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Characterization of the Sequence Element Directing Translation Reinitiation in RNA of the Calicivirus Rabbit Hemorrhagic Disease Virus[▽]

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The calicivirus minor capsid protein VP2 is expressed via reinitiation of protein synthesis after termination of translation of the preceding VP1 gene. A sequence element of about 80 nucleotides denoted "termination upstream ribosomal binding site" (TURBS) (25) is crucial for reinitiation. Deletion mapping in the TURBS of a rabbit calicivirus identified two short sequence motifs that were crucial for VP2 expression. Motif 1 is conserved among caliciviruses and is complementary to a sequence in the 18S rRNA. Single-residue exchanges in this motif severely impaired reinitiation when they affected the putative rRNA binding, whereas an exchange preserving complementarity had only a minor effect. Single exchanges in motif 2 were rather well tolerated, but the introduction of double exchanges almost blocked VP2 expression. In contrast, the deletion analyses showed that the RNA between the two motifs is of minor importance. The distance between motif 2 and the start site was found to be important, since deletions of increasing length in this sequence or upstream positioning of the start codon reduced VP2 expression stepwise to low levels, whereas multiple-nucleotide exchanges in this region were tolerated. The low flexibility of the arrangement of TURBS motif 2 and the start codon stand in marked contrast to the requirements with regard to the location of the stop codon of the preceding VP1 gene, which could be moved far downstream with continuous reduction, but without loss, of VP2 translation. The sequence mapping resulted in a refined model of the reinitiation mechanism leading to VP2 expression.

Caliciviruses represent a family of nonenveloped viruses that cause gastrointestinal diseases in humans and a variety of sometimes fatal syndromes in animals (10). The rabbit calicivirus rabbit hemorrhagic disease virus (RHDV) belongs to the genus *Lagovirus* and is the causative agent for rabbit hemorrhagic disease, a fatal illness of domestic and wild rabbits. RHD was first described in China in 1984 and spread from there to many countries worldwide.

The calicivirus genome is a nonsegmented single-stranded RNA of positive polarity. It has a length of about 7.5 kb and contains two or three functional open reading frames (ORFs) for members of the genera *Lagovirus* and *Sapovirus* or *Vesivirus* and *Norovirus*, respectively (10). The genomic RNA is polyadenylated at the 3′ end and carries a viral protein, VPg, that is covalently linked to the RNA 5′ end via a tyrosine residue. In the infected cells, a subgenomic mRNA (sgmRNA) that is 3′ coterminal with the viral genome is also found. Like the genome, the sgRNA carries VPg and a 3′ poly(A) tail and is packaged into virus particles (13, 26). The sgRNA codes for a major capsid protein, VP1, of ca. 60 kDa and a minor capsid protein, VP2, of ca. 8 to more than 20 kDa (in RHDV, formerly named VP60 and VP10, respectively). VP2 is expressed from a small 3′-terminal ORF that overlaps with the preceding VP1-encoding ORF (reviewed in reference 10).

The absence of a 5′-terminal cap structure at the 5′ ends of calicivirus RNAs indicates that translation initiation has to occur via a cap-independent mechanism. Recently, VPg was shown to interact with the translation initiation factors eIF4E and/or eIF3 in different caliciviruses and seems to serve as a cap substitute in translation of the 5′-terminal ORFs in the viral RNAs (3, 5, 8). The 3′-terminal ORF, which gives rise to the minor capsid protein VP2 (34, 35), was shown to be translated from the bicistronic sgmRNA (12, 24, 25). Translation initiation of the 3′-terminal ORF is achieved via a translation termination/reinitiation process (24, 25). Translation of VP2 via this process was shown to be dependent on an RNA element of about 80 nucleotides located upstream of the start/stop site. This element was named "termination upstream ribosomal binding site" (TURBS) in a recent publication (25). Deletion mapping in this region of the feline calicivirus (FCV) sequence allowed the identification of two short sequence motifs in the region (24). The upstream motif 1 contains a pentanucleotide sequence conserved among calicivirus RNAs. Moreover, motif 1 was found to be complementary to 18S rRNA, leading to the hypothesis that hybridization between viral RNA and 18S rRNA is important to prevent release of posttermination ribosomes and thus to increase the chances for reinitiation. In contrast, the sequence of motif 2 is not conserved, so that it is difficult to determine by sequence comparison whether and where an equivalent element is present in different calicivirus RNAs.

In the present report, the mapping of the TURBS of RHDV via deletion and mutation analyses is described. Both motifs 1 and 2 were identified, and their importance for reinitation was verified. Moreover, the importance of the arrangement of the two motifs and the signals for VP1 translation termination and VP2 translational start was analyzed, leading to a refined model of the reinitiation mechanism.

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MATERIALS AND METHODS

Cells and viruses. BHK-21 cells (kindly provided by T. Rümenapf) and CRFK cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acids.

Vaccinia virus MVA-T7 (36) was kindly provided by B. Moss (National Institutes of Health, Bethesda, MD) and the FCV vaccine strain 2024 by K. Danner (Hoechst Roussel Vet GmbH).

Construction of recombinant plasmids. Restriction and subcloning were done according to standard procedures (30). Restriction and modifying enzymes were purchased from New England Biolabs (Schwalbach, Germany) and Fermentas GmbH (Sankt Leon-Rot, Germany).

All constructs were established on the basis of plasmid pRmRNA (25). Point mutations and deletions were introduced by standard PCR-based site-directed mutagenesis methods using thermostable *Pfu* polymerase (Promega, Heidelberg, Germany) and synthetic primers purchased from Invitrogen (Karlsruhe, Germany) or Metabion (Munich, Germany). The cloned PCR products were all verified by nucleotide sequencing with the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Weiterstadt, Germany). Sequence analysis and alignments were done with Genetics Computer Group software (6). Details of the cloning procedure and the sequences of the primers are available on request.

