Complex signals in the genomic 3' nontranslated region of bovine viral diarrhea virus coordinate translation and replication of the viral RNA

OLAF ISKEN,^{1,2} CLAUS W. GRASSMANN,² HAIYING YU,² and SVEN-ERIK BEHRENS^{1,2}

¹Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111, USA

²Institute for Virology, Justus-Liebig-Universität Giessen, 35392 Giessen, Germany

ABSTRACT

The genomes of positive-strand RNA viruses strongly resemble cellular mRNAs. However, besides operating as a messenger to generate the virus-encoded proteins, the viral RNA serves also as a template during replication. A central issue of the viral life cycle, the coordination of protein and RNA synthesis, is yet poorly understood. Examining bovine viral diarrhea virus (BVDV), we report here on the role of the variable 3'V portion of the viral 3' nontranslated region (3'NTR). Genetic studies and structure probing revealed that 3'V represents a complex RNA motif that is composed of synergistically acting sequence and structure elements. Correct formation of the 3'V motif was shown to be an important determinant of the viral RNA replication process. Most interestingly, we found that a proper conformation of 3'V is required for accurate termination of translation at the stop-codon of the viral open reading frame and that efficient termination of translation is essential for efficient replication of the viral RNA. Within the viral 3'NTR, the complex 3'V motif constitutes also the binding site of recently characterized cellular host factors, the so-called NFAR proteins. Considering that the NFAR proteins associate also with the 5'NTR of the BVDV genome, we propose a model where the viral 3'NTR has a bipartite functional organization: The conserved 3'C portion (3'C) is part of the nascent replication complex; the variable 3'V portion (3'V) is involved in the coordination of the viral translation and replication. Our data suggest the accuracy of translation termination as a sophisticated device determining viral adaptation to the host.

Keywords: RNA virus; translation termination; pestivirus; hepatitis C virus; NFAR proteins

INTRODUCTION

The genomes of positive-strand RNA viruses exert two roles in the cytoplasm of the infected host cell. Initially, they operate as mRNAs to yield the viral proteins. Concomitant with the translation process, the nascent viral as well as cellular proteins are assumed to assemble with the RNA to form the viral replication complex. At a certain stage, the viral genomes switch roles and RNA replication initiates at the 3' terminus to yield complementary negative-strand intermediates, which, in a second step, act as templates for the production of progeny positive-strand RNA molecules. Defining the molecular determinants that regulate protein and RNA synthesis is of central importance for the understanding of the virus life cycle (Agol et al. 1999).

The pestivirus bovine viral diarrhea virus (BVDV) is a prevalent animal pathogen, and, within the *Flaviviridae* family of positive-strand RNA viruses, closely related to hepatitis C virus (HCV), the major causative agent of chronic liver disease in man (Lindenbach and Rice 2001). Because studies on the human pathogen are hampered by a low rate of viral replication and the lack of infectious cell culture systems, BVDV serves as a valuable surrogate model for HCV.

The BVDV genome has a length of ~ 13 kb. Mimicking a eukaryotic mRNA, it encodes a long open reading frame (ORF), which is flanked by nontranslated regions (NTRs) at the 5' and 3' ends. Translation initiates at an internal ribosomal entry site (IRES) that involves a major part of the 5'NTR and the 5' portion of the ORF. The resulting polyprotein (NH₂-N^{pro}, C, E^{ns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B-COOH) is co- and post-translationally processed by cellular and viral proteases to give rise to the structural (C, E^{ns}, E1, E2, p7) and nonstructural (N^{pro}, NS2-NS5B) viral proteins (Lindenbach and Rice 2001; Fig. 1A).

Reprint requests to: Sven-Erik Behrens, Institute for Cancer Research, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA; e-mail SE_Behrens@fccc.edu; fax: (215) 728-3105.

Article and publication are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.7290904>.

Studies aimed at characterizing the factors and elements involved in viral translation and replication were considerably facilitated by the finding that subgenomic RNA molecules, which consist essentially of the NTRs, the 5' portion of the ORF, and the genetic units encoding NS3 to NS5B, are capable of autonomous replication in transfected host cells. As a major experimental advantage, translation and replication of these replicons occur independently of virus particle formation (Behrens et al. 1998; see also Fig. 1A).

