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Viral Strategies of Translation Initiation: Ribosomal Shunt and Reinitiation

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Due to the compactness of their genomes, viruses are well suited to the study of basic expression mechanisms, including details of transcription, RNA processing, transport, and translation. In fact, most basic principles of these processes were first described in viral systems. Furthermore, viruses seem not to respect basic rules, and cases of "abnormal" expression strategies are quiet common, although such strategies are usually also finally observed in rare cases of cellular gene expression. Concerning translation, viruses most often violate Kozak's original rule that eukaryotic translation starts from a capped monocistronic mRNA and involves linear scanning to find the first suitable start codon. Thus, many

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viral cases have been described where translation is initiated from noncapped RNA, using an internal ribosome entry site. This review centers on other viral translation strategies, namely shunting and virus-controlled reinitiation as first described in plant pararetroviruses (Caulimoviridae). In shunting, major parts of a complex leader are bypassed and not melted by scanning ribosomes. In the Caulimoviridae, this process is coupled to reinitiation after translation of a small open reading frame; in other cases, it is possibly initiated upon pausing of the scanning ribosome. Most of the Caulimoviridae produce polycistronic mRNAs. Two basic mechanisms are used for their translation. Alternative translation of the downstream open reading frames in the bacilliform Caulimoviridae occurs by a leaky scanning mechanism, and reinitiation of polycistronic translation in many of the icosahedral Caulimoviridae is enabled by the action of a viral transactivator. Both of these processes are discussed here in detail and compared to related processes in other viruses and cells.

I. Introduction

Eukaryotic chromatin seems to be very uneconomically used. Large regions are devoid of genes, large distances usually separate individual genes, and large and multiple introns separate the true coding regions of a gene. The situation is fundamentally different with virus genomes. These have evolved to optimize the available genomic space to allow a high density of coding information and *cis*-acting control elements such as promoters, polyadenylators, and replicators (*1*). Noncoding regions are minimized and DNA sequences are often multiply used, that is, several overlapping reading phases of viral DNA are used to encode proteins, and coding regions also contain *cis*-elements. Often, polyproteins are produced that are later cleaved to individual functional proteins, which reduces the numbers of promoters and polyadenylators on the DNA as well as on 3′- and 5′-untranslated regions on the RNA. Apparently, this justifies the cost of the extra proteinase required for polyprotein processing. Furthermore, virus RNAs are often multiply used, either through alternative splicing or by various mechanisms of polycistronic translation. In addition some viral proteins are multifunctional.

The class of reverse-transcribing elements (2), that is, retroviruses, pararetroviruses, and retrotransposons, although related in type and number of genes, have evolved a variety of different strategies of gene economy (Fig. 1). Hepadnaviruses have the highest degree of gene and signal overlap: The envelope-coding region is completely included in a different reading phase of the *pol* gene. Hepadnaviruses also make use of multiple transcription initiation from a single promoter. This leads to two types of capsid protein and three types of envelope protein, distinguished by different N-termini. Complex retroviruses, such as human immunodeficiency virus-1 (HIV-1), are specialists in alternative splicing. This leads to six different mRNAs originating from a single original

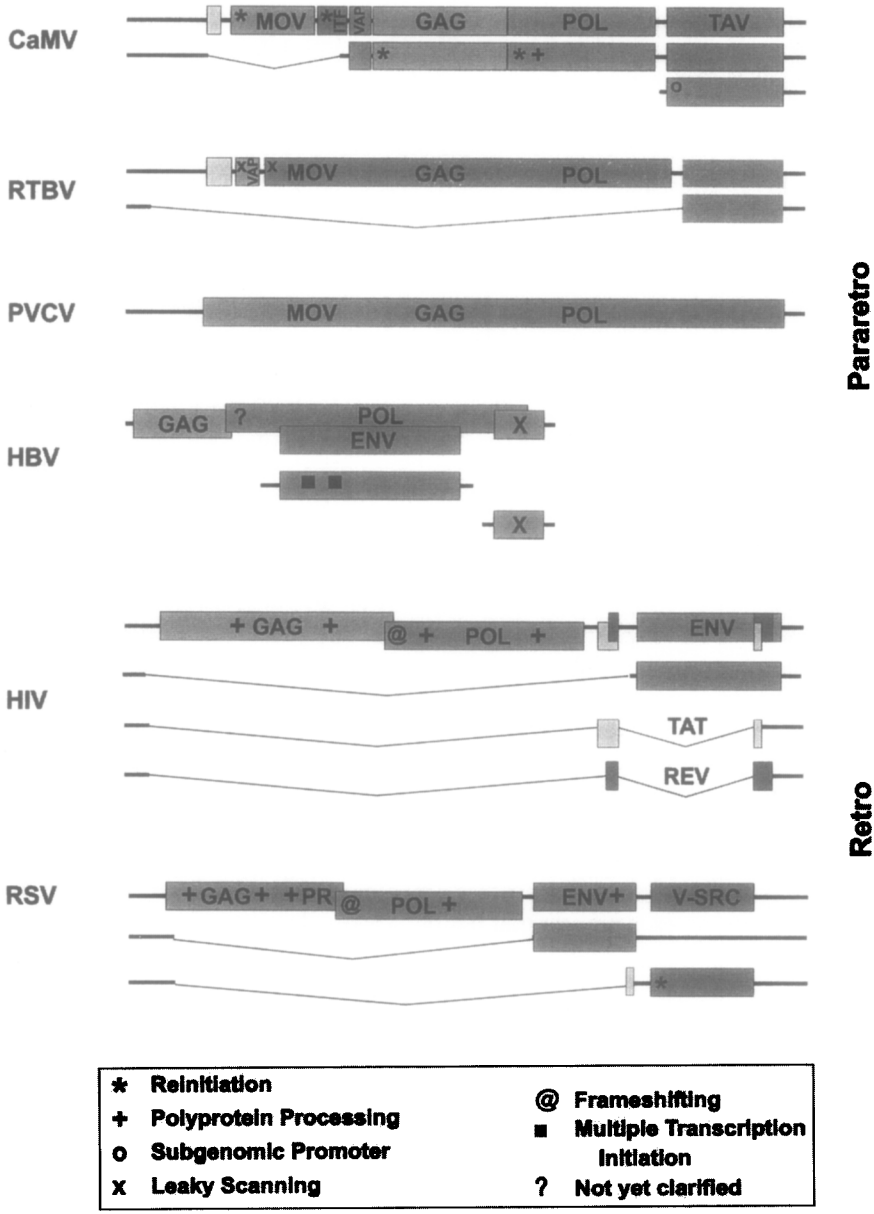

FIG. 1. Schematic representation of pararetro- and retroviral genomes and their expression strategies. The most representative RNAs and features are shown. For more complete information see Refs. *3* and *192*. The processing sites of the PVCV polyprotein are not known. HBV Pol expression does not occur by frameshifting; conflicting results exist regarding whether it is translated via leaky scanning/reinitiation or internal ribosome entry as well.

transcript. This alternative splicing is controlled by a specialized virus protein, Rev, that determines the timing of transport of viral RNA from the nucleus to the cytoplasm (3). Retroviruses also use a frameshift or stop-codon readthrough strategy to produce Gag and Gag–Pol polyproteins from a single RNA, and a polyprotein strategy whereby the Gag protein is processed to yield at least three different structural proteins: the matrix, capsid, and nucleocapsid proteins. In addition, the Gag–Pol polyprotein is processed to yield protease, reverse transcriptase/RNAse H, and integrase. Finally, some of the Caulimoviridae have developed highly sophisticated polycistronic translation mechanisms to produce the maximum number of proteins from a single RNA.

Even among the Caulimoviridae, expression strategies can differ greatly. Petunia vein clearing virus produces a single polyprotein (4), from which the individual proteins are thought to be derived. Badnaviruses and rice tungro bacilliform virus (RTBV) produce a large polyprotein and two small individual proteins by multiple leaky scanning translation (5); the additional open reading frame (ORF) in RTBV is expressed from a spliced RNA. Finally, cauliflower mosaic virus (CaMV) and its closest relatives use a subgenomic promoter for the transactivator/viroplasmin (TAV) protein, splicing and polycistronic translation for expression of six of its ORFs, and the polyprotein strategy to produce protease and reverse transcriptase/RNAse H from a single precursor. Furthermore, at least one of its proteins, TAV, is multifunctional: It both acts as a translation reinitiation activator and directs assembly of virus particles. The "virion-associated protein" (VAP) in Caulimoviridae might also be multifunctional. In addition to being required for insect transmission, it is essential for insect-independent plant infection. Another interesting feature of the Caulimoviridae is that the leader of the pregenomic RNA is long and highly structured and includes packaging and replication signals. The secondary structure of this leader is not melted during the pretranslational scanning process, since most of the leader is bypassed by scanning ribosomes, a process that has been termed shunting.

II. Main Initiation Strategies in Eukaryotes

Mechanisms of translation initiation have been intensively studied and grouped into several categories that differ in the pathway by which ribosomal subunits reach the initiator AUG codon (reviewed by Jackson in Ref. 6).