Expression, detection, and quantification of proteins. Transient expression of plasmids in BHK-21 cells using vaccinia virus MVA-T7, metabolic labeling with [^{35}S]cysteine or [^{35}S]methionine (ICN, Eschwege, Germany), preparation of cell extracts, and recovery of immunoprecipitates with double precipitation were done as described previously (25). Briefly, VP2 expression efficiency was quantified after sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation of VP1 and VP2 precipitated with the antisera "antiRHDV-M" and "anti-RHDV-N," respectively (35). Double precipitation was used to ensure quantitative recovery of the proteins as tested before (25). The precipitates were combined, and aliquots thereof were separated by 10% PAGE using a gel system published previously (31). The gels were analyzed with a Fujifilm BAS-1500 phosphorimager, and the intensities of the signals were determined with TINA 2.0 software (Raytest, Straubenhardt, Germany). The molar ratio of VP1 and VP2 was calculated based on the number of labeled residues within the proteins and the measured radioactivity. For comparison of expression efficiencies of different constructs, the VP2 expression level of the wild-type (wt) construct pRmRNA was defined as 100%. The amounts of VP2 expression of the other constructs were normalized to the values determined for VP1 as an internal standard. The corrected value for VP2 was then used for calculation of the expression efficiency, given as a percentage of the wt value. In order to exclude the effects of different mRNA stabilities on the outcome of the experiments, four selected constructs showing considerably reduced VP2 expression and the wt plasmid were analyzed in duplicate for intracellular steady-state mRNA levels via Northern blotting after transfection of MVA-T7-infected BHK-21 cells. The detected differences with regard to the data for the wt construct were within the expected experimental variation and much smaller than the effects observed on the protein level (not shown). The data presented here represent the averages of at least three independent experiments.

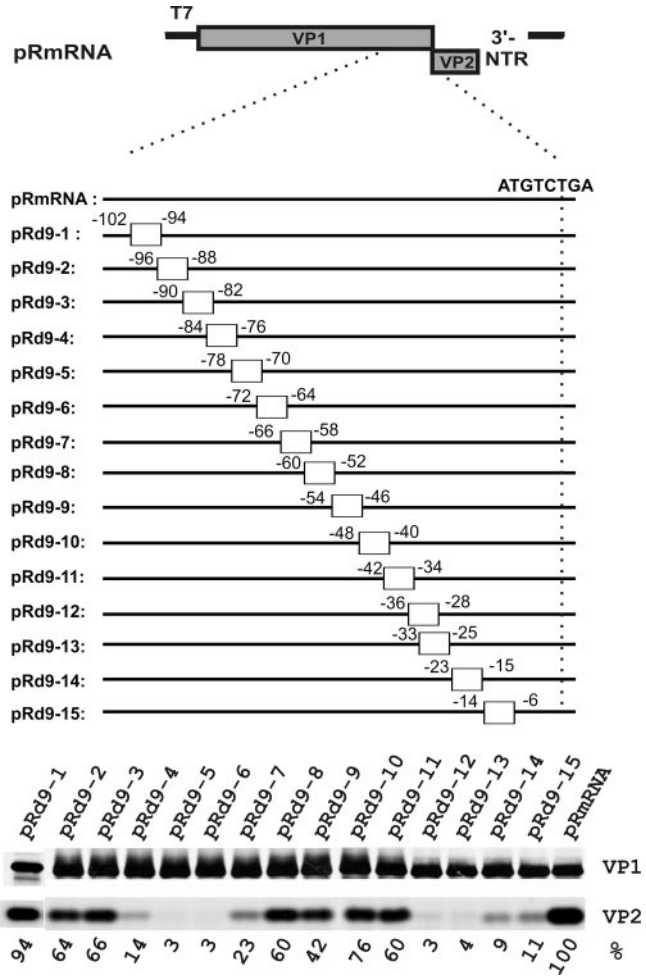

FIG. 1. Influence of 9-mer deletions in the TURBS region on VP2 expression. On top, a schematic representation of the basic structure of the pRmRNA construct is shown (25) (not drawn to scale). Below, the location of the start stop/site (ATGTCTGA) and the 9-nucleotide deletions (white boxes) in the different mutant constructs are indicated (the numbers refer to the "T" of the ORF1 TGA, indicated by the dotted vertical line). At the bottom, an autoradiograph shows the expression products labeled with ^{35}S amino acids and precipitated with antisera specific for VP1 (upper gel) and VP2 (lower gel). Below the gel, the VP2 expression levels relative to the pRmRNA wt control are given (mean values of at least three independent experiments; the data are normalized relative to the expression levels of VP1). T7, phage T7 promoter; 3′ NTR, 3′ nontranslated region.

RESULTS

Identification of essential motifs in the RHDV TURBS. Previous analyses had shown that the 84 3′-terminal residues of ORF1 represent a TURBS that is crucial for translation of ORF2 via a termination/reinitiation mechanism (25). A similar observation was made for the corresponding region of the FCV genome (24). Deletion analysis showed that the FCV TURBS contained two short sequence motifs that were crucial for translation reinitiation, whereas the spacer sequence separating the two motifs was without obvious function. A pentanucleotide core sequence of motif 1 is conserved among caliciviruses and was therefore easily detected in the RHDV RNA (24). In contrast, a sequence homologous to motif 2 could not be identified. To investigate whether the RHDV TURBS is also composed of two motifs separated by a spacer sequence, a series of deletion mutants was established and analyzed with regard to VP2 expression efficiency (Fig. 1). These constructs were based on the plasmid pRmRNA (25). As described above, analysis of VP2 expression was done via transient expression, quantitative immunoprecipitation, and phosphorimager quantification of the radiolabeled proteins after PAGE. Expression of VP1 from the preceding ORF1 in the same plasmid served as an internal control for normalization of transfection efficiency and the general expression level. A series of 9-mer deletion mutants was established and analyzed for VP2 expression. Similar to the results published before for FCV, the deletion mapping resulted in identification of two essential regions in which the deletions blocked VP2 expression almost completely. The region close to the 5′ end of the TURBS (about residues −78 to −64 with respect to the ORF1 termination codon) is specified