A focal point of recent experimental studies has been the viral 3'NTR, because this region is believed to play a key role in the assembly of the replication complex and for the coordination of translation and RNA replication. As a general feature, all pestiviral 3'NTRs consist of a conserved region at the 3' end (3'C) and a variable region (3'V), which is located downstream from the translational stop-codon (Deng and Brock 1993; Fig. 1B). The 3'C region encodes pronounced RNA motifs, the most prominent of which are a highly conserved single-stranded sequence stretch (SS) and a stable stem-loop structure (SLI) at the immediate 3' terminus. These elements were suggested to be major functional components of the negative-strand promoter of the initial replication complex (Yu et al. 1999).

In contrast to the 3'C portion, the 3'V region displays a remarkable heterogeneity in size and nucleotide composition between different virus strains. Despite this fact, certain structure and sequence motifs were found to be common to all pestiviruses. Thus, 3'V generally forms two unstable stem-loop structures, SL_{stop} and SLII, and it harbors at least one copy of a moderately conserved 12-nt consensus sequence designated as UGA box motif (Isken et al. 2003; see also Fig. 1B). Correct formation of the RNA structure and the presence of UGA box motifs were previously indicated to be critical for the association of a set of cellular host factors, the NFAR proteins, with the BVDV 3'V region (Isken et al. 2003). The NFAR group includes three types of cellular proteins, namely different isoforms of NF90/NFAR-1 (Saunders et al. 2001), NF45 (Kao et al. 1994), and RNA helicase A (RHA; Claude et al. 1991). Interestingly, the NFAR proteins bind also to the BVDV 5'NTR, and they were indicated to be essentially involved in the viral replication process, possibly by supporting an interaction of the 5' and 3' termini of the viral RNA (Isken et al. 2003).

In this report we present experimental evidence demonstrating that accurate formation of the complex structure of the BVDV 3'V region is critical for the viral RNA replication process, NFAR protein binding, and, most interestingly, for efficient termination of translation at the ORF stop-codon. Thus, especially in view of the proposed NFAR-protein-mediated interaction of the 3'NTR with the 5'NTR, these data suggest that 3'V plays a pivotal role during the regulation of viral protein and RNA synthesis.

RESULTS

Characteristics of the 3'V region of a replicative BVDV RNA

To investigate the role of the 3'V region during translation and replication of the viral RNA, we took advantage of the BVDV replicon DI9c (Fig. 1A). DI9c RNA exhibits the same organization as subgenomic viral RNA molecules that occur during a natural BVDV infection (Tautz et al. 1994); via cDNA constructs, functional DI9c transcripts can be easily generated and mutagenized in vitro (Meyers et al. 1996). Previous studies established genetics on DI9c in combination with appropriate biochemical assay systems as a powerful experimental tool that allowed insights into the function of viral and host proteins as well as a detailed charac-

FIGURE 1. (Legend on next page)

terization of RNA encoded replication and translation signals (see Isken et al. 2003 and references therein). Along this line, the DI9c 3'V region was shown to form the SL_{stop} and SLII structures and to encode several UGA box motifs. Thus, one element, designated as the 5'UGA box, resides in the upstream portion of 3'V (residues U₁₉–A₃₀ of the 3'NTR). A second element spanning residues U₄₆–A₅₇ was called UGA_{pos.cons.} box due to its nearly identical position in all pestiviral genomes. A third sequence stretch resides in the downstream part (residues A₇₉–U₉₀) of 3'V. It was termed 3'UGA-like box, because it largely resembles the UGA box consensus sequence (Isken et al. 2003; Fig. 1B).

Sequence alignments of the genomes of representative pestiviral strains (data not shown) revealed another conspicuous feature of the BVDV DI9c 3'V region that is conserved among all viruses. It concerns the distance between the UGA box motifs and the viral ORF, which, interestingly, can be measured in triplet units. That is, the 5'UGA box is situated 15 nt (5 triplets) downstream from the translational stop-codon, and the UGA_{pos.cons.} box and 3'UGA-like box are spaced by 45 (15 triplets) and 75 nt (25 triplets), respectively. This peculiarity, which prompted the speculation that some kind of translational reading frame is maintained in the 3'NTR (see below), includes also triplets that resemble translational stop-codons. The DI9c 3'V contains three of what we call here “pseudo stop-codons” (first, U₂₈A₂₉A₃₀, second, U₅₅A₅₆A₅₇, and third, U₆₁A₆₂G₆₃), the upstream two of which are constituents of the 5'UGA box and of the UGA_{pos.cons.} box, respectively (Fig. 1B).