A. Cap-Dependent Scanning

The majority of eukaryotic mRNAs are monocistronic. Their 5′-leader sequences are capped, short, and unstructured, and they direct initiation from the AUG codon nearest to the capped 5′-end via a cap-dependent ribosome-scanning mechanism (7, 8). The 40S ribosomal subunit, together with associated

initiation factors, engages the mRNA at the capped 5′-end and migrates linearly until it encounters the first AUG codon. This occurs in several steps: the m^7 GpppX cap at the 5′-end of most mRNAs is recognized by subunit 4E of eukaryotic initiation factor (eIF) 4F, which contains in addition eIF4G, eIF4A (an ATP-dependent helicase), and ATP. Ribosomes separate into 40S and 60S subunits before binding mRNA. The 40S subunit binds eIF3 and the eIF2–GTP–initiation methionyl transfer RNA (Met-tRNAi) ternary complex (for review, see Refs. *9, 10*). eIF5 has been implicated in bridging eIF3 and eIF2 (*11*). The complex requires also eIF1 and eIF1A to begin scanning to search for the correct AUG start codon (*12*). At the AUG codon, which is recognized by base pairing with the anticodon in Met-tRNAi, the 40S subunit stops, a 60S subunit joins to form an 80S ribosome in the presence of factor eIF5B (*13*), and elongation begins.

The mechanism of cap-dependent scanning has been elaborated in mammalian and yeast cell systems, but most of its features are also valid for plants (*14, 15*). Scanning can be strongly affected by *cis*-acting elements and *trans*-acting factors: (1) strong secondary structures (below −50 kcal/mole) completely block scanning, causing stalling of 40S ribosomes at the 5′-side of the hairpin (*16–19*); (2) multiple AUG start sites upstream of the main ORF reduce efficiency of downstream initiation (*20, 21*); and (3) RNA-binding proteins can specifically block scanning or 40S loading on the capped 5′-end (*22, 23*).

About 10% of eukaryotic mRNAs (particularly those encoding growth-related factors, tumor suppressors, transcription factors, and protooncogenes) and many viral RNAs contain long, often structured leaders with one or multiple small ORFs upstream of the main coding region, which would interfere with the normal scanning process. To account for this, alternative modes of translation initiation have been proposed that allow the presence of inhibitory elements in the leader to be overcome: (1) "leaky scanning" (*8*), (2) internal initiation (*24–26*), (3) reinitiation after small ORF translation (see reviews by Hinnebusch, Ref. *27*, and Morris and Geballe, Ref. *28*), and (4) nonlinear ribosomal scanning or ribosomal shunting (*29–34*).

B. Leaky Scanning

Leaky scanning allows the bypass of the upstream AUG codon in at least two cases. A start codon located close (<10 nucleotides, nt) to the cap site (*35–37*) or an AUG in a suboptimal nucleotide context for initiation (*20*) (or a non-AUG start codon in an optimal context) is poorly recognized by ribosomes. The latter effect can be suppressed by a structural element located 14 nt downstream of the start codon (*38*). Thus, in some cases, upstream ORFs might be bypassed by leaky scanning, obviating the need for reinitiation at the downstream AUG start site.

C. Internal Initiation

Internal initiation involves direct binding of the 40S ribosome to a region (internal ribosome entry site, IRES) at, or upstream of, the authentic AUG codon, and is mainly used by viruses which have developed a strategy to shut down cap-dependent initiation in a host cell (for review see Refs. *39, 40, 10*). This mechanism does not require a cap structure on the mRNA and thus does not depend on eIF4E and the N-terminal part of eIF4G containing the eIF4E-binding domain (*41*). Therefore, in contrast to cap-dependent initiation, internal initiation is not impaired by cleavage of eIF4G by viral proteases. The best-characterized IRESs are from picornaviruses and hepatitis C virus (HCV), classical swine fever virus (CSFV), and bovine viral diarrhea virus (BVDV) (*42, 43*).

D. Reinitiation

After translating an upstream ORF, terminating ribosomes might remain associated with the mRNA, continue scanning, and initiate at a downstream start codon with an efficiency depending both on mRNA *cis*-elements and *trans*-acting factors (*28*). Reinitiation requires the recruitment of initiation factors and, in general, can occur in eukaryotic RNAs when the upstream ORF is short (2–30 codons), with reinitiation frequency increasing with the distance between the short ORF (sORF) and the "main" ORF (*44, 27, 45, 46*). The requirement for scanning 40S ribosomes to recruit initiation factors, including the ternary complex eIF2–GTP–Met-tRNAi, is the limiting step for reinitiation of translation (*27*). The decrease of reinitiation frequency with the increase of the length of the sORF suggests that, during the short translation event, some initiation factors remain associated with the translational machinery after termination of translation and can be used for reinitiation (*44, 19*).

Scanning posttermination ribosomes might be affected by the mRNA structure surrounding the stop codon of the sORF (*47*), the structure of the sORF-encoded peptide (*48, 49*), and, in some special cases, by degradation of the mRNA (*50, 51*).

After translating an upstream ORF longer than about 30 codons, the terminating ribosome is believed to dissociate from the mRNA. However, there are a few reports suggesting reinitiation at AUG codons located a short distance upstream of the long ORF termination codon in artificial RNA constructs in mammalian cells (*52, 53*).

E. Shunting

Ribosome shunting, first described in CaMV, is a nonlinear scanning mechanism in which initially scanning ribosomes are transferred directly from a 5′-donor site to a 3′-acceptor site without linear scanning of an intervening region (*30*). Shunting will be described later in detail.

III. Translation and Translation-Dependent Strategies of Pararetroviruses and Retroviruses

The plant viral family Caulimoviridae and its animal counterpart Hepadnaviridae, including the well-known human hepatitis B virus (HBV), have been proposed to be classified into a suborder Pararetrovirinae of the order Retrovirales of reverse-transcribing elements (*2*). The members of Pararetrovirinae—also known as pararetroviruses—contain DNA in mature virions, in contrast to the RNA-containing retroviral virions. It is worth mentioning that virions of spumaviruses—a separate genus of Retrovirinae (or retroviruses)—can also contain DNA (*54*). This, together with other striking similarities to pararetroviruses (reviewed in Refs. *55, 56*), places them between the pararetro- and orthoretrovirus suborders of the Retrovirales. The DNA of pararetroviruses is targeted to the nucleus of infected cells, where host RNA polymerase II transcribes a terminally redundant pregenomic RNA, which is eventually reverse-transcribed by the viral reverse transcriptase complex to produce the DNA genome. Retroviruses and pararetroviruses use their (pre)genomic RNA and its spliced versions as polycistronic mRNAs that can be translated into more than one protein. Strategies of polycistronic translation include: (1) frameshifting (in most retroviruses; reviewed in Refs. *3, 57*), (2) leaky scanning [e.g., RTBV (*5*); HIV-1 (*58*)], and (3) reinitiation activated by a viral transactivator [discovered first in CaMV (*59*); see below]. In the controversial case of the HBV Pol ORF (the second cistron following the overlapping Core ORF), translation is believed to occur by leaky scanning combined with reinitiation after translation of a short ORF within the Core ORF (*60–62*) (see Fig. 1). However, alternative initiation mechanisms such as the internal ribosome entry suggested in earlier studies (*63*) and ribosome shunting have not been convincingly ruled out. Moreover, in duck hepatitis B virus, which represents a distinct genus of Hepadnaviridae, a mechanism other than leaky scanning, with some, but not all features of internal, initiation, has been implicated in Pol translation (*64*).

A. The Interplay of Translation and Packaging of Viral RNA

To regulate the multiple usage of pregenomic RNA for replication as well as translation, pararetroviruses have adapted to versatile properties of the eukaryotic translation machinery. In HBV, passage of the translating 80S ribosome (but not the scanning 40S ribosomal subunit) through the RNA packaging signal (ε) disrupts its secondary structure, thereby preventing encapsidation/reverse transcription of the pre-Core RNA (*65*) (Fig. 2). On the other hand, the reduced ability of the 40S ribosome to scan through such secondary structures (*18*) accounts for efficient functioning of the ε signal for packaging of the shorter Core