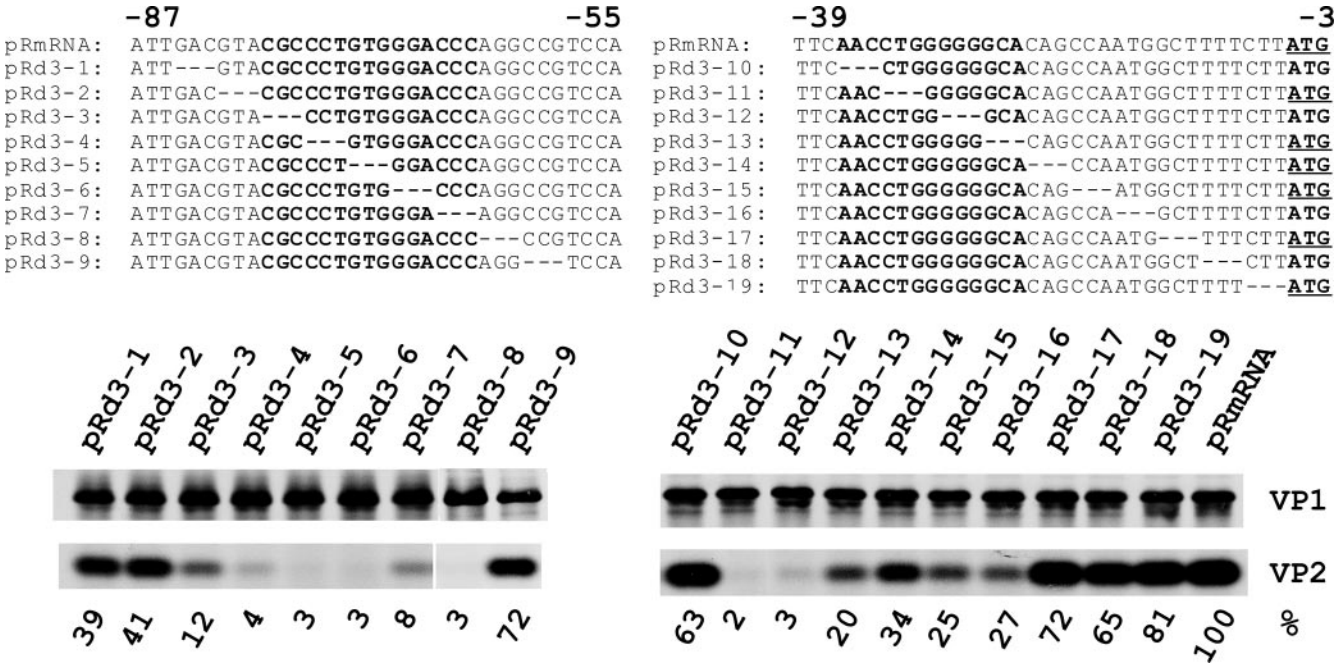

FIG. 2. Mapping of the TURBS with 3-nucleotide deletions. The sequence on top represents regions of the 3′-terminal end of ORF1, with the two essential regions determined by the 9-nucleotide deletions written in boldface. The numbers indicate the locations of the sequences with respect to the ORF1 TGA. Below, the positions of 3-nucleotide deletions in the first (left) and second (right) motifs are given. The gels show the proteins precipitated after transient expression, with the calculated VP2 expression efficiencies indicated below the autoradiographs (relative to wt pRmRNA, normalized to the expression level of VP1).

by the deletion mutants pRd9-5 and pRd9-6, which both show VP2 expression at only 3% of the wt level. This region is equivalent to motif 1 of the FCV TURBS and contains the conserved sequence. The second essential region starts with mutant pRd9-12 (with deletion starting at residue −36) and extends toward the 3′ end of the TURBS. The strongest effect with only 3 to 4% of wt activity was seen when deletions affected positions −36 to −25 upstream of the ORF1 stop codon, whereas downstream of it, the VP2 expression efficiency increased again to about 10% of the wt level.

To narrow down the locations of the two essential sequence motifs, different 3-nucleotide deletions were introduced into these regions (Fig. 2). All the 3-mer deletion mutants showed reduced levels of VP2 expression. The greatest effect with only 3 to 8% of wt activity was found for deletions in a stretch of 15 nucleotides that included the conserved pentanucleotide of motif 1. The second region in which the deletions reduced VP2 expression significantly below 10% of the wt level was composed of only six residues (−33 to −28). For deletions downstream of that region, the ORF2 translation activity increased again to 20% or more until it reached nearly wt level for a deletion directly upstream of the ORF2 start codon. Interestingly, the effect of a 3-mer deletion at the 3′ end of motif 1 had a more severe effect than the corresponding 9-mer deletion that included the deleted 3-mer (compare the expression levels of constructs pRd3-8 [Fig. 2] and pRd9-7 [Fig. 1]). This result could be due to the fact that another sequence is fused to the motif 1 core region in the 9-mer deletion mutant that, on a functional level, might be more similar to the genuine sequence than the corresponding sequence in the 3-mer deletion mutant. In the 9-mer deletion mutant, AAA is found instead of the genuine AGG, so that at least the 5′-terminal A and the presence of purine residues are conserved. The 3-mer deletion mutant pRd3-8 contains a CCG here. According to the results obtained with the 3-mer deletions, the motif 1 core sequence is made up of the 15 nucleotides located at positions −78 to −61.

Taken together, the results of the mapping analyses allowed the identification of two important motifs in the RHDV TURBS in which single-codon deletions led to nearly complete abrogation of VP2 translation. The two motifs are connected by a less important spacer sequence. A second spacer region that can tolerate single-codon deletions separates motif 2 and the start/stop site.

Importance of sequence integrity of the TURBS motifs. To further analyze the two crucial motifs identified in the RHDV TURBS by deletion analysis, point mutations were introduced into the respective sequences. Motif 1 contains the conserved sequence that was found before to be complementary to a short sequence in the 18S rRNA. Determination of the VP2 expression efficiencies of mutants with point mutations in the respective region showed that the sequence of motif 1 is very critical for ORF2 translation (Fig. 3). Four out of six point mutants abrogated VP2 translation nearly completely. A change of T to C close to the 5′ end of the motif (pRmm1-1) allowed residual activity of 15%, whereas for mutant pRmm1-6, with an A-to-G mutation at position −67, VP2 was detected at 70% of the wt level (Fig. 3A). The latter mutation would preserve the hypothesized rRNA hybridization, although with somewhat reduced binding force, via a G-U pairing instead of the wt A-U pairing. Importantly, the A-to-C

