Structure and function of a cap-independent translation element that functions in either the 3’ or the 5’ untranslated region

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ABSTRACT
Barley yellow dwarf virus RNA lacks both a 5’ cap and a poly(A) tail, yet it is translated efficiently. It contains a cap-independent translation element (TE), located in the 3’ UTR, that confers efficient translation initiation at the AUG closest to the 5’ end of the mRNA. We propose that the TE must both recruit ribosomes and facilitate 3’-5’ communication. To dissect its function, we determined the secondary structure of the TE and roles of domains within it. Nuclease probing and structure-directed mutagenesis revealed that the 105-nt TE (TE105) forms a cruciform secondary structure containing four helices connected by single-stranded regions. TE105 can function in either UTR in wheat germ translation extracts. A longer viral sequence (at most 869 nt) is required for full cap-independent translation in plant cells. However, substantial translation of uncapped mRNAs can be obtained in plant cells with TE105 combined with a poly(A) tail. All secondary structural elements and most primary sequences that were mutated are required for cap-independent translation in the 3’ and 5’ UTR contexts. A seven-base loop sequence was needed only in the 3’ UTR context. Thus, this loop sequence may be involved only in communication between the UTRs and not directly in recruiting translational machinery. This structural and functional analysis provides a framework for understanding an emerging class of cap-independent translation elements distinguished by their location in the 3’ UTR.

Keywords: 5’ cap; luteovirus; poly(A) tail; RNA secondary structure

INTRODUCTION
The 5’ m7GpppN cap and a 3’ poly(A) tail on eukaryotic mRNAs interact synergistically to facilitate translation initiation (Gallie, 1991; Tarun & Sachs, 1995). The structural foundation for this synergy is the circularization of the mRNA by protein factors that interact with the cap and the poly(A) tail (Wells et al., 1998). Specifically, eukaryotic initiation factor (eIF) 4E binds the 5’ cap (Carberry et al., 1991; Marcotrigiano et al., 1997), poly(A) binding protein (PABP) binds the poly(A) tail (Deo et al., 1999), and each of these proteins binds a different site on the large, adaptor protein, eIF4G (Mader et al., 1995; Tarun & Sachs, 1996; Le et al., 1997). Various isoforms of these factors and other proteins participate in this process (Browning, 1996; Craig et al., 1998; Gradi et al., 1998), but the important notion is that the above interactions bring the cap and the poly(A) tail together in a closed loop mRNA (Hentze, 1997). By mechanisms that are not clear, this structure greatly enhances the recruitment of the 43S ribosomal subunit initiation complex to the 5’ UTR of the mRNA. The 43S complex scans in the 3’ direction to the first AUG codon, at which point the 60S subunit joins, initiation factors are released, and protein synthesis begins (Kozak, 1989; Gray & Wickens, 1998; Dever, 1999; Preiss & Hentze, 1999).

The 5’ cap and poly(A) tail also play roles in mRNA stability. Deadenylation and decapping are triggers for mRNA degradation (Beelman & Parker, 1995). Many capped, polyadenylated eukaryotic mRNAs contain elements in their UTRs that modulate interactions of the mRNA with translation or degradation machinery. Examples include mRNAs translationally regulated by iron responsive elements (Rouault et al., 1996), and numerous mRNAs whose translation and localization are controlled by the 3’ UTR in early embryo development of Drosophila (Gunkel et al., 1998; Sonoda & Wharton, 1999), Caenorhabditis elegans (Goodwin & Evans, 1997), and vertebrates (Gray & Wickens, 1998).

mRNAs of many RNA viruses lack a cap and/or a poly(A) tail, but they compete effectively with host capped and polyadenylated mRNAs for the translation machinery. These viral RNAs often have special UTRs...
that substitute for a cap or a poly(A) tail. The best-characterized sequences that confer cap-independent translation are the internal ribosome entry sites (IRES) of mRNAs of viruses in the *Picornaviridae* family (reviewed by Jackson & Kaminski, 1995; Ehrenfeld, 1996), *Pestivirus* genus (Poole et al., 1995), and related hepatitis C viruses of the *Flaviviridae* family (Teukiyama-kohara et al., 1992; Wang et al., 1993; Reynolds et al., 1995). Some cellular mRNAs also have an IRES, even though they are capped (Macejak & Sarnow, 1991; Johannes & Sarnow, 1998; Chappell et al., 2000). An IRES is normally hundreds of bases long, and is a highly structured 5' UTR that can facilitate ribosome binding with the assistance of many canonical initiation factors (Jackson & Kaminski, 1995; Pestova et al., 1996; Kolupaeva et al., 1998). A variety of different RNAs can have IRES activity. The two classes of IRES in the *Picornaviridae*, the HCV IRES, those of cellular genes, and a short sequence in a *Tobamovirus* that purportedly acts as an IRES (Skulachev et al., 1999) all bear little resemblance to each other in primary or secondary structure. Despite considerable research, including identification of IRES-binding proteins (Belsham & Sonenberg, 1996), the exact mechanisms by which these complex structures recruit the ribosome is still a mystery.

In contrast to the above RNAs, Tobacco mosaic virus (TMV) RNA has a 5' cap but no poly(A) tail. A pseudoknot-rich domain in the TMV 3' UTR substitutes for a poly(A) tail (Gallie & Walbot, 1990), perhaps by binding a common factor that also binds to the TMV 5' UTR in a cap-dependent manner (Tanguay & Gallie, 1996). In the case of *Rotavirus*, which also has capped, nonpolyadenylated mRNAs, viral protein NSP3A interacts with the viral 3' UTR as well as the human elf4GI in a complex with elf4A and elf4E (Vende et al., 2000). Thus, NSP3A substitutes for PABP in the interaction between two ends of the mRNA.