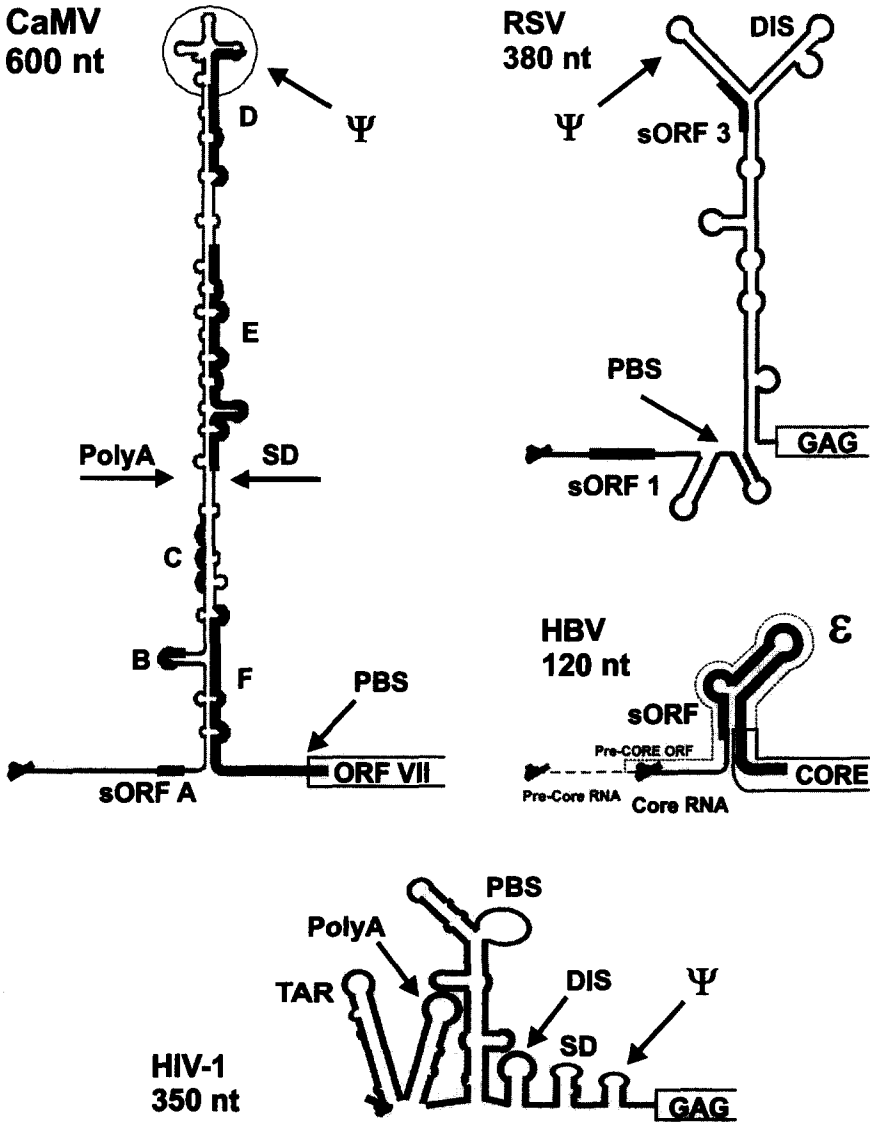

FIG. 2. RNA leaders of pararetro- and retroviruses. The most representative leaders and features are shown. DIS, Dimerization signal; PBS, primer-binding site; polyA, polyadenylation signal; SD, splice donor; TAR, Tat-responding element; Ψ or ε, packaging signal.

RNA, which lacks the 5′ portion with the AUG start codon of the pre-Core ORF (*65*) (see Fig. 2). The ε structure-mediated retardation of the scanning ribosome heading toward the Core start codon might allow the formation of the ε–Pol complex and initiation of reverse transcription on a subpopulation of Core RNAs. Moreover, possible collision of the Pol-nascent DNA complex with the scanning ribosome may help to relocate this complex to the 3′-end of Core RNA in the poorly understood process of template switching that is required for a complete cycle of reverse transcription (*66–68*). Interestingly, despite its negative effect on scanning, and therefore on initiation of polycistronic translation of the Core RNA, the ε structure plays a positive role in pre-Core RNA translation, because it ensures the recognition of the upstream pre-Core AUG, which is in a suboptimal context (*69*), in accordance with Kozak's rule (*38*). This should ensure complete exclusion of pre-Core RNA from the packaging process.

Plant pararetroviruses such as CaMV and RTBV exploit the shunting property of the scanning ribosome (*31, 70*) (see below in greater detail) to allow the bypass of a large region of the pregenomic RNA leader containing a putative packaging signal (for CaMV, see Fig. 2). We speculate that regulation of shunting efficiency may affect conformations of the intervening structure and thereby modulate the interaction of the viral coat protein and the putative packaging signal. In the case of CaMV, this specific RNA–coat protein interaction has been demonstrated by using the yeast three-hybrid system (*71*). Computer-aided analysis of the pregenomic RNA leaders with regard to the consensus shunt configuration (see below) predicts that ribosome shunting operates in most plant pararetroviruses sequenced so far (Ref. 72 and our unpublished results for *Mirabilis* mosaic virus). Similar configurations of shunt elements have also been recognized in the (pre)genomic RNAs of HBV and human foamy spumavirus (our unpublished predictions).

The sorting of genomic RNA for translation and replication has been studied in retroviruses. In some studies, the existence of two separate pools of RNA, one for translation, the other for packaging, was proposed (e.g., *73*). However, in many complex retroviruses, such as lentiviruses (e.g., HIV-1) and alfaretroviruses (e.g., Rous sarcoma virus, RSV), RNA for packaging seems to be sequestered away from the pool of translated RNAs (see, e.g., Ref. *74*). The case of RSV illustrates the complexity of possible interplay between the host translation machinery and viral *cis*- and *trans*-acting factors involved in RNA packaging. The 379-nt-long leader of RSV RNA preceding the Gag ORF contains three phylogenetically conserved sORFs and extensive secondary structure (Fig. 2). The 5′-proximal sORF, sORF 1, is the major ribosome-binding site (*75, 76*). sORF 3 is also translated, and the efficiency of this translation event is thought to regulate a conformational switch in the structure of the RNA packaging signal, Ψ (*77–79*). The conflicting reinitiation/leaky scanning models of sORF 3 and Gag ORF translation deduced from those early studies have been recently revisited by

the group of J.-L. Darlix (*80*), who identified a bipartite IRES driving the two initiation events. A new model proposes that the translating ribosomes stall at sORF 1, most likely due to the stable downstream structure and/or a particular feature of the encoded peptide (MAGPLIP), which resembles the mammalian S-AdoMet decarboxylase regulatory peptide (MAGDIS), which blocks proper termination (*28, 81*) thereby preventing reinitiation at downstream start codons. On the other hand, the Gag AUG start codon is recognized by ribosomes entering internally at the 3′ IRES. Thus, both events would preserve the intervening structure containing the Ψ signal. In addition, ribosomes can enter at a somewhat weaker IRES, roughly mapped to the intervening region upstream of the sORF 3 AUG start codon. This latter ribosome binding followed by sORF 3 translation might interfere with Gag binding to the Ψ signal. The same study (*80*) also proposes a mechanism for translation initiation on the RSV spliced v-Src mRNA. The splicing event creates a new sORF of 30 nucleotides, where the Gag ATG is in-frame with a stop codon located behind the splice junction (see Fig. 1). Presumably, IRES-driven recognition of the Gag AUG would lead to translation of this sORF followed by reinitiation at the v-Src AUG. In fact, the 66-nt region between the sORF and the v-Src ORF favored efficient reinitiation when placed after a large cistron on an artificial bicistronic mRNA (*80*), thus supporting the reinitiation model.

The current revision of RSV translation mechanisms is in line with identification of IRESs in lentiviruses simian immunodeficiency virus (SIV; *82*) and HIV-1 (*83*) as well as in other genera of Retroviridae [murine leukemia virus (*84*), murine sarcoma virus (*85*), avian reticuloendotheliosis virus (*86*)]. However, in these cases the usage of viral RNA for translation and replication may be regulated by means differing from those proposed for RSV. For example, the HIV-1 IRES is located just downstream of the Gag ORF start codon (*83*) and the preceding leader sequence containing the Ψ signal does not seem to exhibit any IRES activity (*87*) (Fig. 2). Scanning ribosomes migrating through the Ψ signal may interfere with the packaging process, unless they are retarded by the leader secondary structure as in the case of HBV Core RNA described above.

Notably, the structural configuration of the RSV RNA leader with a 5′-proximal sORF terminating in front of an extended stem–loop structure resembles the shunt configuration found in plant pararetroviruses (*72*). The experimental evidence does not necessarily exclude the possible occurrence of shunt-dependent initiation events on RSV RNA as suggested in earlier work by Darlix and coworkers (*75*).

B. Retroelement Gag–Pol Translation

A tandem array of Gag (or Core, in the case of HBV) and Pol genes represents the core of reverse-transcribing elements that provides the structural and enzymatic functions essential for replication. The expression of Gag and Pol, and

their ratio, are tightly regulated at different stages of replication. Translational control plays a major role in this regulation. In most retroviruses, Pol is expressed from genomic RNA as a Gag–Pol fusion protein (e.g., *88*), which is thought to be coassembled into the virion together with the much more abundant Gag protein, and eventually processed to release the mature reverse transcriptase. The correct ratio of Gag protein to Gag–Pol protein is critical for viral infectivity (e.g., 20:1 for HIV-1; *89*). The mechanisms driving Gag–Pol translation in retroviruses are (1) programmed ribosome "−1" frameshifting (reviewed in Refs. *90, 91*) and (2) stop-codon readthrough [in type C retroviruses, e.g., UAG readthrough in murine leukemia virus (*92*); reviewed in Ref. *93*]. In both cases, 5–10% of the translating ribosomes produce the Gag–Pol fusion protein, and the others terminate at the Gag stop codon. A structural stem–loop or pseudoknot element downstream of the "slippery" heptamer in the case of frameshifting, or the suppressed stop codon in the case of readthrough, is believed to pause 80S translating ribosomes to increase the efficiency of these events about three orders of magnitude above background. Interestingly, transient pausing of translating (or scanning) ribosomes at various structural elements in mRNA seems to be a common feature of unusual translation mechanisms such as frameshifting, readthrough, hopping (see Ref. *94* and references therein), and shunting (see below).