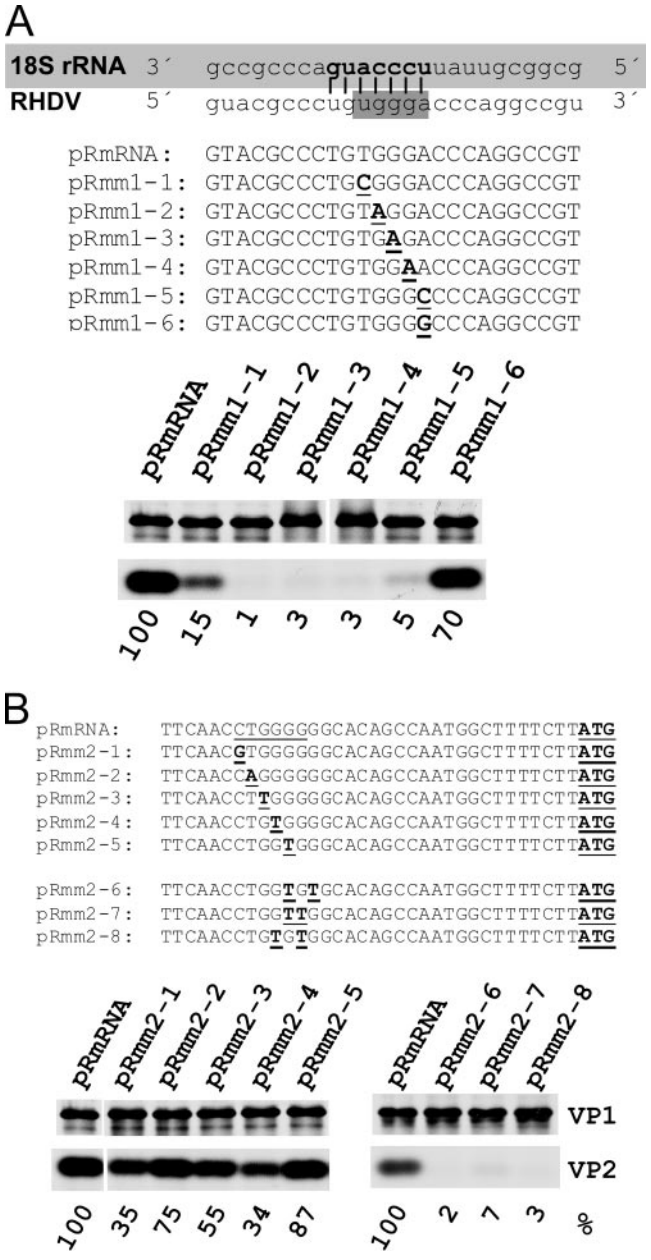

FIG. 3. Effects of mutations in the core regions of the crucial TURBS motifs on VP2 expression. (A) At the top, a part of the rabbit 18S rRNA sequence (nucleotides 1101 to 1125, highlighted in light gray) is given in 3′–5′ orientation. Possible base pairing between the 18S rRNA and the RHDV TURBS motif 1 RNA sequence is indicated by vertical lines. The pentanucleotide conserved among calicivirus sequences is highlighted in gray. Below the RNAs, DNA sequences of the pRmRNA construct and mutants thereof with exchanges in the motif 1 region are given, with the exchanged residues highlighted. Below are gels with the proteins precipitated after transient expression of the indicated constructs. (B) Similar to panel A, with single (left gel) and double (right gel) exchanges in the motif 2 region. In both panels, the calculated VP2 expression efficiencies in percentages are given below the autoradiographs (relative to wt pRmRNA, normalized to the expression levels of VP1).

transversion at the same position, which would prevent rRNA binding, nearly abrogated VP2 expression.

The result of point mutations within motif 2 was less clear. All exchanges reduced VP2 translation but to various extents, and the reductions were less pronounced than with the motif 1 mutations (Fig. 3B). Since triplet deletions in the motif 2 region were able to almost block VP2 expression, the importance of the primary sequence was further analyzed by double mutations. All three tested double mutants showed dramatically reduced VP2 translation, so it can be concluded that the integrity of motif 2 has to be preserved, not only at the level of the number of residues, but also with regard to the sequence, but with somewhat more flexibility than in motif 1.

Relevance of the spacer between motif 2 and the start/stop site. The results of the 3-mer deletion mapping mimicked those obtained with the larger deletions, but with more precise specification of the crucial regions. However, a major difference between the two analyses was observed for the last ca. 20 nucleotides upstream of the start/stop site, where the 9-mer deletions almost abrogated VP2 translation whereas single-codon deletions reduced the expression level much less. To further analyze this point, two more series of mutants with either 12-mer or 6-mer deletions were established (Fig. 4A). The 12-mer deletions reduced VP2 expression efficiency to below 6% when the crucial motifs were affected. Once again, the deletions located between motif 1 and motif 2 had only quite moderate effects. However, 12-mer deletions downstream of motif 2 had even more dramatic effects than the 9-mer deletions in the same region, so that translation of VP2 was basically abrogated for all 12-mer mutants starting downstream of position −37. In contrast, the 6-mer deletions were able to differentiate between the essential motif 2 and a less important spacer region located between motif 2 and the start/stop site with results similar to those obtained with the 3-mer deletions. To check, if the primary sequence of this spacer region or the distance between motif 2 and the start/stop site was important, we established a mutant with the length of the spacer region preserved but with multiple exchanges. Expression of this mutant resulted in detection of VP2 at nearly wt level (88%) (Fig. 4B).

These results showed that the spacer between motif 2 and the start/stop site has to place these two elements at a certain distance. This was verified with a series of constructs in which the ORF2 translational start codon was moved upstream in single-codon steps. It had already been shown in a previous publication that a naturally occurring AUG at codon position −4 with regard to the genuine ORF2 start codon was not able to initiate significant VP2 translation in the absence of the genuine start codon, since a double mutant with both AUGs destroyed worked as well as the single mutant with only the original start site changed (25). New AUG codons were introduced by mutations at codon positions −1, −2, and −3 (Fig. 5). In all cases, two versions of the mutants were established, one with preservation of the original ORF2 start site and one with a mutation changing the AUG to UGU, a codon not used as an initiation site in this sequence context (25). The results of the latter constructs with regard to VP2 expression were normalized to the values obtained for the respective constructs containing both the original and the newly introduced AUG in order to exclude secondary effects due to the sequence change within the TURBS. Moving the start site upstream by one codon reduced VP2 expression by ca. 25%, a two-codon step accounted for a ca. 50% drop in VP2 translation, and a three-codon move resulted in a reduction by 75% that was very