RNAs of viruses and satellite viruses in the *Luteovirus* (Allen et al., 1999) and *Necrovirus* (Lesnaw & Reichmann, 1970; Danthinne et al., 1993; Timmer et al., 1993) genera, and the large *Tobamovirus* family (Qu & Morris, 2000) lack both a 5' cap and a 3' poly(A) tail. However, they can be translated efficiently, owing to different translation enhancement sequences residing in their 3' UTRs (Danthinne et al., 1993; Timmer et al., 1993; Wang & Miller, 1995; Wang et al., 1999; Wu & White, 1999; Qu & Morris, 2000). These differ from IRES in two fundamental ways: they do not confer internal ribosome entry, and they are located in the 3' UTR. The structures and mechanism of action of these sequences are unknown.

To investigate this novel translational control via 3' UTR, we use the 5,677-nt genomic RNA of Barley yellow dwarf virus (BYDV), a member of genus *Luteovirus* that has a particularly complex 3' end. Various domains within the 3'-terminal 869 nt of the BYDV genome have the following functions: (1) cap-independent translation element (3' TE) (Wang et al., 1997), (2) substitution for a poly(A) tail (this report), (3) two subgenomic RNA promoters (Koev & Miller, 2000), (4) a small ORF, and (5) the origin of (-)-strand synthesis (Koev, 1999). At the 5' end of this 869 nt sequence resides the 105-nt 3' TE (TE105, nt 4814–4918, gray box in Fig. 1A), which facilitates translation of both genonomic and subgenomic RNAs cap independently in wheat germ S30 extract (Wang & Miller, 1995; Wang et al., 1999). This sequence can also facilitate cap-independent translation when located in the 3' or 5' UTRs of reporter genes (Wang et al., 1997, 1999). In plant cells, a longer sequence of at most 869 bases at the 3' end of the genome (TE869, nt 4809–5677, Fig. 1A) is necessary for full translation activity. It enhances the translation of uncapped mRNA at least 100-fold compared to mRNA lacking a functional 3' TE (Wang et al., 1997). The ability of portions of the 3' UTR of BYDV to substitute for a cap and a poly(A) tail makes it unlike any known IRES sequence or 3' translational control element of capped mRNAs. Thus, the 3' TE, and probably similar elements in the 3' UTRs of uncapped plant viruses (Danthinne et al., 1993; Timmer et al., 1993; Wu & White, 1999; Qu & Morris, 2000), represent a new class of cap-independent translation elements and 3' UTR translational control elements.

Given that ribosomes scan from 5' to 3', and that peptide synthesis initiates at the 5'-proximal AUG codon on BYDV RNA, the 3' TE must interact with the 5' UTR either directly or indirectly. Thus, we predict that the 3' TE has at least two functions: communication with the 5' end of the mRNA, and recruitment of the translation machinery. In this report, we reveal the highly structured nature of the TE sequence, and roles of domains within the TE in cap-independent translation.

**RESULTS**

### The 105-nt 3' TE confers cap-independent translation in vivo, and additional viral sequence functionally substitutes for a poly(A) tail

The 105-nt 3' TE sequence, spanning nt 4814–4918 (TE_4814–4918 or TE105), is sufficient for cap-independent translation in wheat germ extract (Wang et al., 1997, 1999). However, additional sequence from the viral 3' UTR is necessary for translation in oat protoplasts (in vivo) (Wang et al., 1997, 1999). Because TE105 alone provides translational activity equal to a cap in vitro (Wang & Miller, 1995), we hypothesize that the extra sequence required in vivo might provide a functional equivalent to a poly(A) tail. Presence of a poly(A) tail has little effect on translation of mRNAs in wheat germ extracts (Doel & Carey, 1976), whereas it greatly stim-
ulates translation of capped mRNAs in vivo (Gallie et al., 1989; Gallie, 1991).

To test the above hypothesis, we constructed luciferase-encoding mRNAs containing TE105 with all possible combinations of a cap and/or a 60-nt poly(A) tail. These transcripts were tested for the ability to express luciferase in vivo (oat protoplasts). As a positive control, we used mRNA containing the viral 5' UTR and a 3' UTR comprised of 869 viral bases, from base 4809 to the 3' end of the genome, base 5677 (5' UTR-LUC-TE869). As shown previously (Wang et al., 1999), this mRNA was translated efficiently in oat protoplasts. Its efficiency as a translation template was not stimulated significantly by addition of a cap and/or a poly(A) tail (Fig. 1B). In contrast, uncapped mRNA lacking viral sequences gave virtually undetectable translation in the absence of either cap or poly(A) (vec50-LUC-vec58; Fig. 1B). Presence of both these modifications gave translation equal to that of uncapped, nonpolyadenylated 5'UTR-LUC-TE869. Uncapped, nonpolyadenylated transcript, containing viral 5' UTR and only the TE105 in the 3' UTR (5' UTR-LUC-TE105) gave no detectable translation. Addition of a poly(A) tail stimulated translation at least 50-fold. Thus, the additional viral sequence in the 869-nt 3' UTR (outside of TE105) that is needed for cap-independent translation in vivo can be replaced significantly by a poly(A) tail alone, but not by a cap alone (Fig. 1B). However, this was still four- to fivefold below the luciferase expression obtained with a cap and a poly(A) tail, or that obtained with the 869-nt viral 3' UTR in the absence of a cap. As a negative control, we introduced a four-base duplication in the BamHI4837 site within TE105 (TE105BF), which showed previously eliminates cap-independent translation (Wang et al., 1997). As expected, this completely abolished translation of uncapped mRNAs, but had little or no effect on capped and polyadenylated mRNAs (Fig. 1B). In summary, although TE105 and a cap are interchangeable in vitro, these in vivo results suggest that it may be an oversimplification to assume that one sequence element perfectly mimics a cap and that another mimics a poly(A) tail.