In hepadnaviruses and many plant pararetroviruses, the Gag and Pol genes are uncoupled and Pol is translated from its own AUG start codon (*95–97*). However, different translation strategies are used to initiate Pol translation. Whereas hepadnaviruses seem to rely on leaky scanning or internal entry abilities of the 40S ribosome (as discussed above), plant caulimoviruses, including CaMV and two other genera of Caulimoviridae, code for a transactivator protein (transactivator/viroplasmin, TAV) that allows polycistronic translation of viral RNAs by a reinitiation mechanism (see below). In CaMV, the Gag and Pol genes (ORFs IV and V, respectively) are located internally both on the pregenomic RNA and its spliced derivatives. Their expression in plant protoplasts requires the presence of TAV (*98*). ORF IV (Gag) is, most likely, translated as a second cistron from one of the three spliced RNAs (*99*), although its translation from pregenomic RNA is not excluded. The mechanism of Pol translation has not been investigated in sufficient detail to draw any definitive conclusion (*97*; also M. Schultze, unpublished PhD thesis, University of Basel, Basel, Switzerland). Theoretically, ORF V (Pol) could be translated in the presence of TAV from the ORF IV-expressing spliced RNA as the third cistron, or from pregenomic RNA as the sixth cistron. Alternatively, a monocistronic RNA species (either subgenomic or spliced RNA) for ORF V might exist. Indeed, there have been occasional, unconfirmed reports of a 22S RNA in CaMV-infected plants (e.g., *100*). In this regard, an additional link has been established between plant caulimoviruses and animal spumaviruses (see above); in human foamy virus, Gag and Pol translational events are also uncoupled (*101*) and Pol is expressed from a separate, spliced RNA (*102*).

In contrast to the three genera of Caulimoviridae coding for TAV (CaMV-like, soybean chlorotic mottle virus-like, and, possibly, cassava vein mosaic virus-like viruses; see Ref. *103*), the three other genera of plant pararetroviruses (RTBV-like, badna-viruses, and PVCV-like viruses) do not possess any homology to TAV consensus sequences. Strikingly, their genome organization further differs in that they encode Gag and Pol within a single polyprotein, thus predicting a 1:1 ratio upon proteolytic processing. The paradox of the apparent surplus of Pol protein that might be expressed by these viruses remains to be understood. Interestingly, the *Schizosaccharomyces pombe* retrotransposon Tf1 also contains a single ORF for Gag and Pol without any obvious means for overexpressing Gag protein (*104*).

IV. Shunting Mechanisms

A. sORF-Dependent Shunt in Caulimoviridae

The RNA leaders of CaMV and other members of the Caulimoviridae contain all three types of elements inhibitory for scanning: (1) a low-energy elongated hairpin (*105*), (2) several sORFs (*72*), and (3) a putative packaging signal (*72*) that, in the case of CaMV, interacts with the viral coat protein (*71*). These would make translation initiation at a downstream ORF difficult (Figs. 2 and 3A). However, translation downstream of the leader of CaMV occurs with reasonable efficiency in plant protoplasts (*106, 30*), in several different *in vitro* systems [wheat germ extract (*107*), reticulocyte lysate (*81*), and yeast extract (*108*)], and in transgenic plants (*109*). This cannot be explained by internal ribosome entry, since initiation at the AUG of ORF VII downstream of the CaMV leader is strongly cap-dependent (*107, 110*).

Originally, it was found that certain parts of the leader support downstream translation, whereas others are inhibitory (*30*). This analysis suggested a mechanism termed the "ribosomal shunt" by which ribosomes bypass the inhibitory leader elements during normal cap-dependent scanning (*30, 31*). Antisense oligonucleotides strongly inhibited translation if directed against 3'- and 5'-proximal parts of the leader, but not if directed against the central region (*107*), again indicating bypass of the central part of the leader.

The ribosomal shunt hypothesis was confirmed in a series of transient expression experiments in plant protoplasts. A strong stem structure inhibited downstream translation if positioned close to the cap site, whereas it had a little effect if placed within the central part of the leader. This result shows that the capped 5'-end is involved in initiation, excludes internal ribosome entry, and suggests that the central stem structure is bypassed. A dicistronic mRNA with

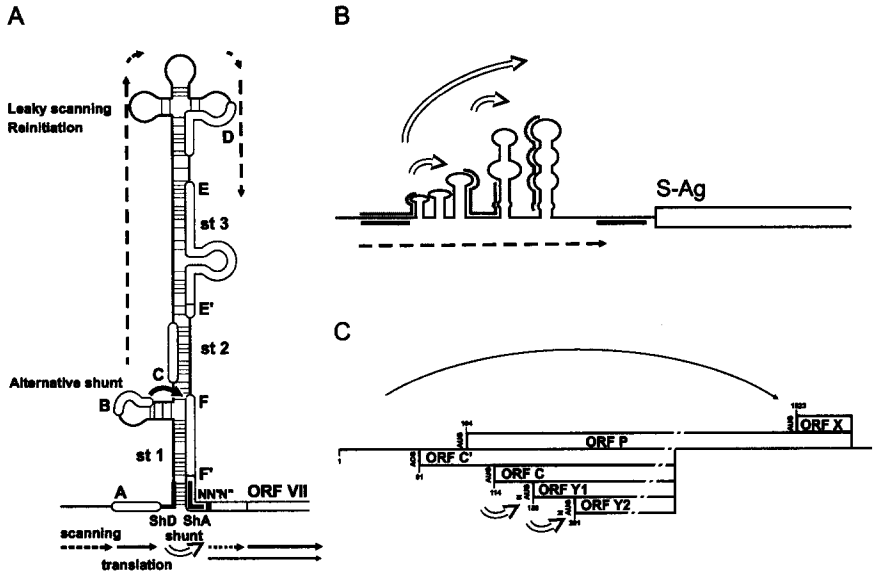

FIG. 3. Schematic representation of shunt-mediating leaders of (A) CaMV, (B) adenovirus, and (C) paramixovirus. (A) The secondary structure of the CaMV leader followed by ORF VII. Black solid lines indicate the positions of the shunt donor (ShD) and shunt acceptor (ShA) sites. Arrows show migration of ribosomes by scanning (dashed), translation (black), main shunting (open), and alternative pathways (solid black). CaMV initiation strategies: Main mechanism, shunt via stem section 1 (st 1; open arrow); alternative shunting via stem section 2 (st 2; solid black arrow) and linear ribosome migration along the leader via leaky scanning and reinitiation (dashed black arrows). Reinitiation can occur at non-AUG codons (N, N′, N″), but the majority of shunting ribosomes reinitiate at the AUG of ORF VII. (B) The secondary structure of the tripartite leader (*33*). Arrows show migration of ribosomes by linear scanning (dashed) and possible shunting pathways (open). The locations of the complementarity regions are schematically shown (thin lines) on the leader structure, as are potential shunt take-off and landing sites (solid black lines). (C) Linear schematic presentation of the Sendai virus P/C mRNA. ORFs are shown as boxes. The AUG or introduced non-AUG (N) start sites are indicated. Arrows show AUGs recognized by shunting ribosomes initiating at Y1 and Y2 ORFs (open) and ORF X (solid black).

a β-glucuronidase (GUS) ORF inserted into the central portion and a chloramphenicol acetyltransferase (CAT) ORF downstream of the leader resulted in translation of both ORFs. Thus, one population of ribosomes might scan into the central hairpin to reach GUS (see later), whereas a second subpopulation might shunt the hairpin to translate CAT (*31*).

The ribosomal shunt was reconstructed by providing the leader in two RNA molecules transcribed from separate plasmids (*31*). The first, spanning residues 1–300, contains the shunt "take-off site" and the second contains the "shunt

landing site" (see below) and a reporter ORF. If these two RNA molecules anneal, the elongated hairpin could form, reconstituting the shunt structure. That the shunt can indeed occur from these two separate molecules was demonstrated in plant protoplasts, albeit with lowered efficiency (*31*).

1. "Take-off" and "Landing" Sites

Extensive analysis of the effects of insertions of a strong stem interfering with scanning ribosomes, as well as start codon insertions, revealed shunt take-off and landing sites flanking the bypassed region of the leader. It is assumed that formation of the leader hairpin structure promotes shunting by bringing the shunt landing site upstream of the first main ORF into close spatial proximity with a shunt take-off site downstream of sORF A, the first 5′-proximal sORF (*110*). Indeed, the CaMV shunt landing site has been mapped on the 3′-proximal part of the elongated hairpin to between positions 548 and 562 (*31*). In RTBV, the shunt landing site has been precisely mapped to just downstream of the leader hairpin (*70*). CaMV sORF A turns out to be a key element promoting shunting in CaMV and other pararetroviruses. The first indication of the importance of sORF A for shunting came from experiments where replacement of the sORF A AUG start codon with an UUG codon nearly abolished translation downstream of the CaMV leader (*31*). Extensive mutational analysis of sORFs within the 35S RNA leader, separately and in different combinations, revealed that only mutations in sORF A frequently reverted on passage of the respective CaMV mutants *in planta* (see Table 1 in Ref. *111*). In addition, mutations leading to the disruption of the elongated hairpin also reverted. Moreover, a striking correlation has been found between the efficiency of ribosomal shunt and viral infectivity; point mutations in sORF A which reduced the level of shunt-dependent expression also reduced infectivity of the virus in turnip plants (*112*). Mutational analysis of sORF A confirmed its important role in ribosomal shunt in different *in vitro* systems (*81, 108*) and in plant protoplasts (*113*).

Comparison of sORF A and several artificial sORFs of different length revealed that the optimal length of the 5′-proximal sORF for efficient shunting was between 2 and 10 codons; longer sORFs significantly reduced shunt-mediated translation (*108, 110, 113*). The peptide sequence of sORF A in general was not important for shunting or infectivity of the virus (*112, 114*). A notable exception is that a one-codon (start–stop) ORF does not promote shunting at all (*113*). Note, however, that in special cases the sORF of optimal length promotes shunting with only a low efficiency (*81*).