FIGURE 1. Genomic and subgenomic BVDV RNAs; characteristics of the BVDV DI9c 3'NTR. (A) Physical map of the full-length BVDV genome and of the subgenomic replicon “DI9c”. The NTRs are indicated as single lines; genetic units encoding the viral proteins are represented as shaded boxes. Known enzymatic activities associated with the viral proteins are indicated (Lindenbach and Rice 2001). The proteolytic cleavage sites in the polyprotein are marked as follows: (arrow) cleavage by the viral protease complex NS3/NS4A; (circle) cleavage by cellular signal peptidases; (A) autoproteolytic; (?) uncertain. Note that with BVDV DI9c, the pestiviral autoprotease N^{pro}, which has no further function during RNA replication, generates the N terminus of NS3 (Behrens et al. 1998). (B) (Top) Sequence and structure characteristics of the BVDV DI9c 3'NTR. The depicted region corresponds to nucleotides 12090–12281 of the genome of the DI9c ancestor strain BVDV CP7 (Meyers et al. 1996); numbering starts at the translational stop-codon (italics). The UGA segments and pseudo stop-codons are boxed. The arrow marks the border between the variable (3'V) and the conserved (3'C) regions of the BVDV 3'NTR as proposed by Deng and Brock (1993). Residues that are 100% conserved in all pestivirus genomes are underlined. The depicted secondary structure summarizes the results of experimental probing data obtained by chemical modification and RNase digestion that were superimposed on an *mfold* structure prediction of the DI9c 3'NTR at lowest $\Delta G_{\max}$ (Yu et al. 1999; Isken et al. 2003). (Bottom) Consensus sequence of the pestiviral UGA box motifs. The consensus sequence is shown in comparison with the sequence of the 5'UGA box, the UGA_{pos.cons.} box (corresponding exactly to the consensus), and the 3'UGA-like box (strong homology to the consensus) of BVDV DI9c. Conserved residues are underlined; pseudo stop-codons are boxed.

Mutagenesis of 3'V

The conservation of sequence and structure motifs in the pestiviral 3'V region suggested an important function of these features in the viral life cycle. Hence, we decided to modify these elements by a systematic pattern of mutations and to investigate the effect of these mutations on the translation and replication process of the viral RNA. In the course of these studies, it turned out that the introduction of defined nucleotide exchanges, which left the size of 3'V essentially intact, was the most suitable strategy to gain conclusive insights into the complex organization and functions of the 3'V region (see Table 1 for an overview).

With the initial series of mutations we wanted to evaluate the impact of the primary sequence composition of the UGA boxes and of the pseudo stop-codon motifs on the function of the DI9c RNA. Accordingly, we introduced point mutations into these elements that were aimed at changing the sequence of the RNA while having no effect on the structure of the 3'V region (see Table 1; Fig. 1B). We focus here on the description of only three of these structure-neutral mutations; however, it should be noted that other comparable point mutations in the UGA boxes and/or pseudo stop-codons had similar minor effects on the translation and replication process of the viral RNA (data not shown; see Fig. 3 and Discussion). First, we inactivated the upstream two pseudo stop-codons, which are part of the 5'UGA box and of the UGA_{pos.cons.} box, by changing UAA into CAA (mutant 1). With the second mutant (mutant 2), we modified an additional conserved residue in each of the 5'-terminal UGA box motifs. The third mutation involved a single nucleotide exchange that altered the sequence of the third pseudo stop-codon from UAG to UGG (mutant 3). Mutations 1–3 had definitely no effect on the structure of the 3'V region. This was demonstrated by chemical structure probing of transcripts that corresponded either to the individual 3'NTRs or to the full-length replicon RNAs and where we compared the structure of the mutant 3'NTRs with that of the wild-type (wt) DI9c 3'NTR (data summarized in Fig. 2; for technical details of the procedure, see Yu et al. 1999 and below).