The TE must recruit ribosomes to the 5'-proximal AUG via some form of 3'-5' communication. This 3'-5' communication is ovated when the TE is in the 5' UTR. When the TE was moved to the 5' UTR in combination with a 3' poly(A) tail, it gave significant cap-independent translation in protoplasts (TE105-LUC-vec15; Fig. 1B). This mRNA produced about two-thirds as much luciferase activity as when the TE105 was located in the 3' UTR (Fig. 1B, compare uncapped, polyadenylated forms of 5' UTR-LUC-TE105 and TE105-LUC-vec15). This agrees with previous observations in wheat germ extract (Wang et al., 1999). This result can potentially allow us to separate sequences or structures in the TE needed only for 3'-5' communication from those needed for other aspects of recruitment of the translation apparatus, if it is possible to separate these functions.

The TE105 sequence has a cruciform secondary structure

To identify functional domains in TE105, we first determined its secondary structure. Using the program
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MFOLD (Zuker, 1989), TE105 is predicted to fold in a cruciform structure with three stem-loops (SL-I, SL-II, and SL-III) and base pairing (Stem-IV) between the ends of the RNA (Fig. 2A). A transcript comprised only of TE105 was used for structural probing. We ensured that this transcript is biologically active by showing that it can inhibit cap-independent translation in trans (Fig. 2B, TE105 as competitor). End-labeled TE105 and the nonfunctional TE105BF mutant were probed under nondenaturing conditions with imidazole, ribonuclease T1, and ribonuclease T2, which cleave single-stranded nucleotides, and with ribonuclease V1, which preferentially cuts double-stranded and base-stacked regions. The cleavage pattern (Fig. 2C) supported most of the computer-predicted structural elements, with the exception of the predicted 3 bp predicted at the proximal end of Stem-IV, which cleave strongly, indicating that they are predominantly single stranded. Also, the two predicted base pairs at the proximal end of SL-III gave ambiguous results (Fig. 2C,D). Ribonuclease probing, performed in the actual buffer conditions used for in vitro translation, supported the base pairing, whereas imidazole cleavage (0.4–1.6 M imidazole) did not. Interestingly, the GAUC insertion in nonfunctional mutant TE105BF had only minor effects on the secondary structure. The GAUC bases themselves simply extended the single-stranded region at the Bam HI 4837 site (Fig. 2C,D). More subtle changes are apparent elsewhere in the TE at the different imidazole concentrations. The mostly single-stranded bases on the proximal side of SL-III (between SL-II and SL-III and between SL-III and Stem-IV), and a bulge in Stem-IV cut more strongly in TE105BF RNA than in TE105 RNA (Fig. 2C,D).

Alignment of the TE105 sequence with those of other isolates supports the existence of much of the secondary structure (Fig. 2E). However, the sequence is so highly conserved that only a few variations exist to evaluate phylogenetic conservation of the secondary structure. All aligned sequences can form the same cruciform structure as the wild-type TE105. The co-variation of two A:U pairs in Stem-III of MAV-PS1, the G:C base pair substitutions (in isolates 2t, 3b, 13t, and PAV129; Fig. 2E) for the G:U pair in the proximal ends of SL-III of PAV6, support the formation of the 2 bp at the proximal end of the bulged SL-III structure, that were unclear from structure probing (Fig. 2C,D). An unusual isolate, PAV129, has two 11-base insertions in Stem-III, which can form 8 bp and extend Stem-III for a full helical turn (lower case letters, inset in Fig. 2A). Base pair substitutions in isolates FHv2, MAV-PS1, and PAV129 also support the Stem-IV structure. Predicted loop regions have relatively more sequence variation. Thus, the multiple sequence alignment provides additional support for the conservation of the TE105 secondary structure shown in Figure 2A.

The conserved sequence of SL-I is essential for translation initiation

As mentioned above, we assume that the TE105 sequence has at least two functions. It must participate in recruitment of the translation initiation factors and/or the 40S ribosomal subunit to facilitate initiation of translation. It must also facilitate communication with the viral 5’ UTR, to ensure initiation at the 5’ AUG of the mRNA. When the TE105 sequence is located in the 5’ UTR, the communication function should be unnecessary. These two different functions may be associated with different portions of TE105. To map such domains, deletion and point mutations were introduced into TE105, and the ability of the TE mutants to mediate cap-independent translation from either the 3’ or the 5’ UTR contexts was assessed. Mutations that knock out only the communication function should reduce translation only when the TE is in the 3’ UTR, and have no effect when it is in the 5’ UTR. In contrast, mutations that prevent ribosome recruitment (directly or by blocking initiation factor binding), would reduce translation in both contexts. Based on this logic, some mutants that did not function in the 5’ UTR were not tested in the 3’ UTR. For the in vivo functional assay, the 60-nt adenine tract was added to the 3’ end of in vitro constructs by cloning.

To determine the roles of the major internal structures in the TE, sequences encompassing each of the three stem-loops in the TE were deleted individually. Every stem-loop deletion destroyed the function of the TE at either the 3’ UTR or the 5’ UTR (Fig. 3C), mutations ∆SL-I, ∆SL-II, and ∆SL-III, indicating that either the sequence or the secondary structure of these regions was critical for translation initiation.