Another critical parameter was found to be the spacing between the stop codon of sORF A and the base of the leader hairpin. For optimal shunting this distance should be about 5–10 nucleotides (*110, 113*). Moving the position of sORF A closer to the hairpin base would allow a corresponding number of additional stem base pairs to melt during translation of sORF A, and, as a

consequence, the shunt landing site is shifted to a position further upstream (*81*). Shifting of sORF A further away from the hairpin base precludes melting of the hairpin; the landing site is moved to position further downstream, and the shunting efficiency is strongly diminished. Thus, the position of the shunt landing site depends on the take-off site, which is itself determined by the end of the 5′-proximal sORF.

2. Role of the Secondary Structure between the Take-off and Landing Sites

The stem structure downstream of the 5′-proximal sORF supports near-optimal shunting; a reduction in the number of base pairs reduced its efficiency (*110, 115*). *In vitro* analysis of the strength of the CaMV elongated hairpin, in particular its most stable lower part (so-called stem section 1), suggested the importance of base pairings rather than primary sequence of stem section 1. Physical disruption of stem section 1 by mutations in the left arm of the hairpin structure resulted in a significant reduction of shunt expression, but this could be restored by compensatory second-site mutations that restored secondary structure (*110, 113, 115*). Moreover, an artificial strong stem positioned downstream of sORF A was well able to replace the CaMV hairpin, and an artificial shunt structure could be assembled from the Kozak stem mentioned above with an artificial sORF upstream (*108, 116*). It was therefore proposed that the combination of sORF followed by a stem could be a universal signal promoting the bypass of internal leader region. In line with this, the sORF/stem combination, but little of the primary sequence, is conserved in most of the Caulimoviridae (*72*).

3. Fidelity of Initiation by Shunting Ribosomes

Shunting ribosomes seem to initiate with low fidelity, since they can initiate at non-AUG start codons if such codons are positioned within or near the shunt landing site. After some scanning in the 3′-direction, fidelity improves significantly and non-AUG codons are no longer well recognized (*81*). These results indicate that, upon landing, shunting ribosomes might be deficient in factors responsible for correct AUG start codon recognition. The situation is similar to that in the case of IRES-mediated initiation, where enhanced recognition of non-AUG codons placed at the entry site has been reported (*117*).

In the case of RTBV, ORF I lacks a proper AUG initiation codon and shunt-dependent translation is initiated at an AUU codon (*70*). Shunting ribosomes most likely land directly on this non-AUG codon, apparently without scanning. An AUG start codon introduced at the position of the AUU codon gives a six- to sevenfold increase in the level of ORF I expression, whereas an AUG placed 9 nt upstream of the authentic start site is not recognized. The efficiency of ORF I start codon recognition is low, and effective leaky scanning through the AUU codon as well as through further downstream start codons in suboptimal context

allows polycistronic translation of three of the four large ORFs on the RTBV RNA (5) (see Fig. 1).

4. Shunting Is a Type of Reinitiation

Translation downstream of the CaMV leader could occur either via reinitiation by shunting ribosomes or via an IRES induced after 5′-proximal sORF translation. To distinguish between these two possibilities, sORF A was replaced by an artificial, six-amino-acid-long sORF, MAGDIS, which is known to be inhibitory for downstream start codon recognition (*49, 118*). This sORF caused stalling of the translating ribosome near the sORF stop codon (*81*), thus preventing scanning toward the 3′-end and downstream reinitiation in a bicistronic construct. The stalling event strongly represses expression of a downstream reporter gene in wheat germ extract, reticulocyte lysate, and plant protoplasts, in both linear and shunt-mediating constructs, suggesting that shunting involves a reinitiation process (*81*). The inhibitory effect of the MAGDIS sORF demonstrates that the 5′-proximal sORF must be translated before shunting occurs. Mutant sORFs MAGDI or MAGRIS, which did not cause stalling, allowed efficient progression toward the 3′-end. Thus, CaMV-type shunting can be considered as modified reinitiation.

Further support for this hypothesis comes from the fact that the CaMV-encoded reinitiation factor TAV significantly enhances shunt-dependent expression in plant protoplasts (*31, 113*).

The host protein requirement for shunting is not clear. However, the efficiency of shunting varies significantly between protoplasts originating from host and nonhost plants (*106*). On the other hand, shunting functions well in reticulocyte lysate and yeast, indicating that plant-specific factors are not required.

For the CaMV-type shunt mechanism, the 5′-proximal sORF translation event leads to the bypass of the structural element downstream. This could indicate that the short translation event might selectively remove some of the canonical initiation factors associated with the 48S initiation complex that may be inhibitory for ribosomal shunt, such as the initiation factor eIF4F-associated eIF4A helicase, and eIF4B, which could melt the secondary structure of the stem. However, other factors might still be associated with the ribosome to promote shunting, for example, eIF3.

The current shunt model (Fig. 3A) can be summarized as follows: (1) Ribosomes start cap-dependent linear scanning from the cap until they reach a 5′-proximal sORF start codon located just upstream of a strong structural element. (2) The sORF is translated and terminated, rendering 40S ribosomes shunt- and reinitiation-competent. (3) The ribosomes bypass the structured region and are able to reinitiate at an AUG or a non-AUG start codon immediately downstream or resume scanning and reinitiate at an AUG start codon located further downstream (*81, 113*).

5. Other Initiation Events Operating on the CaMV Leader

After translation of sORF A, a fraction of ribosomes shunt, but others can continue to migrate linearly toward the center of the CaMV leader (*113, 119*). Here, they can recognize some of the central sORFs, reinitiate according to a distance-dependent mechanism, then dissociate from the mRNA after termination of translation (Fig. 3A; *119*). In reticulocyte lysate, the fraction of shunting ribosomes greatly exceeds the fraction of linearly migrating ribosomes (*119*).

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 stem section 1, the combination of sORF B and stem section 2 can support shunting *in vivo* and *in vitro* (*119*) (Fig. 3A). The function of the second shunt might just be to contribute to a tighter protection of the central leader structure, which is thought to be required for packaging. This alternative shunt pathway, although not essential, may increase viral fitness *in planta*.

B. Shunt in Animal Viruses

sORF-dependent shunting has not been reported for viruses other than Caulimoviridae. The molecular details of shunting or of related phenomena proposed for other viruses remain obscure. However, three intensively studied cases of shunting might shed some light on general mechanism of ribosomal shunting.

1. Adenovirus

The adenovirus tripartite leader is a 200-nt-long noncoding region, which facilitates translation of viral mRNAs late in infection (Fig. 3B). The adenovirus late mRNAs transcribed from the major late promoter all contain the tripartite leader. Structural analysis of the leader predicted that the 5′-end is unstructured, whereas the 3′-part includes several moderately stable stem–loops (*120*) (Fig. 3B). As was shown for Caulimoviridae, translation initiation starts at the 5′-end of the tripartite leader, which does not function as an IRES (*121*). In uninfected cells, initiation downstream of the tripartite leader occurs by both scanning and shunting mechanisms with about similar efficiencies (*33*), again resembling the situation in CaMV, where a significant proportion of scanning ribosomes migrates toward the central part of the leader (*113, 119*).

The shunting phenomenon in adenovirus has been demonstrated mainly by insertion of strong hairpin structures (−80 kcal/mole) or AUG codons within the leader, either close to its 5′-end or in the middle part, where both types of insertion would affect the level of translation at the downstream ORF if this region is scanned (*33*). These insertions are inhibitory in the unstructured leader regions comprising the first 80 nt and about last 35 nt and, accordingly, these regions are believed to be scanned linearly. In contrast, insertions into

the structured middle part of the leader are not inhibitory and therefore are considered to be potentially shunted. However, it was found recently that this structured part contains elements essential for efficient shunting. These regions are complementary to the 3′-end of 18S ribosomal RNA (*34*) (see Fig. 3B). The first of these is located within the first 80 nucleotides of the tripartite leader, while the other two are located in the central structured domain. A high level of ribosomal shunting was found to be dependent on the presence of any two of these three regions. Some kind of redundancy exists, since the removal of each of these regions separately had little influence on shunting and scanning efficiency. However, the combination of the second and the third complementarities are functionally dominant, that is, their combined removal significantly impaired shunting. Thus, the shunt take-off and landing sites are apparently degenerate and might be affected by the particular complementarity region present.

The functional role of the complementarity regions in ribosomal shunt remains to be understood. Despite lack of direct evidence for prokaryotic Shine–Dalgarno-type interactions in eukaryotes, a growing number of examples are accumulating where interactions between 5′-noncoding mRNA and 18S RNA regions either promote internal initiation (*122*) or facilitate the binding of 40S ribosomes to RNA leaders (S. Zhanibekova, R. Akbergenov, and B. Iskakov, personal communication). Similar sequences are found at uORF4 of the yeast GCN4 leader (*47*) and close to the shunt landing site in CaMV (*98*). These complementarity regions might induce direct binding of ribosomes or rebinding of dissociated scanning ribosomes, or they might cause stalling of scanning ribosomes migrating within these regions. In this regard, an additional link might be established between adenovirus and CaMV shunt: In both cases shunting might be induced by stalling, mediated by the complementarity region in the case of adenovirus and the stem structure in the case of CaMV.

Consistent with this idea, replacement of sORF A by one of the complementarity regions from the adenovirus leader promotes shunting in the context of the CaMV leader with an efficiency similar to that of the wild-type leader (L. Ryabova and T. Hohn, unpublished). The use of 18S RNA complementarities as shunt elements might be universal, since similar complementarities in the case of another adenovirus RNA, the IVa2 mRNA, which is synthesized during the intermediate phase of viral infection, promote bypass of internal leader regions during late stages of viral infection (*34*).

