactivator, TAV, which promotes reinitiation at internal ORFs on polycistrionic mRNA (34). (TAV does not function in our in vitro system.) TAV stimulates the main shunt event about 2–3-fold (27). We observed that TAV also promoted about a 2-fold increase in the initial level of ribosomal shunting via stem section 2 (Table II). Thus, in vivo expression of the CAT ORF by shunting ribosomes via stem section 2 can also be inferred to require translation of sORF A\(^{A'}\)B, and, as in the case of the main shunting event, TAV stimulates reinitiation.

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Removal of sORF E\(^{+}\), in addition to D and E, in both contexts resulted in a strong additional increase of scanning-dependent expression of F::CAT (Fig. 3C, lanes 4 and 8), indicating that this 3-amino acid sORF, which is in a perfect initiation context, caused a significant fraction of linear migrating ribosomes to dissociate after its translation.

Taken together, these results indicate that scanning ribosomes that escape shunting get held up by all three central sORFs (D, E, and E\(^{+}\)) with increasing order of efficiency. Strikingly, the level of linear ribosome migration through the elongated hairpin of the CaMV leader is comparable with the level of shunting, and it is the start codons in internal regions of the CaMV leader rather than the complex secondary structure that are the main blocks of linear ribosome migration.

Second Shunt and Linear Ribosomal Migration in RRL—The shunt mechanism promoted by the CaMV leader can function in RRL (26), meaning that plant-specific factors are not required. Removal of stem section 1 (La\(^{A}\)B) reduced CAT ORF expression but increased expression from F\(^{+}\) ORF (Fig. 3D, lane 2), indicating that the second shunt pathway also operates in RRL. Removal of the F\(^{+}\) AUG led to further scanning and restoration of reinitiation at the AUG of CAT (Fig. 3D, lane 3).
Experiments similar to those performed in WGE showed that alternative shunting also in RRL is promoted by sORF B and stem section 2 (data not shown).

We also analyzed the level of linear migration in the RRL cell-free system (Fig. 3D). A leader lacking sORFs D, E, and E′ produced at least 6-fold less F::CAT polypeptides in RRL than in WGE, both in the context of the full-sized leader-containing RNA (lanes 5, 6, and 7) and of a deletion of stem section 1 (lanes 9, 10, and 11). Moreover, removal of the E′ AUG, in addition to those of D and E, decreased scanning-dependent translation of F::CAT ORF. Thus, in RRL mainly shunting is operating as opposed to linear ribosomal migration via central regions of the leader.

**DISCUSSION**

The classical scanning model of translation initiation predicts that the strong elongated hairpin with its multiple sORFs should block linear scanning along the CaMV leader. Indeed, translation downstream of the leader is largely achieved by a bypass of this strong secondary structure under the control of a translational event at sORF A located just upstream of it (ribosome shunt; Fig. 4A; see Refs. 26 and 27). The arrangement of an sORF followed by a stem structure occurs twice in the CaMV leader; if the main shunt is impaired or ribosomes manage to scan through the main shunt donor site, the combination of sORF B and stem section 2 can also support shunting (Fig. 4B). This alternative shunt pathway operates in vitro and also in vivo in plant protoplasts. That it also functions in the viral context in planta can be inferred from the strong conservation of the 2 codon sORF B in strains of CaMV, with the single exception of strain CM1841, where its AUG is replaced by a non-AUG start codon (25). Analysis of the requirement for the 35 S RNA leader sORFs in planta has shown that in a CaMV mutant lacking sORFs A, B, and C, besides fast restoration of sORF A, there was also a tendency to restore sORF B (25). Thus, sORF B might be an important determinant in planta where it is used by ribosomes to bypass stem section 2. Thus, the alternative shunt pathway, although not essential, may increase viral fitness in planta.

sORF A and stem section 1 of the CaMV RNA leader can be exchanged for other natural or synthetic sORFs and stable energy-rich hairpin (Kozak stem), respectively, without greatly affecting the efficiency of shunting (22, 26, 27, 35). Note, however, that in the special case of sORFs whose products repress the downstream termination process shunting is promoted with only a low efficiency (26).

The scanning distance between sORF A and sORF B is 43 nt, which seems to be too short to allow significant reinitiation at the sORF B AUG, although reinitiation might be enhanced significantly by stem section 2 because of pausing of scanning ribosomes (3). The combination of sORF B and stem section 2 would then allow those ribosomes that did not shunt at stem section 1 to shunt at stem section 2 and reinitiate at the AUG of F′ (Fig. 3B). The alternative shunt becomes apparent when the primary shunt has been impaired. The function of the second shunt might be just to contribute to a tighter protection of the central leader structure, which is thought to be required for packaging (36).