To determine whether the sequences or the secondary structural domains contribute to TE function, the stem-loops were mutated at higher resolution. SL-I is part of a 17-nt tract (bases 4837–4853) that is completely conserved (Wang et al., 1997: Fig. 2E) in all members of genus Luteovirus (with the exception of BYDV-PAV129, which has an A at position 4838), soybean dwarf virus (SDV, an unclassified member of Luteoviridae that is the closest sequence relative to BYDV; Rathjen et al., 1994), as well as more distantly related Tobacco necrosis virus (TNV) RNA (genus Necrovirus), which also has uncapped, nonpolyadenylated RNAs (Lesnaw & Reichmann, 1970). All mutations that we introduced into the 17-nt tract were defective both in vitro and in vivo (Fig. 3A,C). Changing GGAAA4845–4849 to UUCC or to GA GA (a GNRA tetraloop) eliminated cap-independent translation (Fig. 3A,C, mutations LI-m1 and LI-m2), indicating that the loop sequence of SL-I is important for TE function.

An interesting feature of the 17-nt conserved region is that sequence GAUCCU4838–4843 can potentially base pair to the AGGAUC sequence located five bases from
FIGURE 2. See caption on facing page.
the 3' end of 18S rRNA (Fig. 3A; Wang et al., 1997). This is the location of the anti-Shine–Dalgarno sequence (mRNA-binding site) in prokaryotic 18S rRNA. Although three of the GAUCCU<sub>4838-4843</sub> bases are base paired in stem I (Fig. 3A), we can consider the possibility of RNA “breathing” or helicase-melting of SL-I. Moreover, the arrangement of these bases resembles that of the ribosome binding site of the extremely efficiently translated coat protein gene of bacteriophage Qβ, in which the three 3' bases of the sequence are in a small stem structure (Priano et al., 1997). A set of mutations that disrupted potential base pairing to 18S rRNA or within Stem-I or both was constructed. All mutations in this set abolished cap-independent translation with TE105 in the 5' UTR (Fig. 3C, SI-m1, SI-m2, and SI-r), including one that may enhance 18S rRNA binding by disrupting SL-1 (SI-m2). However, a natural variant (PAV129; Fig. 2E), contains a G-to-A transition in the second position of the conserved 17-nt tract that allows only five bases to pair to 18S rRNA. The 3' TE of PAV129 confers cap-independent translation on reporter genes (L. Guo, unpubl. observation), and virus containing the PAV129 TE is infectious (S. Liu, pers. comm.). This suggests that a Shine–Dalgarno-like interaction may not be necessary, but more definitive evidence is required to rule out such a mechanism. Because all of the mutations in SL-1 destroyed TE activity, we conclude that its sequence is essential, but we can draw no conclusion about the role of secondary structure of SL-I.

The secondary structures of SL-II and SL-III are necessary for full cap-independent translation

We next tested the roles of SL-II and SL-III. Only the loop sequence varies among BYDV isolates (Fig. 2E). Mutants with disrupted Stem-II lost all (in vivo) or most (in vitro) of the cap-independent translation activity with the TE in the 5' or 3' UTR contexts (SII-m1 and SII-m2; Fig. 3B,C). The double mutation that restored Stem-II structure regained the full function of the TE at either end of the mRNA both in vitro and in vivo (SII-r; Fig. 3B,C). Thus, the secondary structure but not the primary sequence of SL-II is necessary for the function of the TE.

To test the function of SL-III, we replaced SL-III of PAV6 (wild type) with SL-III of isolate PAV129. SL-III of isolate PAV129 contains two 11-base insertions that are predicted to extend the helix, with three mismatches (Fig. 3B). The resulting hybrid TE had 50% of wild-type activity in vitro in both the 5' UTR and 3' UTR settings. In vivo, this hybrid TE regained full activity of the wild-type TE (Fig. 3C, SL-III swap). Comparing the hybrid TE with the nonfunctional ΔSL-III mutation (50% versus 4.5% in vitro, 99% versus 4% in vivo) suggests that the stem of SL-III also plays a role in translation initiation.