With the second series of mutants we wanted to further change the sequence of the UGA boxes and, at the same time, affect the structure of SL_{stop} or SLII. Using mutant 2 as a starting point, we exchanged for this purpose a third conserved nucleotide in the 5'UGA box and in the UGA_{pos.cons.} box. In contrast to the former point mutations, the newly introduced nucleotides were such that they interfered with the formation of the stem of SL_{stop} (mutant 4). With a similar kind of intervention we altered the sequence of the 3'UGA-like box at two central positions, and these point mutations were intended to prohibit simultaneously the proper formation of SLII (mutant 5). Experimental structure probing demonstrated that mutations 4 and 5 had the desired effect on the structure of the 3'V region. Thus, in

TABLE 1. Mutations in the BVDV 3'V region and their functional characteristics

Mutations	Name	Effects on sequence and structure motifs of 3'V	Replication (% wt)	Translational readthrough
U ₂₈ ► C and U ₅₅ ► C	1	sequence of 5'UGA box and UGA _{pos.cons.} box (1st and 2nd pseudo stop-codon)	~95	n.d.
A ₂₀ ► G; U ₂₈ ► C and A ₄₇ ► G; U ₅₅ ► C	2	sequence of 5'UGA box and UGA _{pos.cons.} box (1st and 2nd pseudo stop-codon and add. nucleotide)	~85	n.d.
A ₆₂ ► G	3	sequence of 3rd pseudo stop-codon	~110	n.d.
A ₂₀ ► G; U ₂₄ ► C; U ₂₈ ► C and A ₄₇ ► G; U ₅₁ ► C; U ₅₅ ► C	4	sequence of 5'UGA box and UGA _{pos.cons.} box, (1st and 2nd pseudo stop-codon and 2 add. nucleotides) structure of SL _{stop}	~30	?
U ₈₀ ► G; U ₈₂ ► G	5	sequence of 3'UGA-like box, structure of SLII	~65	n.d.
combination of 3 and 4	6	sequence of 3rd pseudo stop-codon sequence of 5'UGA box and UGA _{pos.cons.} box, structure of SL _{stop}	~15	→
combination of 3, 4 and 5	7	sequence of 3rd pseudo stop-codon, sequence of 5'UGA box and UGA _{pos.cons.} box, structure of SL _{stop} sequence of 3'UGA-like box, structure of SLII	~10	→
U ₁ ► G	wt (-stop)	ORF stop-codon	~50	no
G ₂ ► A	wt (UAA)	ORF stop-codon	~120	no
G ₂ ► A; and insertion of U ₄ A ₅ A ₆	wt (UAAUAA)	ORF stop-codon	~130	no
7 and U ₁ ► G	7 (-stop)	ORF stop-codon	0	→
7 and G ₂ ► A	7 (UAA)	ORF stop-codon	~30	?
7 and G ₂ ► A; and insertion of U ₄ A ₅ A ₆	7 (UAAUAA)	ORF stop-codon	~30	?

The mutations are listed on the left; the numbering of nucleotides refers to that of Figures 1 and 2. (►) Nucleotide substitution. Note that nucleotide exchanges were performed such that the generation of novel pseudo stop-codons was avoided. The middle columns indicate the names of the mutations and the effects of the mutations on the sequence and structure of the diverse 3'V motifs, respectively (see text and Fig. 2). The consequences of the mutations for RNA replication and translation termination are summarized on the right. Replication of the mutant RNAs was calculated with respect to that of the wild-type BVDV D19c RNA (considered 100%; see also Fig. 3). Effects on translation termination (see Figs. 5 and 6) are indicated as follows: (→) stop-codon read through clearly detectable; (no) stop-codon read through undetectable; (?) stop-codon read through at detection limit; (n.d.) not done.

the case of mutant 4, SL_{stop} was replaced by a weaker hairpin structure whereas SLII and the residual 3'NTR remained unaffected. Mutation 5 had the inverse effect, that is, the structure of SLII was significantly modified whereas the structure of the residual 3'NTR stayed in the original wild-type conformation (Fig. 2).