It is interesting that the basal shunting activity identified in the absence of all three complementarity elements is found to be approximately 5% of the shunting efficiency of the original tripartite leader and considered to represent a basic shunt, the mechanism of which is unknown (*34*). The tripartite leader confers the ability to eliminate or gradually reduce the normal requirement for eIF4E or eIF4F (*121, 123*). Late adenovirus mRNAs have a low requirement for eIF4F

and are preferentially translated late in infection or under heat shock conditions when eIF4F is inactivated (*123*). Significantly, ribosomal shunt mechanisms in adenovirus also function exclusively in conditions when eIF4F is inactivated, during late adenovirus infection or heat shock conditions (*33*).

Like the CaMV shunt, which is activated by TAV (*112*), adenovirus shunt can also be influenced by a viral protein, L4-100K, which is involved in the preferential translation of adenovirus late mRNAs (*124*). Its mechanism of action in the shunt process and whether this is specific for adenovirus late RNA translation remain to be clarified.

Two molecular models have been suggested to explain the phenomenon of ribosome shunt in adenovirus (*125*):

1. *Dissociation model.* The loss of eIF4F RNA unwinding activity would accelerate a high dissociation off-rate of the 40S ribosomes loaded at the 5′-unstructured end, followed by direct rebinding of these ribosomes nearby, or at the start codon, via sequential leader–18S RNA interactions.
2. *Nondissociating or translocation model.* The lack of unwinding activity would block the 40S complex from further scanning into the structural region and promote instead translocation of the tethered 40S complex to the start codon via RNA–RNA interactions or initiation factors recruited to the shunting elements.

2. SENDAI VIRUS

The Sendai virus, a paramyxovirus, contains a nonsegmented minus-strand RNA genome of 15.3 kb, from which six mRNAs are transcribed (*126*). One of these, the polycistronic P/C mRNA, contains two overlapping ORFs and is known to initiate protein synthesis at multiple start codons (*127*). The order of these initiation start sites from the capped 5′-end is ACG^{81} of the C′ ORF, AUG^{104} of the P, V, and W ORFs (moderate initiation context), AUG^{114} of the C ORF, $AUGs^{183/201}$ of the Y1/Y2 ORFs (these two AUGs are in the weakest initiation context), and AUG^{1523} of the X ORF (Fig. 3C). Three proteins (P, V, and W) contain the same N-terminal 317 amino acids (aa), the X protein represents approximately the C-terminal 95 aa of the 568-aa-long P protein, and C, C′, Y1, and Y2 (215, 204, 183, and 175 aa, respectively) represent a nested set of four C proteins with a common C-terminus (*32, 128, 129*). Translation of the P/C mRNA is cap-dependent. Accordingly, its function is inhibited by cap analogs, insertion of a 5′-proximal stem–loop, and poliovirus infection (*130*). ORFs C′, P, and C are translated by linear leaky scanning, while the Y1/Y2, and presumably X, start sites are accessed by shunting.

No specific shunt "take-off" site could be identified by deletion mutagenesis. Moreover, artificial unstructured leaders fused to the start site of C′ ORF mediated efficient shunting, leading to normal levels of Y1/Y2 expression (*130*).

Both Y1 and Y2 expression yields are not affected by mutation of these AUGs to ACGs, suggesting that the fidelity of start codon recognition is strongly reduced. This is reminiscent of the situation found in CaMV and RTBV, although in the latter cases AUGs in the landing site are much more efficiently recognized than non-AUGs. AUGs inserted in two positions in front of the Y1 and Y2 start sites were not recognized as start sites and thus had little effect on the yield of Y1/Y2 proteins, suggesting that shunting ribosomes are directed precisely to the non-AUGs, much as in the case of CaMV and RTBV. Thus, the shunt landing site might be located between the two start sites of the Y1 and Y2 ORFs.

It can be speculated that shunting on Sendai virus RNA might also be induced by stalling of the scanning ribosome, which may be caused by the possible combination of unknown leader *cis*-elements and downstream initiation events at the C′, P, and C initiation codons. Pausing of the ribosome during an initiation event (*131*), further induced, for example, by secondary structure, might create an obstacle for the following ribosome; such pausing then would induce shunting of this ribosome past the stalled first ribosome, allowing the former to initiate further downstream.

Another candidate ORF, the AUG of which is reached by discontinuous ribosome scanning, appeared to be ORF X located at the 3′-end of the P/C mRNA (*29*) (see Fig. 3C).

3. PAPILLOMAVIRUSES

Papillomaviruses are small, double-stranded DNA (dsDNA) viruses that replicate extrachromosomally in the nuclei of infected cells (reviewed in Ref. *132*). Primary transcripts of early and late virus RNAs overlap and are processed by complex splicing and polyadenylation events. Human papillomavirus RNAs, like those of the Caulimoviridae, appear to be polycistronic, that is, encode more than one functional protein (*133–135*). However, posttranscriptional gene regulation in this case is poorly understood and requires further investigation.

Two pathways of initiation of translation have been suggested for expression of the polycistronic papillomavirus RNA containing three long ORFs, E6, E7, and E1, reading from the 5′-end: "extreme" leaky scanning for expression of E7 and ribosomal shunt for E1 (*136, 137*). Despite the very tight arrangement of ORFs E6, E7, and E1 (only 6-bp intergenic space) and 5′-dependent expression of all these ORFs, translational coupling via readthrough or reinitiation is apparently not used. Using the established criterion of examining the effect of insertion of stable stem–loops and upstream strong start codons, both linear scanning to reach E7 [without initiating at any of the 13 AUGs preceding the E7 ORF (*137*)] and nonlinear scanning (ribosomal shunt) for E1 initiation (*136*) have been demonstrated. In the latter case, the shunt "take-off" site is presumably located within the first half of ORF E6, and shunt "landing" would occur somewhere just upstream of the ORF E1 AUG. This indicates that shunting is

not a randomly organized process in papillomavirus, although the specific set of conditions, such as secondary structure or protein requirements, that can trigger the bypass mechanism are not known.

4. Possible Combinations of Shunting and Internal Initiation

Hepatitis C virus mRNAs under control of the HCV IRES were inefficiently translated compared with capped and polyadenylated mRNAs. Addition of a cap and a polyA tail on the HCV mRNAs revealed that these structures interacted with the HCV IRES in a synergistic manner to load ribosomes onto the HCV mRNAs, thereby strongly enhancing translation. The positive effect of the cap and the polyA tail on initiation of translation at the initiator AUG embedded in the HCV IRES might have been the result of a discontinuous scanning, or shunting, mechanism where ribosomes are translocated from the cap site to the IRES (*138*).

Internal initiation in poliovirus might also include a shunting step to bypass the intervening region between the upstream AUG of the IRES used for direct ribosome binding and the downstream AUG used as the translation start site (*139*).

A combination of internal ribosome entry, leaky scanning, and/or shunting was also proposed for the expression of ORF 3b from polycistronic porcine transmissible gastroenteritis coronavirus (TGEV) mRNA 3 (*140*).

C. Candidates for Shunting in Cellular mRNAs

The type of shunting mechanism employed by adenovirus might also be used by the cellular mRNAs encoding hsp70 and c-*fos* (*34*); the leader sequence of both these RNAs contains a single element of complementarity to 18S rRNA and promotes initiation downstream via shunting and scanning mechanisms (*34*).

One unconfirmed report suggested an "internal ribosome repositioning" mechanism to explain differential expression of Myc1 and Myc2 isoforms in the human c-*myc* 5′-UTR (*141*). However, alternative mechanisms such as internal initiation have not been ruled out (*142, 143*).

V. Polycistronic Translation Strategies

After translating an upstream ORF of more than about 30 codons, the terminating ribosome is believed to dissociate from the eukaryotic mRNA in a scanning- and reinitiation-incompetent state. Although we have some knowledge of the first steps of the termination process in eukaryotes and the factors associated with it, our understanding of termination is far from complete. The ribosomal release factor (RRM), which triggers 50S or 70S ribosome release from the mRNA in prokaryotes (*144*), is not found in eukaryotes. Thus, the

fate of the eukaryotic ribosome after translation termination remains unclear. However, following sORF translation, some ribosomes can be assumed to remain associated with the mRNA, continue scanning, and reinitiate. Very little is known about factor requirements for reinitiation of translation. The GCN4 case clarified the role of eIF2 in recognition of the second start site for the reinitiating 40S subunit (*27*). These experiments also suggest that eIF2 is not essential for the 40S scanning process in yeast. eIF3 would apparently also be required to promote binding of the ternary complex to the 40S ribosome. The role of eIF3 and other canonical initiation factors in reinitiation remains to be clarified.

Several groups have reported the existence of potentially bicistronic gene structures in eukaryotes, which could require reinitiation steps for their expression. Phylogenetic analysis of the SNRPN (small nuclear ribonucleoprotein N, SmN) mRNA reveals a highly conserved coding sequence SNURF (SNRPN upstream reading frame, 71 amino acids in length) upstream of the SNRPN ORF; both SNURF and SNRPN are produced from a bicistronic transcript in normal human and mouse tissues (*145*). In mammals, active LINE 1 elements of retroviral origin produce two proteins from a single transcript (*146–148*). Several bicistronic messages have been reported in *Drosophila melanogaster,* at the *stoned* locus (*149*), at the *Adh* locus (*150*), and from the *mei*-218 gene (*151*). Interestingly, the overlapping stop–start codon UGAUG, found in embryonic exons as a result of alternative splicing during development, converts the monocistronic adult-type message encoding glutamic acid decarboxylase (GAD) into a bicistronic one coding for a 25-kDa leader peptide and a 44-kDa enzymatically active truncated GAD (*152*). It remains to be seen if any of these mRNAs use internal initiation, reinitiation, or leaky scanning. Also, for each case of a potentially polycistronic RNA, careful analysis of additional promoter regions, splice sites, and degradation mechanisms is required (*153*).