**FIGURE 2. (facing page)** A: Computer-predicted (Mfold) secondary structure of TE105 and nonfunctional TE105BF RNAs. The inset shows the predicted SL-III structure of isolate PAV129, with the two 11-base insertions indicated in lower case. B: Effect of adding wild-type or mutant TE transcripts on translation of uncapped 5' UTR-LUC-TE105 mRNA (0.1 pmol) in wheat germ extract. Subscripts indicate BYDV bases present in the transcript. TE105 indicates the TE<sub>4814-4918</sub> transcript. TE105BF is TE105 with a four-base (GAUC) duplication in the BamHI<sub>4907</sub> site. Relative translation is defined as percent of relative light units obtained in absence of competitor, measured after 1 h. C: Partial hydrolysis of 5' end-labeled TE105 and TE105BF RNAs with structure-sensitive chemicals and nuclease. RNase treatment and imidazole concentrations are indicated above each lane. Nuclease digestion was performed in the non-denaturing, ionic conditions used for in vitro translation (see Materials and Methods), except the T1 lane, which was done in denaturing conditions (55°C, 7 M urea). Vertical bars beside gels indicate regions where TE105 and TE105BF differed slightly in imidazole sensitivity. D: TE105 secondary structure superimposed with the sites cleaved by double-strand specific RNase V1 (arrow), single-stranded G-specific RNase T1 (strong cut: uppercase T; weak cut: lower case t), and the single-strand specific chemical imidazole (strong cut: solid arrowhead; weak cut: open arrowhead) enzymatic and chemical cleavage sites. Curved lines indicate regions that were cut more strongly by imidazole in TE105BF. E: Multiple alignment of TE105 region of different BYDV isolates with the following Genbank accession numbers: PAV6 (X07653), P (D11032), JPN (D85783), FHv1 (AJ007491), FHv2 (AJ007492), 131 (X80050), MAV-PS1 (D11028), and PAV129 (AF218798). Sequences 2t, 3b, cloutier, and RG are from Chalhoub et al. (1994). Stem-loops (SLs) are indicated by converging arrows above the aligned sequences. With bulges as dashed line. Base-paired regions are shaded. Box indicates 17-nt tract (bases 4838–4853 in BYDV-PAV6) conserved in Luteovirus and Necrovirus genera, and in soybean dwarf virus.
tions indicate that these eight bases could be part of Stem-IV, or part of a pseudoknot by base pairing between UUGGC\textsubscript{4913–4917} and GUCAA\textsubscript{4885–4889} in Loop-III (Fig 4B). Both base-pairing possibilities were prevented by mutating bases UUG\textsubscript{4913–4915} to AAC. This mutation did not affect the function of the 3’ TE (Fig 4A, TE\textsubscript{4830–4912}PM). Therefore, neither the potential pseudoknot nor the terminal portion of Stem-IV is necessary for either function of the TE. In fact, deletion of the 3’ terminal six bases of the 3’ TE still gave substantial translation (Fig 4A, TE\textsubscript{4830–4912}), defining the 3’ extremity of the TE between nt 4912–4913.

An interesting question arises: how can deletion of two bases (G\textsubscript{4911} and U\textsubscript{4912}) abolish the TE function in the 3’ UTR while still allowing function of the TE in the 5’ UTR context (compare TE\textsubscript{4830–4910} with TE\textsubscript{4830–4912} in Fig 4A)? This was addressed by comparing the predicted secondary structures of the functional 5’-located TE\textsubscript{4830–4910}, including the linker sequences introduced by cloning, with the nonfunctional 3’-located TE\textsubscript{4830–4910}. We found that the linker sequence of TE\textsubscript{4830–4910} in the 5’ UTR context strengthens Stem-IV and the overall cruciform secondary structure (Fig 4C). The predicted free energy of TE\textsubscript{4830–4910} in the 5’ UTR context is much more negative (more stable) than that of the 3’-located TE\textsubscript{4830–4910} (Fig 4D). MFOLD predicted several alternative structures for the 3’-located TE\textsubscript{4830–4910}, none of which was as stable as the 5’-located TE\textsubscript{4830–4910}. In addition, Stem-IV and SL-I were absent in the computer-predicted structure of 3’-located TE\textsubscript{4830–4910} (Fig 4D). To test the role of stem IV, three guanosine residues (lower case, bold, Fig 4E) were inserted into the 5’ end of the nonfunctional 3’-located TE\textsubscript{4830–4910}. This should strengthen Stem-IV by pairing with the CCC sequence at the 3’ end, and restore the cruciform structure as in the fully functional TE\textsubscript{4814–4918} (3’-located TE\textsubscript{4830–4910}CLAMP, Fig 4E). Indeed, this structure provided as efficient cap-independent translation in the 3’ UTR context as the functional TE\textsubscript{4814–4918} (Fig 4A). In summary, these data demonstrate that Stem-IV is essential for the TE to function in either the 3’ or 5’ UTR context. This provides a reminder that flanking sequences must be carefully considered when studying RNA structure and function out of context.

Loop-III sequence is required only in the 3’ UTR context

In contrast to the above terminal deletion mutants, we obtained one internal substitution mutant that clearly affected cap-independent translation much more in the 3’ UTR than in the 5’ UTR context. Alteration of the loop of SL-III (Loop-III) from CUGUCAA\textsubscript{4883–4889} to AGC GACC in the 89-nt 3’ TE reduced luciferase activity by fourfold (TE\textsubscript{4830–4912}mL3, Fig 4A). In contrast, with the TE in the 5’ UTR, the Loop-III mutant gave about 80% of the activity of constructs with wild-type TE\textsubscript{105} (or

![FIGURE 3. Effect of mutations in TE105 on translation activity. The mutated bases are shown in bold with the effects on base pairing indicated by boxed regions. A: Mutations in SL-I. The sequence of the 3’ end of 18S rRNA and potential base pairing is shown in gray. B: Mutations in SL-II and SL-III. C: Relative luciferase activity (to relative light units produced by constructs with viral 5’ UTR and TE105 in 3’ UTR) produced by translation of mRNAs containing the indicated mutants of TE105 in the UTRs indicated by the maps at top of table. The 3’ UTR in vivo reporters have an additional 60-nt poly(A) tail downstream of TE105.](image-url)
In protoplasts, using an mRNA construct with the full 869 nt viral 3' UTR, the mL3 mutation reduced luciferase expression to 0.8% of wild-type levels (data not shown). In contrast, in an mRNA with the TE 4830–4918 in the 5' UTR, combined with an (A) 60 tail, the mL3 mutation gave 10 times as much luciferase activity (as the 3' UTR-located mL3 mutant TE). This was about half the luciferase expression that was obtained with the wild-type TE 105 in the 5' UTR. Thus, the mL3 mutation in Loop-III has much more deleterious effects in the 3' UTR than in the 5' UTR in vivo. A construct containing the wild-type TE in the 3' UTR and a plasmid vector sequence in place of the viral 5' UTR gave 21.2% (in vitro) and 0.2% (in vivo) as much luciferase activity as with viral 5' UTR. This reduction is similar to that caused by the 3' mL3 mutant with the viral 5' UTR (above). These results are consistent with a 5' UTR communication function for Loop III, with only a minor role in recruiting the translational machinery.