The third set of mutations was aimed at modifying the diverse 3'V motifs in concert. To this end, we combined, for example, mutations 3 and 4. Thus, we changed the sequence of SL_{stop}, and removed all pseudo stop-codons (mutant 6). Finally, we created a DI9c derivative that combined mutations 3, 4, and 5. Applying a sum of nine defined point mutations we modified in this way all conserved features of the BVDV 3'V region, namely the consensus sequence of each of the UGA boxes, the structure of SL_{stop} and SLII, and the sequence of each of the three pseudo stop-codons (mutant 7). Experimental structure probing of the 3'NTR of mutants 6 and 7 confirmed that the combination of the different mutations had no unexpected effects on the RNA secondary structure. In other words, the structure of, for example, the 3'NTR of mutant 7 reflected exactly the sum of effects of the individual mutations 3, 4, and 5 (Fig. 2).

Taken together, we conducted this mutational approach to dissect the role of each of the previously defined sequence and structure motifs of the BVDV DI9c 3'V region. Along this line, examination of the RNA secondary structure turned out to be a valuable tool to demonstrate straightaway that each mutation (or combination of mutations) had none but the intended effect on the structure of the RNA (Fig. 2). As an important control we ensured that under the conditions of the subsequently applied translation and replication assay systems the stability of the mutant RNAs was the same as that of the wild-type RNA (data not shown; for experimental details, see Grassmann et al. 1999).

Significance of sequence and structural features of 3'V for the formation of the viral RNA/NFAR protein complex

Next, we determined the effect of the various 3'V mutations on the binding behavior of the NFAR proteins, because these factors were indicated to be important functional components of the BVDV replication process (see Introduction). For this purpose, we applied transcripts encoding the mutant and wild-type 3'NTRs to a UV cross-linking

assay with cytoplasmic extracts of BVDV host cells (see below), that is, to the same assay system that previously enabled the detection of the viral RNA-binding proteins p130, p120 (RHA), p110 (NF110/NFAR-2), p84 (NF90/NFAR-1), p64, and p45 (NF45; Isken et al. 2003; Fig. 3A). We found that 3'V mutants with intact UGA box motifs and unaltered RNA structure (e.g., mutant 3; data of other mutants not shown) yielded mainly the same pattern of RNA-charged proteins as the wild-type RNA. In comparison, the binding of the NFAR proteins was diminished with RNAs where the consensus sequence of the UGA box motifs had been modified or where the RNA structure was different from the wild-type situation. Although the effects were minor with structure-neutral modifications (mutants 1–3), we measured stronger reductions of the cross-linking signals with viral RNA variants where the sequence and structure of 3'V were changed (mutants 4, 5). The most prominent negative effects on NFAR protein binding were observed with the combinations of mutations, which altered the sequence and structure of the 3'V region most drastically (mutants 6 and 7).

These experiments confirmed in a systematic manner our previous supposition that the UGA box consensus sequence and a proper fold of the SL_{stop} and SLII motifs of the 3'V region represent important determinants of the viral RNA/cellular protein interactions (Isken et al. 2003). Moreover, the data revealed that binding of the NFAR proteins to the viral RNA demands a synergistic interplay of the diverse 3'V encoded elements (see Discussion).

FIGURE 2. (Continued on next page)