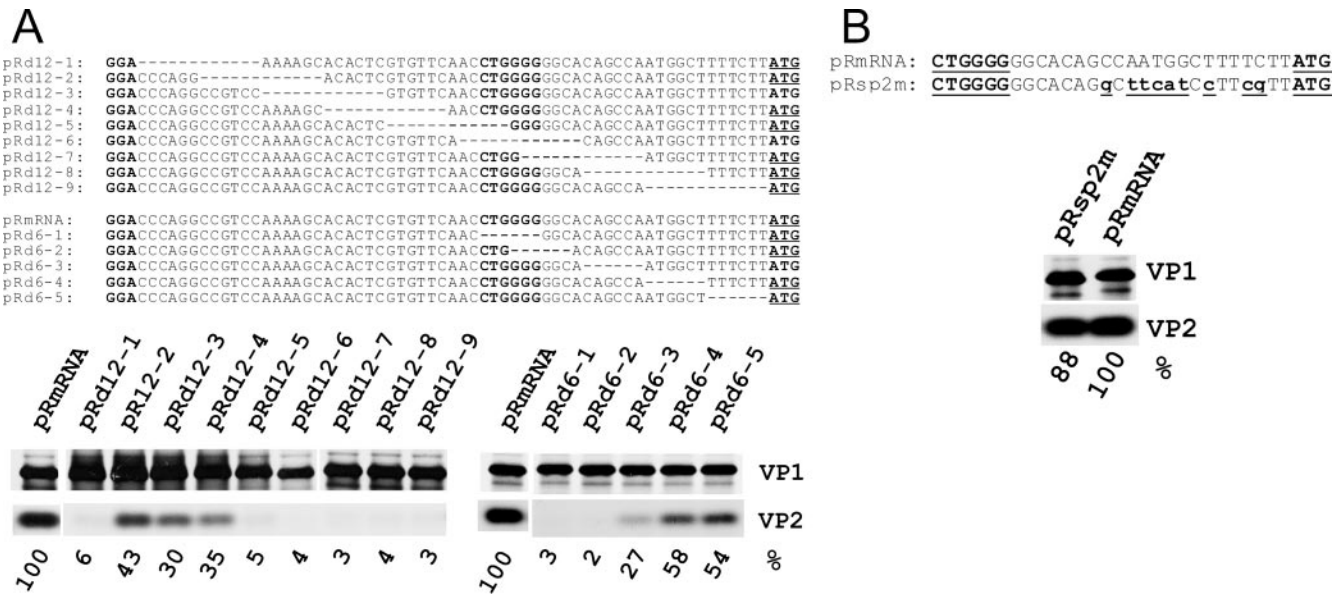

FIG. 4. Importance of the spacer regions connecting motif 1, motif 2, and the start/stop site. (A) The sequence on top represents the region of the 3′-terminal end of ORF1 starting with the three 3′-terminal residues of the rRNA-complementary part of motif 1 (in boldface) and extending to the AUG (boldface and underlined) of ORF2. The core region of motif 2, as specified by the 3-mer deletions, is also shown in boldface. Below, the positions of 12- and 6-nucleotide deletions are given. The gels show the proteins precipitated after transient expression, with the calculated VP2 expression efficiencies indicated below the autoradiographs (relative to wt pRmRNA, normalized to the expression levels of VP1). (B) Influence of multiple-nucleotide exchanges in the spacer region between motif 2 and the ORF2 start site on VP2 expression. The changed residues are highlighted (lowercase and boldface underlined). The core region of motif 2 is underlined.

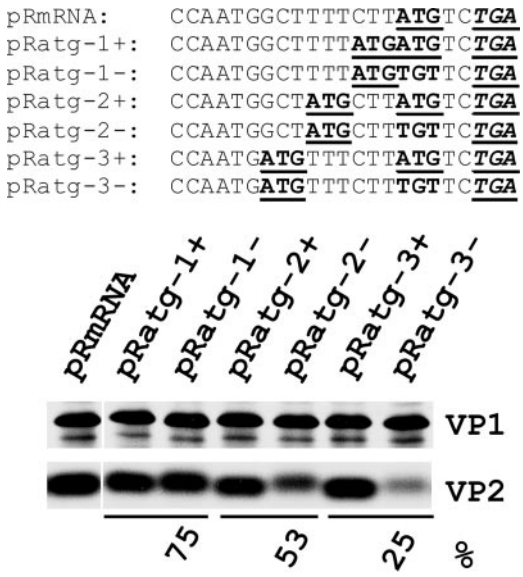

FIG. 5. Influence of upstream initiation codons on VP2 expression in the presence (+) or absence (−) of the genuine ORF2 start codon. The genuine or newly generated ATG codons and the ORF1 TGA are highlighted (boldface and underlined). The mutated genuine ATG is shown in boldface but not underlined. Note the in-frame ATG at position −4 already present in the wt sequence (25). The gels below the sequences show the proteins precipitated after transient expression, with the calculated VP2 expression efficiencies of the mutants without genuine ATG indicated below the autoradiographs (relative to the amount of VP2 expressed from the corresponding construct of the + series containing the same upstream ATG, together with the genuine ORF2 ATG).

similar to the residual activity observed before for the natural −4 variant (with the AUG in the start/stop site mutated) or the double mutant with both AUGs at codon position −4 and at the start/stop site mutated (25). These findings fit quite nicely with the results of the deletion analysis showing that 3-nucleotide deletions within the region close upstream of the start/stop site reduced VP2 translation by 35% or less whereas

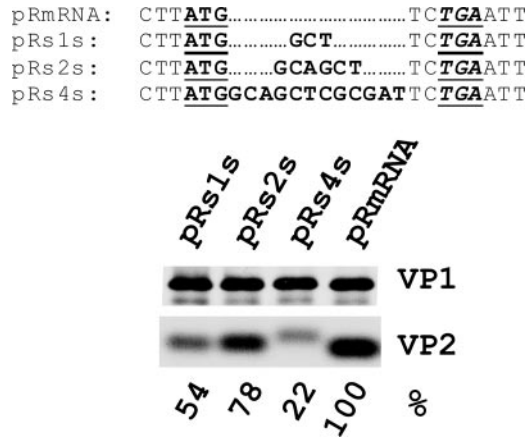

FIG. 6. Influence of insertions between the ORF2 start and ORF1 stop signals on VP2 expression. ATG and TGA are highlighted (boldface and underlined). The inserted sequence is given in boldface. The gels below the sequences show the proteins precipitated after transient expression, with the calculated VP2 expression efficiencies of the mutants (relative to wt pRmRNA, normalized to the expression levels of VP1).