**DISCUSSION**

The 3' UTR of BYDV can substitute for a poly(A) tail

Gene expression from an mRNA with the 105-nt in vitro-defined 3' TE 4814–4918, combined with a 60 nt poly(A) tail, was about 22% of that from an mRNA with the full 869-nt 3' UTR of BYDV. This is more than 50-fold higher than with the same construct lacking the
poly(A) tail. We found previously that mRNA with TE105 and a 30 nt poly(A) tail translated only one-eighth as efficiently as mRNA with the full 869-nt BYDV 3′ UTR (Wang et al., 1997). This shows the importance of poly(A) tail length. The packing density of PABP on a poly(A) tail is about 25 As per PABP in yeast (Sachs et al., 1987), so a 30-nt A tract can be bound by only one PABP, whereas the 60-nt tail can be bound by at least two. In a poly(A)-dependent yeast in vitro translation system, Preiss et al. (1998) showed that a minimum of two PABPs was required for the cooperative interaction with the cap in translation. The longer poly(A) tail also serves to enhance stability of mRNA in vivo (Beelman & Parker, 1995). It is possible that a typical 100–200-nt poly(A) tail could completely restore translation to the level conferred by the 869-nt 3′ UTR. However, addition of a cap did restore full translation to the TE105/A60 construct, suggesting that some BYDV sequence outside of TE105 is needed for cap-independent translation only in vivo. Alternatively, the cap may increase luciferase expression by conferring stability on the mRNA that the TE cannot provide (Beelman & Parker, 1995). It is likely that the sequences in the 3′ UTR facilitate cap-independent and poly(A)-independent translation by a mechanism that cannot simply be broken down into discrete units that precisely mimic a cap or a poly(A) tail.

The ability of a heteropolymeric tract of RNA in the 3′ UTR to replace a poly(A) tail has been shown for other viruses (Gallie & Kobayashi, 1994), including TMV in which a series of pseudoknots was shown to facilitate translation in the absence of poly(A) (Leathers et al., 1993). In mammalian histone mRNAs, which are not polyadenylated, a simple stem-loop can suffice (Gallie et al., 1996). Extensive computer analysis of the BYDV 3′ UTR reveals no pseudoknot-rich domain resembling that in TMV, but, of course, numerous stem-loops of unknown function are predicted in the 869-nt 3′ UTR. Future deletion analysis will allow us to localize the sequence that obviates the requirement for a poly(A) tail.

Structure and potential functions of domains within the 3′ TE105

Essential structures and sequences within the TE105 suggest intriguing functions. All mutations within the absolutely conserved SL-1 sequence (Wang et al., 1997; Fig. 2E) were nonfunctional, supporting the essential role of the region. We proposed (Wang et al., 1997) that a sequence that overlaps with SL-1, GAUCCU_{4838–4843}, may base pair to 18S rRNA to promote translation initiation by a prokaryote-like mechanism (Fig. 3A). Consistent with this model, all introduced mutations that destroyed this potential base pairing, including those that maintained the secondary structure of the TE, abolished cap-independent translation. However, the natural variant PAV129 has a G-to-A mutation at nt 4838 that would weaken the base pairing to 18S rRNA (Fig. 2E). The complete lack of activity in the TE105BF mutant with the GAUC insertion in this region (Wang et al., 1997) is consistent with this model. However, the GAUC insertion did cause additional, if minor, alterations to the secondary structure in the TE (Fig. 2C,D) that may be crucial for its function. Moreover, differences in migration in nondenaturing gel electrophoresis suggests differences in tertiary structure between TE105 and TE105BF (E. Allen, unpubl. observation).

Direct base pairing between mRNA and 18S rRNA has been proposed for poliovirus (Pilipenko et al., 1992) and other IRES function (reviewed by Scheper et al., 1994). Recent evidence indicates that translation of mRNAs can be affected positively (Chappell et al., 2000) or negatively (Hu et al., 1999; Verrier & Jean-Jean, 2000) by base pairing to different sites in 18S rRNA. Chappell et al. (2000) showed that repeats of a nine-base tract complementary to 18S rRNA bases 1132–1124 can function as an IRES when incorporated into a dicistronic mRNA. However, although TE105 confers cap-independent translation, it does so only at the 5′-proximal AUG, and it does not confer internal initiation like an IRES does (Allen et al., 1999). We cannot conclude that the underlying mechanism for recruiting translation machinery is based on this base pairing to 18S rRNA because we have mutations only on the mRNA side of the proposed mRNA–18S rRNA interaction. This awaits direct demonstration of interactions between the two RNAs.

Instead of (or in addition to) direct base pairing, we favor a model in which recruitment of ribosomes to the TE is mediated by protein factors. SL-I has features of a known stem-loop that interacts specifically with two proteins. Its loop sequence, GGAAA_{4845–4849}, fits the consensus of a GNRA pentaloop, a structure that forms a GNRA tetraloop fold with the fourth base (N) protruding from the loop (Legault et al., 1998). This pentaloop structure in Escherichia coli boxB mRNA is bound specifically by the bacteriophage λ N protein and host NusA protein in the transcription antitermination process. Replacement of the SL-1 loop in the TE with a tetraloop or a completely different five-base sequence inactivated the TE. Thus, SL-1 may be involved in recruiting one or more proteins to the TE in a fashion similar to that of E. coli boxB RNA.