FIGURE 2. Effects of 3'V mutations on the BVDV 3'NTR structure. (A) Summary of structure probing data that were obtained with different 3'V mutants. The secondary structures of the mutant 3'NTRs were determined by single-strand specific chemical modification and compared to the chemical modification pattern obtained with the wild-type 3'NTR (structure probing data of most mutant RNAs not shown; B shows the structure probing data of the wild-type 3'NTR and of the most significantly modified mutant 7 3'NTR): (dark gray) highly accessible to chemicals; (light gray) slightly accessible (indicating unstable stem structures; Isken et al. 2003); (no indication) protected. Asterisks mark the individual point mutations. As also described in the text, mutations 1, 2 (not shown), and 3 had no effect on the overall structure of the BVDV 3'NTR. The effects of mutations 1, 3, 4, and 5 are shown as parts of the structures of mutants 2, 6, and 7. Mutation 4 gave rise to a novel hairpin structure near the UGA stop-codon. Mutation 5 modified the fold of SLII, designated as "SLII mod". Note that none of the 3'V mutations affected the fold of 3'C and that the RNA structures of mutant 6 (combining mutations 3 and 4) and of mutant 7 (combining mutations 3, 4, and 5) reflected exactly the sum of effects that were caused by the individual mutations 3, 4, and 5 (see text and B). (B) Experimental probing of the RNA secondary structure of the 3'V region of mutant 7 in comparison with the 3'V region of the wild-type RNA (as summarized in A). Structure probing with the single-strand specific chemicals dimethyl sulfate (DMS) and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMCT). The modification procedures were performed using the protocols, the analogous BVDV DI9c 3'UTR transcript, and primer for reverse transcription as described by Yu et al. (1999). DMS was applied as an A- and C-specific agent and CMCT as a U- and G-specific agent (Black and Pinto 1989); however, a high number of G residues was poorly or not modified by CMCT. The figure shows autoradiographs of structure probing (chemical modification and reverse transcription) experiments performed with whole 3'NTR transcripts of wt(DI9c) and mutant 7 RNA (analytical TBE gel, 7 M urea, 7.5% acrylamide). (–) without treatment; (DMS) after treatment with 1 μ L DMS/ μ g of RNA; (CMCT) after treatment with 210 μ g CMCT/ μ g RNA. The regions that form the stem and loop of SL_{stop} or the novel hairpin formed by the mutant RNA are indicated on the right and left, respectively. As already shown in previous works (Yu et al. 1999; Isken et al. 2003) certain residues in the SL_{stop} stem (e.g., A29–31) were found to be partially accessible to chemicals, indicating a weak constitution of the SL_{stop} stem structure (see also A). Modified regions of SLII are indicated in the same manner. The positions of characteristic sequence stretches are given for orientation. Note that in this particular experiment, residues U₅₉–U₆₁ were only weakly modified, whereas in other experiments these residues were strongly modified with mutant 7. This may point to yet uncharacterized weak interactions of this U stretch with other portions of the viral RNA. As summarized in A, the residual 3'NTR was found to be unaffected by the mutations that were introduced into the 3'V region (some data not shown).

Significance of 3'V for viral RNA replication

Next, we transfected the entire repertoire of BVDV 3'V replicon derivatives into suitable BVDV host cells such as MDBK (bovine kidney) or BHK-21 (hamster kidney) cells and measured the accumulation of viral replication products in the cytoplasm with a quantitative RNase protection procedure (Fig. 3B).

We found that mutants **1** and **3**, which affected the sequence of the 5'-terminal UGA box elements and/or the diverse pseudo stop-codons, replicated at about the same level as the wild-type RNA. Mutant **2**, which, in comparison with mutant **1**, contained an additional point mutation in the consensus sequence of both 5'-terminal UGA box elements (see Fig. 2), replicated at a minimal lower level (at ~85% of the wild-type value). A slightly reduced replication capacity (~60%–70% wild type) was measured with mutant **5**, which encoded a modified 3'UGA box sequence/SLII structure (Fig. 3B; see also Fig. 2). Similar moderate reductions of the RNA's replication capacity were observed with short deletion mutations that removed either the 5'UGA box or the UGA_{pos.cons.} box and that modified the structure of SL_{stop} (data not shown).

From these results we deduced that an intact sequence of the UGA box motifs and of the pseudo stop-codons, a correct architecture of SL_{stop} or SLII, and also the presence of several UGA box copies are not essential for the replication process. However, this constellation evidently guarantees the BVDV RNA genome replicating at high efficiency (see below and Discussion).

The remarkable tolerance of the individual 3'V motifs to mutational changes fueled the idea that they were functional components of a flexible higher order RNA motif. This supposition was considerably strengthened when we tested the remaining set of mutants. Thus, RNA replication was noticeably compromised with mutant **4** (replicating at ~30%–40% wild-type level) where we changed the sequence of both 5'-terminal UGA boxes at three consecutive positions (including the first and second pseudo stop-codon) and where SL_{stop} was replaced by a less stable hairpin structure (see Figs. 2, 3B). Mutant **6** displayed an average replication competence of only 15% wild type and therefore was considerably more defective than mutant **4**. This was particularly interesting considering that mutant **6**, in comparison with mutant **4**, contained only one additional point mutation that inactivated also the third pseudo stop-codon of the 3'V region (for a further discussion, see below). However, the most pronounced decline in replication competence was detected with mutant **7**, where all conserved 3'V features were altered by specific point mutations. This RNA was still functional, but replicated at only 5%–10% of the wild-type value (see Figs. 2, 3B).