The best-studied examples of polycistronic mRNAs are found in the plant viral family Caulimoviridae, which contain up to seven long ORFs within a single RNA. However, the strategy employed to effect polycistronic translation differs in RTBV-like viruses, which use a leaky scanning mechanism, and CaMV-like viruses, which use a reinitiation mechanism activated by a viral protein.

A. Translation of Polycistronic mRNA via Leaky Scanning in Bacilliform Caulimoviridae

The pregenomic RNA of RTBV and other bacilliform Caulimoviridae contains four or three main ORFs, respectively, and serves for translation of at least three proteins, the products of ORFs I, II, and III (*5*). In RTBV, the splicing event between the first sORF and ORF IV provides a mRNA for the production of the ORF IV protein (*154*) (see Fig. 1). The first ORF recognized after shunting in RTBV is ORF I, which begins with the non-AUG start codon AUU (*70*). According to the Kozak scanning model, this non-AUG codon could be easily

bypassed by linear migrating ribosomes or shunting ribosomes landing upstream of ORF I. A remarkable property of ORFs I and II is the complete lack of AUGs in any reading phase to impede scanning ribosomes and preclude downstream initiation. The start codon of ORF II is in an unfavorable sequence context and about half of the scanning ribosomes ignore it and initiate at the start codon of ORF III, which is in a strong initiation context (*5*). Thus, ribosomes loaded at the capped 5′-end of the RTBV RNA first shunt the leader sequence and then scan over a distance of about 900 nt upstream of ORF III, confirming the reported high processivity of scanning ribosomes *in vitro* (*155*). Mutation of the ORF I AUU codon to create a strong initiation codon leads to a drastic decrease in expression of ORFs II and III *in vivo* and *in vitro* (*5*).

B. Activated Reinitiation in Icosahedral Caulimoviridae

The pregenomic RNA of CaMV and its internally spliced derivatives (*99*) serve as polycistronic mRNAs for a number of viral proteins. There are several lines of evidence that these polycistronic RNAs are indeed used for viral protein production in CaMV-infected plants. First, only one subgenomic RNA, the 19S RNA encoding the transactivator/viroplasmin protein (TAV), has been identified. The other ORFs are translated from the 35S RNA or its spliced versions. Second, ORFs I–V are tightly arranged and often overlap by a single base, suggesting that their translation might be linked. Indeed, some mutations within ORFs VII and II are polar, typical for polycistronic prokaryotic mRNAs, meaning that they affect translation of the following ORF (*156, 157*).

1. Polycistronic Translation in Icosahedral Caulimoviridae Is under the Control of TAV

Translation of the 35S RNA is 5′-end-dependent and the first translatable ORF is sORF A within the leader. Thus, translation of major ORFs on the CaMV RNA requires reinitiation. Reinitiation on the polycistronic 35S RNA and its spliced versions is activated in the presence of TAV (*59, 98, 99*), which is expressed early in infection. Transient expression of derivatives of the CaMV genome, each containing the upstream sequence of a specific ORF fused to the CAT or GUS reporter genes, was shown in plant protoplasts (*98*). A reporter gene fusion to the start codon of ORFs VII and II was expressed at 15% of the level of the monocistronic control, but at 100% if a second plasmid expressing the TAV ORF was provided; fusions to the start codons of ORFs I, III, IV, and V were expressed only in the presence of TAV (100% for ORF I, 33% for ORF III, 50% for ORF IV, and 3% for ORF V). TAV-mediated transactivation of expression of all the major ORFs on the polycistronic pregenomic RNA has been also reported for figwort mosaic virus (FMV; *158–160*) and peanut chlorotic streak virus (*161*), and TAV of CaMV and FMV can reciprocally activate polycistronic translation in these viruses (*158*).

The use of partially or completely artificial constructs has confirmed the role of TAV in general activation of polycistronic translation (*19, 45*). When bicistronic constructs, for example, consisting of ORFs VII and CAT, or GUS and CAT, were analyzed in plant protoplasts, the level of expression of the second ORF was always under TAV control, whereas expression of the first ORF was not affected by TAV. Specific *cis* sequence signals are not required for transactivation of second ORF translation, since TAV can activate reinitiation after translation of any first ORF in an artificial bicistronic RNA (*19, 45*). Notably, the presence of a sORF (optimally around 30 codons) upstream of both long ORFs strongly enhances the process, activating expression of the first ORF 2-fold and the second 8- to 10-fold. A long overlap of the major ORFs (130 nt) inhibits transactivation, whereas a short overlap (17 nt) is permissible. In caulimoviruses a short overlap between two ORFs, via an AUGA, is very common, although expression of the downstream ORF is TAV-dependent as well (*19*). A stem structure at the cap site inhibits expression of both reporters, whereas a stem between the two ORFs inhibits only expression of the second (*19*).

Similar results were obtained in yeast when the 35S RNA promoter was used to direct bicistronic RNA transcription (*162*) and in transgenic plants (*163*). Transgenic plants expressing TAV exhibit chlorosis and other virus-like symptoms (*164–168*). TAV expression in these plants also correlates with changes in plant morphology and development, suggesting that TAV might transactivate translation from as-yet-unknown complex mRNAs encoding regulatory proteins involved in development.

2. TAV and Its Interactions with the Host Translational Machinery

TAV is mainly expressed from the 19S subgenomic RNA, but it is also able to transactivate its own expression from the 35S RNA (*169, 170*). TAV is very abundant in the cytoplasm of infected cells and forms a dense matrix, the so-called inclusion bodies or viroplasm. In early phases of infection, these are surrounded by polyribosomes (*171–173*). These "tethered" ribosomes could direct CaMV translation products into the inclusion bodies, where all proteins expressed from CaMV RNAs are found (*174, 175*), including heterologous nonviral proteins encoded by transgenic CaMV RNA (*176*).

TAV has many functions in the life cycle of the virus. In addition to polycistronic translation, it controls virus assembly and replication and determines the host range (see Ref. *177* for review). Virion assembly might be guided by the interaction of TAV with coat protein (*178*). Moreover, it appears that TAV is involved in the stabilization of other viral gene products (*179*).

A basal level of transactivation activity has been demonstrated to be associated with the central portion of the TAV protein (miniTAV or MAV; *180*) (Fig. 4) in protoplasts transfected with a 100-fold excess of DNA encoding this

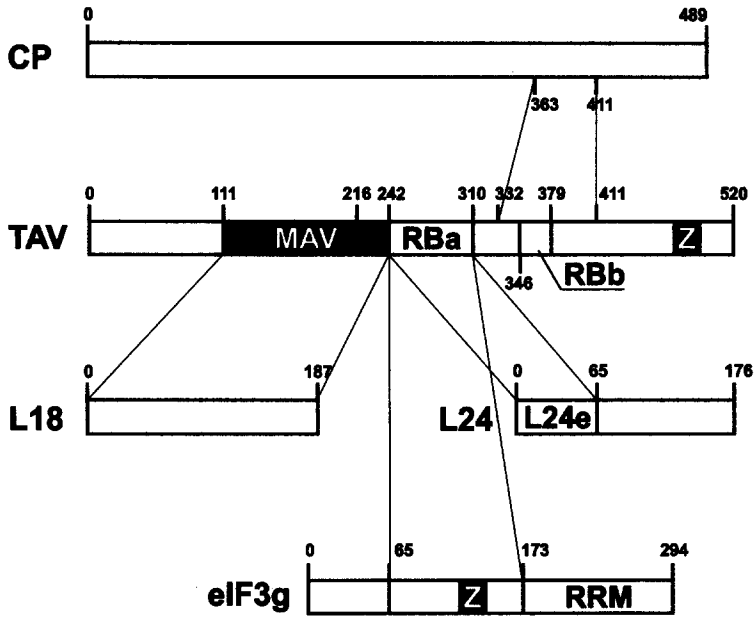

FIG. 4. Protein–protein interactions between TAV and its viral and host partners. CaMV coat protein (CP), ribosomal proteins L18 and L24, and eIF3g are shown as boxes. The interacting regions of the two proteins are connected by thin lines. The MAV, RBa, RBb, and Zn finger of TAV are shown. The L24 region of homology with archaebacterial L24e is indicated. The RRM and Zn finger of eIF3g are also indicated.

polypeptide. In addition to the defect in transactivation, MAV has so far only been found to be active in *Nicotiana plumbaginifolia* protoplasts, in contrast to full-length TAV, which is active in protoplasts of a number of dicot and monocot plants (*59, 158*). The C-terminal part of TAV (CTAV; Fig. 4) can efficiently inhibit the transactivation activity of the entire protein, suggesting the CTAV is able to sequester host factors that are essential for transactivation activity (*180*). Notably, the N-terminal region located upstream of the MAV domain was found also to affect TAV-mediated transactivation activity (*181*).