The base-paired regions of SL-II and SL-III tolerate sequence changes as long as the secondary structure is maintained. SL-II also tolerates changes in the loop and its structure is not conserved outside of the Luteovirus genus, suggesting that SL-II may play no direct role in translation, and that the stem-disruption mutations acted indirectly by causing major rearrangements of the structure elsewhere in the TE. Deletion of SL-II, which could also affect the overall tertiary structure of TE105, reduced cap-independent translation to
structure probing data, this suggests that SL-III exists and is essential.

**Role of 3′-5′ interactions in cap-independent translation**

The only mutation (mL3) that knocked out cap-independent translation in the 3′ UTR but not the 5′ UTR (that cannot be explained by compensating, vector-derived secondary structure as in TE<sub>4830-4910</sub>) was the mutation of the loop in SL-III (Loop III). The simplest explanation is that Loop III is involved primarily in 3′-5′ communication, with little or no role in actual recruitment of the translation apparatus. For this or any other mutation, it might be considered that an RNA stability element, rather than a translation element, has been disrupted. In the case of mL3, it would be a 3′ UTR-specific stability element. However, we showed previously that deletion of the entire 3′ TE had no detectable effect on mRNA stability in vitro (Wang & Miller, 1995) or in vivo (Wang et al., 1997). Therefore, we assume that the mutations introduced here affected functions more directly related to translation. Thus, the loop of SL-III appears to be involved primarily in 3′-5′ communication and not directly in ribosome recruitment.

How the TE interacts with the 5′ UTR or at least the 5′-proximal AUG where initiation takes place, is still unknown. This awaits analysis of the viral 5′ UTR and factors that interact with the 3′ TE. The 5′ UTRs of BYDV genomic RNA and subgenomic RNA1 are both compatible with the 3′ TE (Wang et al., 1999), but a vector-derived 5′ UTR (Results) or the highly active Ω 5′ UTR sequence from TMV (Wang et al., 1997) gave only low levels of cap-independent translation in the presence of the 3′ TE. Thus, there seem to be specific sequences in the BYDV 5′ UTRs that participate in communication with the 3′ TE, although there is little sequence similarity between 5′ UTRs of BYDV genomic RNA and subgenomic RNA1. The possibility of direct base pairing with the 5′ UTR was tested for the 3′ cap-independent translation element of Satellite tobacco necrosis virus (STNV) RNA, which is functionally similar, but bears no significant similarity in sequence (Wang et al., 1997) or predicted secondary structure (Timmer et al., 1993; Danthine et al., 1993) to TE105. Covariation mutagenesis did not support base pairing between UTRs of STNV RNA (Meulewaeter et al., 1998). Another possibility is that the interaction is mediated by proteins that bind each UTR. HCV RNA may provide an example of this. Like BYDV RNA, HCV RNA lacks a cap and a poly(A) tail. HCV differs by having a complex IRES that spans the 5′ UTR and 5′ end of the coding region (Reynolds et al., 1995), but like BYDV, it has an additional sequence (98-nt “X region”) in the 3′ UTR that enhances cap-independent translation (Ito et al., 1998). Pyrimidine tract-binding protein (PTB) binds both the IRES (Ali & Siddiqui, 1995) and the X region (Ito & Lai, 1997), suggesting that PTB may be involved in 3′-5′ UTR communication. However, the X region stimulates translation by only two- to threefold, even in the absence of the internal PTB-binding site. Thus, it is not mimicking a cap or a poly(A) tail.

The biochemical mechanism(s) by which circularization of normal mRNAs, or the above unusual RNAs, facilitates ribosome recruitment is not yet clearly understood. The mechanism mediated by the TE is different than that of capped, polyadenylated mRNAs because this 3′-5′ interaction occurs in wheat germ extract, yet it is insufficient in vivo. Within the 869-nt 3′ UTR, but outside of TE105, exist additional sequences necessary for full cap-independent translation only in vivo, and other sequences that can be substituted by a poly(A) tail, that are also necessary only in vivo. Thus, the poly(A)-like interaction required of normal mRNAs may also be necessary for the TE-mediated cap-independent translation in vivo, but it is also likely that it is an oversimplification to assume sequence domains precisely mimic a 5′ cap or a poly(A) tail. It is clear that BYDV and related uncapped, nonpolyadenylated viruses have evolved a new mechanism for efficient translation initiation while avoiding mRNA degradation. The secondary structural requirements of the BYDV 3′ TE, determined here, will provide a basis for future mechanistic studies of this new class of cap-independent translation elements.

**MATERIALS AND METHODS**

**Plasmid constructs**

All constructs were verified by sequencing on an ABI 377 automated sequencer. Secondary structures of all TE mutants were checked using MFOLD (Zuker, 1989) to ensure that the predicted structures, other than those we intended to alter, were maintained. RNA constructs used in this study were made from the following four groups of plasmids: Set I: in vitro function reporters of TE105 and mutants in the 3′ UTR; Set II: in vitro function reporters of the TE105 and mutants in the 5′ UTR; Set III: plasmids with a poly(A<sub>60</sub>) tail including all in vivo 5′ UTR function reporters and most of in vivo 3′ UTR function reporters; and Set IV: plasmids p5′ UTR-LUC-TE869 as well as pVec50-LUC-Vec58-poly(A<sub>60</sub>).