The observation that mutations, which per se were nearly silent, had a significant negative effect on viral replication when they were stepwise combined substantiated the as-

sumption that the conserved sequence and structural elements of 3'V are cooperative components of a common complex RNA motif. Interestingly, within the limits of accuracy of the applied experimental systems, the mutant RNAs revealed mainly the same kind of behavior in the replication assay as when tested for the formation of the viral/NFAR ribonucleoprotein complex (Fig. 3). This suggested a close link between the formation of the complex and the function of 3'V (see Discussion).

Importance of 3'V for IRES-mediated translation

Experimental evidence that was obtained with different positive-strand RNA virus systems implicated the 3'NTR as an important determinant of the IRES-mediated translation process (Ito et al. 1998; Bergami et al. 2000; Michel et al. 2001).

Accordingly, we addressed the question of whether the replication defect of, for example, mutant **7** RNA was possibly caused by a lower rate of viral protein synthesis. For this purpose, we applied the mutant **7** RNA, that is, the same transcript that was previously tested in the replication assay, to an in vitro translation assay that was based on cytoplasmic extracts of BHK-21 cells. This in vitro translation system was previously shown to reflect the in vivo situation of BVDV protein synthesis rather authentically, because it promotes efficient and accurate translation of the ORF as well as NS3/4A-mediated maturation of the diverse replicon-encoded nonstructural proteins (Grassmann et al. 1999, 2001; Yu et al. 2000; see also Fig. 1A). Translation was measured via incorporation of [³⁵S] methionine into the viral proteins (e.g., N^{pro} or NS3) and evaluated in comparison with the wild-type DI9c RNA and a nonreplicative RNA that encoded a defective replication signal in the conserved SLI motif (Yu et al. 1999; see Fig. 4).

As expected, we found that the modification of SLI or of other entities of the 3'C region (not shown) had no detectable effect on the level of protein synthesis. In comparison, the translation of mutant **7** RNA was observed to be reproducibly reduced to about 50% of the level of the wild-type RNA (Fig. 4). Note that the mutations had no effect on polyprotein proteolysis and that experiments carried out for different time intervals (e.g., ½ h, 1 h, 3 h) yielded identical results excluding stability effects.

These experiments revealed that IRES-mediated translation of the viral replicon RNA is apparently stimulated by the presence of an intact 3'V region. In contrast, the 3'C portion of the 3'NTR was confirmed to act exclusively as a replication signal (see Discussion). Nevertheless, the replication defect of 3'V mutants such as mutant **7** could not be explained by the observed reduction of protein synthesis. This becomes apparent in view of the fact that BVDV replicons with mutations in the 5'NTR, which reduced the rate of translation by a factor of 2–3, still produced sufficient viral protein to allow RNA replication at the wild-type level (Yu et al. 2000; see also Discussion).

FIGURE 3. Characteristics of the BVDV 3'V mutants. (A) Effect of 3'V mutations on the formation of a viral/cellular RNP. Binding of the cellular proteins p130, p120 (RHA), p110 (NFAR-2), p84 (NF90/NFAR-1), p67, p64, and p45 (NF45; not shown) to the BVDV 3'NTR was tested by UV-cross-linking/label transfer using cytoplasmic (S10) extracts of BHK-21 cells and [32 P]-UTP labeled transcripts of the wild-type and mutant DI9c 3'NTRs. (Lane 1) Assay with wild-type 3'NTR; (lane 2) competition experiment performed in the presence of a 50-fold molar excess of nonlabeled wild-type transcript, demonstrating that except for p67 all viral RNA/cellular protein interactions are highly specific (Isken et al. 2003); (lanes 3–9) assays with the 3'NTRs of mutants 1–7. It was confirmed that the observed effects were not caused by differences in the specific activity of the RNA probes (not shown). (B) Effect of 3'V mutations on RNA replication. Replication was measured by RNase protection of newly synthesized positive-strand RNA molecules at different time-points (9, 11, 17 h) post-transfection (pt) into BVDV host cells. Note that different time points and different cell lines yielded identical results and that with all replicative mutants the ratio of negative-strand intermediate and progeny positive-strand RNA molecules was the same as with the wild type (not shown). (Above) Representative experiment; replication was analyzed at 9 h pt. (Lane 1) wild-type DI9c; (lane 2) negative control (RNA expressing NS5B with a defective GDD motif); (lanes 3–9) mutants 1–7. (Below) Bar diagram summarizing the results of four independent transcription/transfection experiments. The amounts of RNA were quantified by phosphorimaging and normalized to the amount of a cotransfected plasmid DNA (Grassmann et al. 1999). The replication ability for each mutant was calculated with respect to that of the wild type (considered 100%). Error bars indicate mean deviations. The possibility that mutant RNAs reverted in the course of the experiment was excluded by RT-PCR amplification and sequencing of the mutated region (not shown).