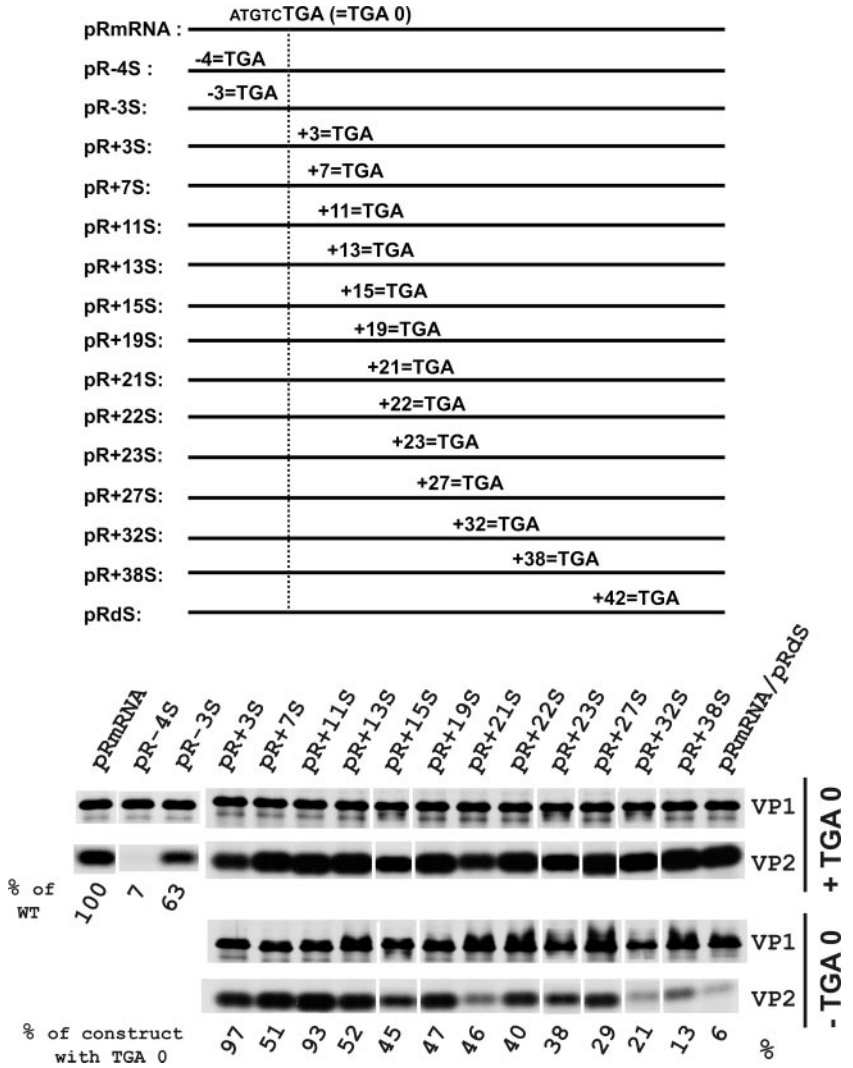

FIG. 7. Influence of translocation of the ORF1 termination signal on VP2 expression. The scheme on top shows the position of the genuine termination signal (vertical dotted line) and the positions of newly introduced stop codons (not drawn to scale; the numbers indicate the codon positions relative to the genuine stop signal). Note that for all the constructs with new stop codons downstream of the genuine termination site, two versions were established, one with the genuine TGA preserved and one with the TGA replaced by CGA. The gels below the sequences show the proteins precipitated after transient expression of the constructs with the original TGA (upper gels) or with CGA instead (lower gels). The calculated VP2 expression efficiencies of the CGA-containing mutants are indicated below the autoradiographs (relative to the expression level of the corresponding construct from the "+TGA 0" series containing both the original TGA and the new downstream termination signal).

6-mer and 9-mer deletions led to about 50% or 90% reductions, respectively.

Effects of separation of the start and stop signals. The results obtained with constructs containing upstream AUG codons could be due to a separation of start and stop signals instead of shortening the distance between TURBS motif 2 and the translational start codon. For FCV, it was shown that downstream transposition of the termination signal by up to six codons had basically no influence on VP2 expression (24). To analyze this issue in detail for RHDV, a series of three constructs with insertions between the start and stop sites was established (Fig. 6). Insertions of one, two, and four codons were tested. The three variants showed reduced VP2 expression efficiencies of 54, 78, and 22% of the wt level for the one-, two-, and four-codon insertion mutants, respectively. Since this result could have been biased by the fact that the pentanucleotide sequence AUGUCUGA of the start/stop region is important for VP2 translation efficiency (reference 25 and unpublished results), a series of mutants was established in which the translational stop codon was moved step by step toward the 3′ end. To this end, the original TGA codon in pRmRNA was replaced by CGA, and new inframe termination signals were introduced at various downstream positions (Fig. 7). The construct with the largest distance between the ORF2 start and ORF1 stop codons was pRmdS, in which ORF1 is terminated by a natural in-frame TGA located 42 codons downstream of the genuine stop signal. To eliminate secondary effects induced by the sequence changes, a second set of constructs was gen-

erated that also contained the new stop codons but with preservation of the original ORF1 termination signal. In addition, two constructs were established with upstream termination signals at codon positions −4 and −3. After quantification of the expression products, the percentages of VP2 expression efficiency of the constructs containing solely a new downstream ORF1 termination signal were calculated in comparison to the corresponding constructs containing both the original and the new stop codons, for which the results were set to 100%. The calculation of the expression efficiencies of the constructs with upstream termination signals was based on the signal of wt pRmRNA, set to 100%. Termination of ORF1 translation upstream of the original start/stop site resulted in a dramatic stepwise reduction of VP2 translation. For pR-3S, the construct with a TGA three codons upstream of the original end of ORF1, considerable amounts of VP2 could still be recovered, whereas a further step to a −4 location of the TGA blocked VP2 expression completely. Since nucleotide exchanges in this region had no considerable effect on VP2 translation (Fig. 4B and unpublished results), this result should be due to the translocation of the stop codon.

For all constructs with downstream location of the termination signal, VP2 expression was detected. With the exception of constructs pR+3S and pR+11S, reduced VP2 expression levels were detected for all constructs, with an overall tendency toward stronger reduction with increasing distance between the TURBS and the stop codon (Fig. 7). The lowest value of about 6% of wt activity was found for the naturally occurring UGA at codon position +42. Taken together, the position of the ORF1 termination signal, and thus the length of the ORF1 and ORF2 overlap, seems to be of rather low importance as long as termination occurs downstream of the ORF2 start site, with a gradient in the efficiency of the system approaching zero with increasing distance.

The decrease of ORF2 translation as a consequence of termination codon translocation could be due to the separation of the termination signal from the TURBS motifs, or it could result from disturbing the coupling between termination and reinitiation by increasing the distance between AUG and UGA. To discriminate between the two possibilities, three different constructs with downstream stop codons were further changed by mutation so that the sequence ATGTC preceded the new stop codon in the DNA construct. By this manipulation, the original start/stop sequence was present at the different downstream positions. Once again, two versions were established for each construct, one with a destroyed original termination codon and a control containing both the original and the new start/stop sites (Fig. 8). The downstream movement of the complete start/stop region in these three constructs had basically the same effect as translocation of the termination signal alone, supporting the conclusion that the decrease in VP2 translation observed for constructs with downstream ORF1 termination cannot be overcome by translocation of the complete start/stop site. The ORF2 translation products obtained in these experiments comigrated in all three cases with the wt VP2 protein, indicating that translation starts at the original start site even when a new AUG is present at the correct distance upstream of the translocated termination signal.