Set I: The parental plasmid pLUC869 (Wang et al., 1999) contains the T7 promoter, viral 5′ UTR, luciferase reporter gene (LUC) and the TE869 (BYDV bases 4809–5677) in the 3′ UTR. Sets of mutagenesis primer pairs (with Acc65I linker on the 5′ primers and SmaI linker on the 3′ primers) were used to amplify TE105 and its mutants from pLUC869. The PCR products were then cut with Acc65I/SmaI and ligated into pLUC869 digested with Acc65I/SmaI to replace the wild-type TE869 sequence.
Set II: PCR-amplified and BssHII-digested TE105 sequence (bases 4814–4918 with BssHII linker) was inserted into the BssHII-linearized pLUC869. (BssHII is a unique site just upstream of the LUC start codon.) This generated an intermediate plasmid, p5’UTR-TE105-LUC-TE869. The entire TE105–LUC fragment was amplified by PCR and cut with Acc65I; the other end was left blunt. This fragment was ligated into the multiple cloning site of pGEM3Zf(+)(Promega, Madison, Wisconsin) that had been cut with EcoICRI (blunt end) and Acc65I. The resulting plasmid is pTE105-LUC. All mutations of the TE105 in the 5’ UTR were made by replacing it, first by digestion with BssHII and Klenow, then by insertion of the PCR products with mutant TE105 sequences from Set I.

Set III: p3’8, which contains a 60-nt poly(A) tract, was a generous gift from Andy White, York University. A VspI site was introduced adjacent to the (A)60 tract by digestion with EcoRI, treatment with Klenow enzyme, and religation. The poly(A) sequence was amplified by PCR and digested with Smal/Sall (introduced in the PCR primers), and then ligated into Smal/Sall-digested pLUC869 to generate p5’UTR-LUC-TE869-(A)60. All other (A)60 plasmids were generated by the same strategy, but used Smal/Sall-digested (A)60 fragment from p5’UTR-LUC-TE869-(A)60 instead of the PCR product.

Set IV: pVec50-LUC-Vce58-(A)60 was generated by ligating the (A)60 fragment into Sstl/Sall-digested pGEM-LUC (Promega).

RNA preparation

The RNAs were synthesized by in vitro transcription with T7 or SP6 polymerase using Megascript (for uncapped RNAs) or mMessage mMachine (for capped RNAs) kits (Ambion, Austin, Texas). Set I plasmids and p5’UTR-LUC-TE869 were linearized with Smal, giving transcripts containing the viral 5’ UTR, LUC, and the TE869 (or mutant derivatives). Set II plasmids were linearized with HindIII, to generate transcripts containing the TE105 (or mutants), LUC, and a 40-base vector sequence as the 3’ UTR. Set III plasmids and pVec50-LUC-Vce58-(A)60 were ligated into VspI with the exception of the Sstl/Sall-digested plasmids cut with XbaI, which is about 30 nt downstream of the LUC AUG. RNA integrity was verified by 1% agarose gel electrophoresis.

In vitro translation

Nonsaturating amounts of RNAs were translated in wheat germ extract (Promega) in a total volume of 25 μL (Wang & Miller, 1995). In RNA competition experiments, the RNAs were mixed with the competitor RNA prior to addition to the translation reaction. After 1 h incubation at 25°C, 3 μL of the translation mix were added to 50 μL Luciferase Assay Reagent (Promega), and measured immediately, as per manufacturer’s instructions, on a Turner Designs TD-20/20 luminometer.

In vivo translation

Three picomoles of RNA transcript were electroporated into 10⁶ oat protoplasts as in Wang et al. (1997). After 5 h, protoplasts were collected and lysed in 100 μL Passive Lysis buffer (Promega) by shaking 15 min at room temperature. Fifty microliters of Luciferase Assay Reagent (Promega) were mixed with 10 μL protoplast lysate supernatant and measured as described. Protein concentration of each sample was measured by the Bradford method to normalize luciferase activity for each sample.

RNA secondary structure probing

Twenty picomoles of RNA were dephosphorylated and 5’ end-labeled with 32P, using polyadenylate kinase and γ-32P-ATP (Dupont-NEN). The labeled RNA was purified on a 6% polyacrylamide, 7 M urea denaturing gel, and the full-length RNA band was eluted (Miller & Silver, 1991). The amount of recovered RNA was determined by liquid scintillation counting. T1 and V1 nucleases probing were performed as described in Miller & Silver (1991) except the reaction buffer was the same but as in wheat germ extract translation reactions: 24 mM HEPES/KOH, pH 7.6, 2 mM MgCl₂, 133 mM KAc, and 0.8 μg/μL RNasin. A T1 sequencing ladder was generated under denaturing conditions as described in Miller & Silver (1991). Structural probing with imidazole was performed in 40 mM NaCl, 1 mM EDTA, 10 mM MgCl₂ with 0.4, 0.8, or 1.6 M imidazole for 15 h as described in Vlassov et al. (1995). Probed products were separated on an 8% polyacrylamide, 7 M urea sequencing gel. The gels were dried and exposed to PhosphorImager screens for 24 h and visualized by a STORM 840 PhosphorImager (Molecular Dynamics).

Secondary structure prediction and multiple alignment

The MFOLD program (Zuker, 1989) in the GCG software package (GCG, Madison, Wisconsin) was used to predict the secondary structure of the TE and all mutants. The default parameters were used, with the exception of the temperature, which was set to 25°C as our in vitro and in vivo translation reactions. Multiple alignments were performed by using CLUSTAL W (Thompson et al., 1994) from NCSA Biology WorkBench (http://biology.ncsa.uiuc.edu/).

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