We also demonstrated in WGE that the fraction of ribosomes continuing linear migration toward the center of the leader is comparable with the fraction of shunting ribosomes (Fig. 4C). The translation event at sORF A is a prerequisite not only for shunting but also for scanning-dependent recognition of the sORF F AUG (26). Efficient translation initiation of this sORF located in close spatial vicinity to the leader hairpin supports the melting of a few base pairs of this structured region. Thus, ribosomes can also resume scanning after sORF A translation, apparently via a leaky scanning and a distance-dependent reinitiation process.

**FIG. 4. A current model for continuous and discontinuous ribosome scanning in the CaMV 35 S RNA leader.** A, main mechanism: shunting via stem section 1. B, alternative shunting via stem section 2. C, migration through the central part of the CaMV leader via leaky scanning and reinitiation. Schematic presentation of the secondary structure of the wt 35 S RNA leader with ORF VII is shown. Arrows indicate migration of ribosomes by continuous scanning, leaky scanning or reinitiation (dashed black lines), shunting (open lines), or translation (solid black lines). Dissociation of ribosomes from the mRNA are shown by openheaded arrows. The thickness of arrows roughly reflects the proportion of ribosomes involved in each process. A, the main shunting event is thought to occur as follows: ribosomes scan from the capped 5′ end of the 35 S RNA until they reach the sORF A start codon. sORF A is translated and terminated and then some fraction of 40 S ribosomes is shunted via stem section 1 and used for reinitiation. AUG codons and non-AUG codons within an AU-rich region are potential start sites for these ribosomes. B, in addition to shunting, post-terminating and perhaps leaky scanning ribosomes can pass through stem section 1 and initiate at the AUG of sORF B, which is in front of another structural element. The second combination of cis-acting shunting elements allows bypassing of stem section 2. C, finally, post-terminating ribosomes melting part of stem section 2 pass it and start migration through the central part of the CaMV leader translating the remaining sORFs.
resulting in reinitiation events. These occur mainly at sORFs E and E' and prevent translation of the F::CAT ORF (Fig. 4C), presumably because ribosomes dissociate from the mRNA following the multiple translation events. In our case the first reinitiation event for linearly migrating ribosomes will be efficient at about 250 nt after the stop codon of sORF A. Removal of sORF C did not affect expression of F::CAT ORF significantly (data not shown). Thus, sORFs D, E, and especially E', rather than complex secondary structures, seem to block linear scanning. It is striking that the short E' ORF is also conserved among different CaMV strains and isolates available in GenBankTM (25). It seems that sORF E' translation ensures concomitant release of ribosomes that have migrated linearly and may allow restoration of stem section 1.

Thus, the following alternative pathways leading to translation initiation in the CaMV leader are equally possible: two shunting events and classical scanning-reinitiation (7). The choice between them might reflect the behavior of different post-translation complexes after having translated the short 5' proximal sORF. The negative correlation between the length of the upstream ORF and the efficiency of reinitiation suggests that ribosomes that translate this ORF may still be associated with some factors required either for translation initiation or for 40 S ribosome scanning along the RNA. There is evidence suggesting that scanning requires ATP hydrolysis (10), where ATP is hydrolyzed by eIF-4A and associated factors that can melt RNA structures. In cap-mediated initiation eIFs 2, 3, 4A, and 4F are sufficient for attachment of 43 S complexes to cap-proximal regions of mRNAs, but eIF1 and eIF1A are required in addition for scanning (37, 38). We assume that the helicase activity of eIFs 4A and 4B, as well as the "scanning-supporting activity" of eIF1 and eIF1A, might favor scanning-reinitiation while inhibiting shunting. On the other hand, the absence of one of these factors but the presence of some other factors of the initial set, especially eIF3 and eIF2 (39), might lead to shunting and reinitiation just downstream of the shunt landing site. In the RRL system, shunting is the predominant process despite the observation that secondary structures seem to be less inhibitory in RRL than in WGE (40). Thus, low scanning reinitiation activity after sORF translation in RRL might reflect a relative deficiency of eIFs 4A, 4B, and/or 1 and 1A.

We now need to address the possible role of these canonical translation initiation factors in shunting, as well as in scanning along the RNA after the sORF translation event.

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