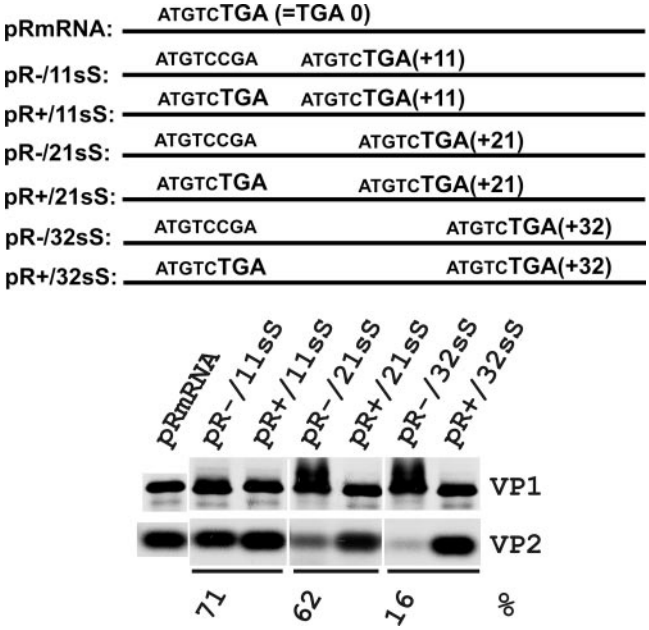

FIG. 8. Influence of complete start/stop site translocations on VP2 expression. The scheme on top shows the positions of the genuine and newly introduced start/stop sites (the numbers indicate the codon positions relative to the genuine stop signal). Note that for all the constructs, two versions were established, one with the genuine TGA preserved (+) and one with the TGA replaced by CGA (−). See the legend to Fig. 7.

DISCUSSION

Viruses rely on the host cellular translation machinery to express their proteins. Thus, viral mRNAs have to be able to interact with the components of the host's protein synthesis system. Moreover, viral RNAs have to compete with a host of cellular mRNAs for the limited translation components to allow efficient viral-gene expression and replication. The decision as to which RNA is translated is made at the step of translation initiation, which is therefore a prominent site of gene regulation (reviewed in references 14 and 18). For most eukaryotic mRNAs, initiation conforms to elements of the ribosomal scanning mechanism (20). Since the small ribosomal subunit starts scanning at the 5′-terminal m7GpppN cap structure of the RNA and then migrates linearly in a 3′ direction, translation usually starts at the AUG codon closest to the 5′ end. In higher eukaryotes, the AUG has to reside in a favorable sequence context (21, 22, 27).

To cope with the different demands on viral RNA translation and also to circumvent the absolute necessity of providing a cap structure, a variety of viruses rely on alternative translation initiation mechanisms, most of which have also been identified later on in the translation of certain cellular mRNAs (reviewed in references 16, 29, and 32). Some of these mechanisms not only allow translation to start close to the 5′ end of the RNA, but can also achieve initiation at far-downstream sites. Among these, internal ribosomal binding via internal ribosome entry sites (reviewed in references 11 and 28), ribosomal shunting, and reinitiation of translation after termina-

tion (reviewed in references 19, 23, and 29) have to be mentioned.

Caliciviruses apparently employ the RNA-bound protein VPg for translation initiation at the 5′ end of the viral RNA (3, 5, 8, 13). Since both the genomic and single sgRNAs were found to carry the viral protein (13, 26), VPg-based translation initiation should be responsible for translation starting close to the 5′ ends of both viral RNA species. However, this mechanism could not readily explain the expression of the minor capsid protein VP2 encoded in a 3′-terminal ORF present in genomic and sgRNA. We showed, first for RHDV (25) and recently also for FCV (24), that VP2 translation is due to reinitiation after termination of translation of the major capsid protein VP1. The biochemistry behind reinitiation by posttermination ribosomes at a new translational start site is not fully understood, but in three cases, the importance of RNA sequences located upstream of the start/stop site was shown (9, 24, 25). For caliciviruses, I proposed the name TURBS (25) for this upstream element, since according to hypothetical considerations, one of the main functions of this element should be to ensure prolonged contact of posttermination ribosomes and RNA to allow the binding of the factors necessary for a new cycle of translation initiation. In the present study, I have dissected the RHDV TURBS into different elements and characterized these elements with regard to primary sequence requirements and position effects.

Deletion analyses allowed the identification of elements of different importance in the RHDV TURBS. Basically, the sequence can be divided into two short essential elements, in which single-codon deletions and most single or double point mutations (motif 1 or 2, respectively) lead to nearly complete loss of VP2 expression, and spacer regions of less importance. In comparison to the results obtained for FCV in similar analyses, it is obvious that in the RHDV system, the regions in which deletions result in considerable reduction of VP2 translation efficiency are larger. The additional sequences might have accessory functions and could be connected with the fact that the RHDV TURBS is about four times more efficient than the FCV TURBS (24, 25). Especially for motif 1, single-codon deletions specify a fragment of about 15 nucleotides as the crucial sequence, whereas in FCV, only 6 residues represent the core region of this motif. Nevertheless, these sequences should be at least generally equivalent, since both contain a 5-nucleotide sequence that is conserved among caliciviruses. For both FCV and RHDV, the conserved sequence, together with flanking residues, shows complementarity to a short element in the 18S rRNA (24). Similar to the results obtained for FCV, mutations in RHDV TURBS motif 1 that disrupt this putative interaction abrogate TURBS function. However, a mutation at position −67 that preserves 18S rRNA complementarity via a G/U pairing instead of an A/U interaction has only minor effects, whereas an A-to-C change at the same position is deleterious. The corresponding sequence in the 18S rRNA represents a region of the RNA that in secondary-structure models would correspond to the apical loop of helix 27 and thus would mostly be part of an unpaired region (15). Moreover, the putative rRNA interaction region is located close to a sequence for which interaction with the so-called translation enhancer element of the mouse homeodomain protein Gtx was shown (7). This interaction was also proposed to be important for translation initiation. Taken together, the mutation analysis and sequence comparison studies support the hypothesis that interaction with 18S rRNA might be important for TURBS function. This binding would allow tethering of the posttermination ribosome to the viral RNA and thus provide the time necessary for binding the initiation factors. However, it has to be stressed that interaction between calicivirus RNA and ribosomal 18S rRNA is still hypothetical. Moreover, this hypothesis cannot explain why sequences flanking the region complementary to the proposed target sequence in 18S rRNA are important for reinitation.