TAV transactivates polycistronic translation by association with host translational machinery, namely polysomes and associated proteins. Indeed, TAV was found in polysomes isolated from CaMV-infected turnip plants, and it cosediments with polysomes isolated from healthy turnip plants (*182*). This interaction is apparently mediated by interaction with key components of the host translational machinery, namely the 60S large ribosomal subunit via at least two ribosomal proteins, L24 (*182*) and L18 (*183*), and with initiation factor eIF3 via its subunit g (*182*). The L18 ribosomal protein interacts with the MAV domain of

NTAV, while both the central segment of eIF3g, including the Zn-finger motif, and the N-terminal half of ribosomal protein L24, compete for the same binding site within one of the RNA-binding domains (RBa) of CTAV (Fig. 4).

It is remarkable that both ribosomal subunits and eIF3 can be found in a complex with TAV *in vitro*, but TAV association with 40S is indirect, that is, mediated by eIF3. In contrast, eIF3 association with 60S is mediated by TAV. Thus, the existence of two ternary complexes, TAV/eIF3/40S and eIF3/TAV/60S, might play an essential role in the transactivation process. Significant accumulation of eIF3 in polysomes isolated from CaMV-infected plants correlates well with TAV accumulation, whereas only traces of eIF3 were found in polysomes isolated from healthy turnip plants (*182*).

Transient overexpression of eIF3g has a strong negative effect on TAV-mediated polycistronic translation in plant protoplasts (*182*), which correlates well with the reported *in vitro* interaction between TAV and complete eIF3, suggesting that a TAV/eIF3 complex is active in transactivation.

Archaebacterial homologs of L24 and L18 are located on the interface and the external surface of the 60S ribosome, respectively (*184, 185*). In eukaryotes, the location of L24 in the main factor-binding site on the interface of the 60S ribosome has also been reported (*186*; R. Beckmann, personal communication). This suggests that, in the eukaryotic 60S ribosome, these proteins are located too far apart to interact with the same molecule of TAV. Thus, the 60S ribosome is presumably capable of binding two TAV molecules simultaneously on its external and internal surfaces.

These observations can explain why transient overexpression of L24 has a strong positive effect on TAV-mediated polycistronic translation in plant protoplasts (*182*). Interaction of TAV with the main factor-binding site on the 60S ribosome via L24 might indeed lead to inhibition of protein synthesis during the late phase of viral infection, promoting a switch to viral assembly. On the other hand, L24 might have an extraribosomal function and a separate protein might be involved in 60S subunit turnover, as shown previously for yeast L24 (*187*). The ribosomal protein L18, due to its location on the external surface of the 60S ribosome, might interact with other partners such as TAV without affecting 80S ribosome formation.

The accumulation of TAV as the main matrix protein in viral inclusion bodies together with the coaccumulation of proteins derived from the polycistronic CaMV RNA suggests that TAV-enhanced translation occurs on the inclusion body surface. The TAV molecules that form the inclusion bodies might present a perfect surface for ribosome and eIF3 recruitment; the concomitant increase in local concentration of these factors might contribute to the increased reinitiation rate observed in the presence of TAV. RNA-binding activities of TAV, mediated by its ssRNA- and DNA-binding domains within its C-terminal part (*180*), and the dsRNA-binding domain within the MAV region (*188*) might further enhance local TAV concentration in polysomes (Fig. 4).

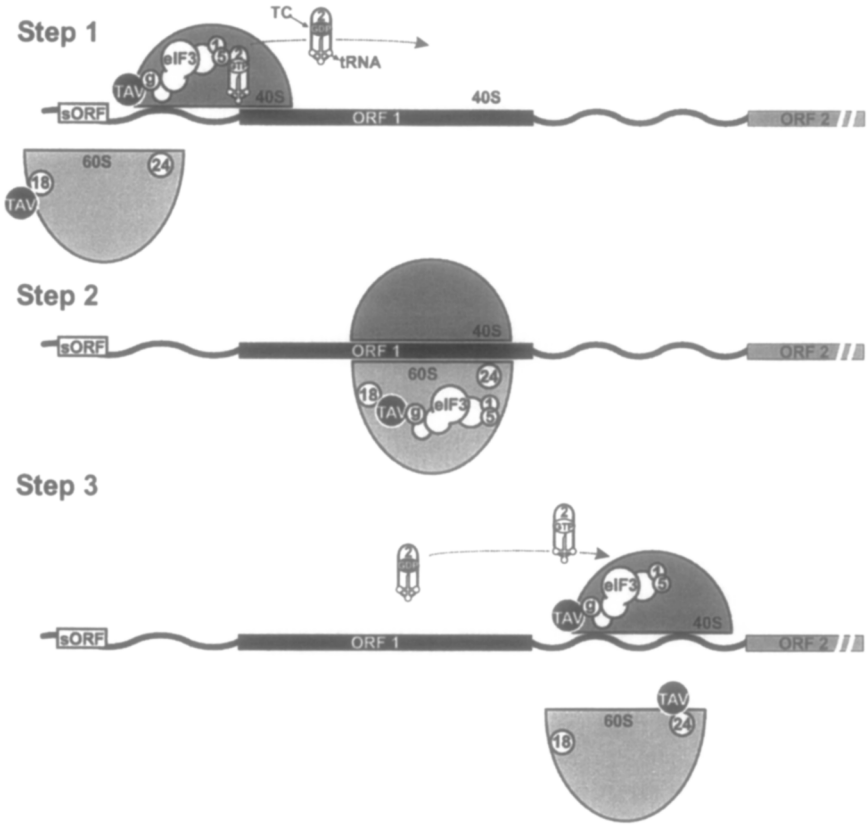

FIG. 5. Model of TAV function during translation of the polycistronic RNA. Steps 1–3 are described in the text. eIF3, its subunit g, eIF1 (1), eIF2 (2), eIF5 (5), tRNA, L24 (24), L18 (18), and TAV are indicated. Recycling of eIF2 is shown by a dotted arrow.

C. Model of TAV-Activated Reinitiation

Despite the abundance of data on TAV molecular interactions with the host translational machinery, we do not yet understand the exact mechanism of TAV-mediated reinitiation of translation. However, the accumulated data allow us to offer the following three-step working model (Fig. 5).

Step 1. Primary TAV acquisition by eIF3. Several observations suggest that TAV is preferentially sequestered by the translational machinery during sORF translation. (1) Ribosomes often translate an sORF and then reinitiate efficiently downstream of it (*44, 28*). This suggests that some of the eIFs are still associated with the translational machinery after sORF translation, but not after translation of larger ORFs. (2) Polycistronic translation under TAV control is enhanced by an sORF (or CaMV ORF VII) positioned in the leader (*19, 45*). (3) TAV

interacts with eIF3 as well as with eIF3 bound to the 40S ribosome (*182*). (4) eIF3 might be associated with the 40S ribosome without interfering with the 80S ribosome formation (*189*). We assume that after sORF translation, the 40S ribosome including certain eIFs remains loosely attached to the mRNA and can resume scanning. We further speculate that eIF3 is one of the remaining factors, whereas eIF2/GDP, and perhaps also eIF4F and eIF4B, are preferentially released. eIF2/GTP/Met-tRNAi can then be reacquired after resumption of scanning via the bridging function of eIF5 interacting with eIF3c (*190*) and TAV could be acquired through its association with eIF3 via its subunit g, perhaps even docking at the same position as other eIF3-interacting proteins. Yeast eIF3g interacts with eIF4B (*191*), and we have confirmed that this interaction also occurs in plants (H.-S. Park, T. Hohn, and L. A. Ryabova, unpublished); TAV could thus be sequestered by eIF3g upon release of eIF4B.

Step 2. Preservation of reinitiation-competent ribosomes during translation of the first large ORF. During translation of a longer ORF in the absence of TAV, eIF3 will be released during the translation elongation step, leaving ribosomes incompetent for reinitiation. In the presence of TAV, however, the interaction of eIF3 with the translating ribosome might be stabilized. We do not know in this case whether eIF3 holds its original position or is moved to a new location within the translation machinery. An exciting possibility is that eIF3 is delivered to the 60S ribosome via the L18 ribosomal protein, which is located at the 60S external surface. We were able to show the existence of both TAV/eIF3/40S and eIF3/TAV/60S complexes *in vitro*. This raises the possibility that eIF3 is transferred to the 60S ribosome apparently via the L18/TAV interaction. We do not think that the alternative L24/TAV interaction is involved at this step, since L24 and eIF3g compete for the same binding site on TAV and L24 is located at the 60S/40S interface.

Step 3. Reinitiation at the second large ORF. We postulate that during the translation termination process, the binding site on the 40S ribosome for eIF3 becomes available again and eIF3 could be transferred back to this site, leading to reacquisition of eIF2/GTP/Met-tRNAi and scanning to find the second ORF start codon.

VI. Outlook

The translation process is the most important step in transforming genetic into functional information. The interplay of initiation factors and regulatory regions on the leaders and trailers of RNA is the most important parameter controlling translation, both quantitatively and qualitatively. Although the basic translation initiation factors and some details of the translation process are known, control of translation initiation varies from case to case and is controlled

by environmental factors, much as is transcription. Scientists try to find rules that make the behavior of biological entities and processes predictable. However, also in biology, rules are there to be broken and often the exceptions lead to deeper understanding of the rule. Viruses are among the best model organisms for studying these parameters due to the rapid evolution of their translation control mechanisms and the high density of regulatory sequences located on their genome. Members of the Caulimoviridae are excellent examples of such viral rule-breakers, circumventing linear scanning by shunting and allowing reinitiation by interactions of a viral protein with the translational machinery. The future will tell whether similar mechanisms occur also during translation of cellular messenger RNAs.

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