TURBS motif 2 cannot readily be identified via sequence comparison because of a lack of significant conservation. The outcomes of the deletion and point mutation analyses, together with the location of the crucial sequence between motif 1 and the start/stop site, support the conclusion that this sequence in the RHDV RNA is equivalent to FCV TURBS motif 2. Proposing a putative function of motif 2 is more difficult than for motif 1. In contrast to the latter, motif 2 has to be located at a defined distance upstream of the start/stop site. Since the position of the termination signal, and thus also the distance between motif 2 and the stop site, allows a rather high degree of flexibility, motif 2 should most likely be involved in positioning the ribosome at the start site for translation initiation. We have shown before for both RHDV and FCV that the presence of an AUG translational start codon is not essential for VP2 translation (24, 25), so other codons can also serve for initiation of translation that nevertheless employs a methionine initiator tRNA (Met-tRNAi). In standard ribosomal-scanning-based translation initiation processes, AUG codon–Met-tRNAi anticodon interaction plays an important role in start site selection. Supported by the activities of a variety of initiation factors, the recognition of AUG and tRNA anticodons seems to induce a conformational change in the initiation complex that is important for the formation of the so-called closed complex in a step involving the release of inorganic phosphate produced earlier by GTP hydrolysis (reviewed recently in reference 2). Since codon/anticodon hybridization can hardly be of significant relevance when Met-tRNA is used for initiation at, e.g., a CUA codon (24), it is obvious that start codon selection has to be achieved by other mechanisms in calicivirus VP2 translation. These considerations, together with the importance of the distance but not the sequence between motif 2 and the start site, clearly argues in favor of the above-proposed function of motif 2 in start site selection, but the mechanism is still obscure. In bacteria, translation initiation is dependent on the so-called Shine-Dalgarno sequence, a motif positioned upstream of the translational start site that hybridizes to a sequence close to the 3′ end of the 16S rRNA and is important both for ribosome recruitment and start site selection (17, 33). Sequence comparison studies have revealed that rRNA also contains sequences complementary to motif 2, so the possibility that motif 2 functions via hybridization to rRNA cannot be excluded. However, it seems quite unlikely that this possibility is relevant because of the absence of sequence conservation in this motif. For FCV, the hexanucleotide sequence AGGAGU was identified as the most important part of motif 2. In contrast, the RHDV analyses led to discovery of the core sequence CUGGGG. Thus, hybridization of the two calicivirus motif 2 sequences to rRNA would be

possible only to different regions of this cellular RNA. Alternatively, motif 2 could function via interaction with translation initiation factors, but in this case also, it would be difficult to imagine how such an interaction could be achieved by a short nonconserved sequence to initiate a defined step in a mechanism that should be equivalent in the different caliciviruses. It is also somewhat puzzling that, in contrast to the results for motif 1, single-residue exchanges in the motif 2 core sequence generally have rather small effects on VP2 translation, whereas the exchange of two residues in this region almost blocks VP2 expression. Start site selection can be influenced by secondary-structure effects (4). It therefore will be important to investigate the structure of the TURBS in order to get an impression of how motif 2 could interact with, e.g., other parts of the TURBS sequence to ensure translation initiation at the correct site.

Taken together, a current model of TURBS activity would propose that it is responsible for tethering posttermination ribosomes to the viral RNA and for positioning these ribosomes properly at the translational start site. Prevention, or at least delay, of the release of the posttermination ribosome would provide time for binding of the necessary initiation factors. These functions of the TURBS seem to be rather independent of the location of the termination site as long as termination does not occur more than three codons upstream of the start site. The position of the termination signal downstream of the start site seems to be rather flexible, since translocation of the UGA toward the 3′ end leads only to a step-by-step reduction of TURBS efficiency that probably approaches zero in an asymptotic way. An attractive hypothesis would be that motif 1 is responsible for tethering the ribosome via hybridization to the ribosomal 18S RNA, whereas the task of motif 2 would be to position the ribosome in the right way by a still-undefined mechanism to ensure the selection of the correct start site. The activity of motif 2 is position dependent, allowing only minor changes with regard to the distance between the motif and the start site. Upstream positioning of the start site by the introduction of deletions or by the creation of new AUGs via mutagenesis is only tolerated within a very narrow range and always results in considerable loss of reinitiation frequency. Similarly, the downstream positioning of a complete AUGUCUGA start/stop site does not lead to shortened versions of VP2 and is also not able to increase reinitiaton efficiency compared to corresponding constructs with only the stop codon moved to the downstream position. This finding shows again that the distance between motif 2 and the start site and not the distance between start and stop signals is important. Further sequence or structural elements have to be involved in start site selection, since motif 2 is not able to induce translation initiation at a given distance on its own, as can be concluded from the reduction of VP2 expression as a consequence of deletions in the spacer region between motif 2 and the start site. If motif 2 were sufficient to define a start site regardless of the sequence or structural context, one would expect that deletions downstream of the motif would simply shift the initiation site toward the 3′ end. The start codon itself does not represent this additional element that is necessary to define a start site, since the system can tolerate a lot of changes in the genuine AUG and still start translation with a Met-tRNAi (24). This observation represents a strong argument for de novo initiation instead of putative continuous translation processes leading to VP2 via frameshifting and subsequent proteolytic processing.

Support of translation initiation by base pairing between mRNA and 18S rRNA, as discussed here as one possible function of TURBS motif 1, could probably be best explained by the establishment or stabilization of a contact between the (small subunit of the) ribosome and the mRNA. Direct interaction between the mRNA and the small ribosomal subunit would be reminiscent of the bacterial Shine-Dalgarno sequence that recruits ribosomes via hybridization to 16S rRNA and positions the preinitiaton complex at the translational start site during primary initiation at 5′-terminal start sites, as well as reinitiation at downstream sites (17, 33). In contrast to the prokaryotic system, binding and start site positioning of ribosomes would be executed by different sequence elements in my model of the TURBS function. The differences with regard to the prokaryotic system are also obvious when the principles of translation reinitiation are compared. In eubacteria, reinitiation of translation is a common process and occurs basically at any initiation site located close to a termination signal, regardless of whether the site is located upstream or downstream of the stop site. If more than one start site is present, a strong preference for the closest site and a weaker preference for downstream versus upstream sites were detected (1). These features of prokaryotic translation reinitiation are fundamentally different from what is found in caliciviruses.

Viral RNAs interact with the same translation machinery that is active on cellular mRNAs. Thus, reinitiation of translation guided by TURBS-like elements might prove to represent one of the special ways that the cell uses to cope with the different demands made on protein expression.

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