3'V is involved in translation termination

The interesting fact that all the pestiviral 3'V regions encode pseudo stop-codons gave rise to the idea that 3'V might have a role in translation termination. This notion was further nurtured by the observation that mutant 6 RNA that carried mutations in all three pseudo stop-codons of the 3'V region, was significantly more defective than the mutant 4 RNA, which encoded two defective pseudo stop-codons (see Fig. 3). Hence, we decided to examine mutants 4, 6, and 7, which revealed the most pronounced replication defects, for a potential leakage of the translational stop-codon. This was done with the aforementioned *in vitro* translation system and nonreplicative mini-RNA constructs that encoded only two proteins, the pestiviral autoprotease N^{pro} (see Fig. 1A) and a ~31-kDa-sized NS3/5B fusion protein, termed NS_{fus} (Fig. 5).

Translation experiments that were performed with the wild-type mini-RNA yielded solely N^{pro} and NS_{fus}, indicating a tight control of translation termination. In contrast, the analogous experiment carried out with mutants 4, 6, and 7 yielded an additional protein product (with mutant 4 at detection limit) that corresponded to up to 5% of the sum of translation products and that migrated slower than NS_{fus} in the analytical SDS gel (Fig. 5). Its molecular weight of ~38 kDa immediately suggested that this novel protein corresponded to a C-terminally extended variant of NS_{fus} (designated here as NS_{fus} + 3') that arose by cotranslation of the 3'NTR (~31 kDa NS_{fus} + ~7 kDa that derived from the cotranslated 3'NTR). This was confirmed by further experimental evidence, some of which is described in the following paragraph.

Accordingly, one possible explanation of the significant replication defect of the mutant RNAs 4, 6, and 7 was that ribosomes passed the ORF stop-codon and continued translation in the 3'NTR (see Fig. 7 and Discussion). At this stage of the study, this notion was supported by the fact that there was an inverse relation of the replication efficiency of mutants 4, 6, and 7 and the amounts of NS_{fus} + 3' that were produced by the respective mini-RNA constructs (Fig. 5).

Efficient termination of translation is a prerequisite for efficient replication of the viral RNA

The following experiments were directed at further substantiating the idea that 3'V supports translation termination and to explore the functional relation of translation termination and replication of the viral RNA. As a first approach addressing these issues, we inactivated the natural translational stop-codon (UGA to GGA; see Fig. 6A) of the wild-type DI9c RNA (wt(-stop)) as well as that of the mutant 7 RNA (mutant 7 (-stop)). Mutant 7 was again chosen, because this RNA showed the most profound replication defect correlating with a significant nonsense read through (Figs. 2, 3, 5). Employing the established assay systems,

FIGURE 4. Mutations in the BVDV 3'V region affect IRES-mediated translation. (A) The figure shows a representative *in vitro* translation assay that was carried out for 1 h with cytoplasmic extracts of BHK-21 cells and the full-length wild-type and mutant replicon RNAs (note that experiments carried out for different time periods yielded identical results). Translation/processing of the viral polyprotein was analyzed by SDS-PAGE. The viral protein products are indicated. (Lane 1) translation assay with wild-type DI9c RNA; (lane 2) with the 3'V mutant 7; (lane 4) with the nonreplicative 3'C mutant SLI_{mod} (Yu et al. 1999; a scheme of this mutation in the 3'-terminal SLI stem-loop is shown on the right; modified nucleotides are indicated by asterisks). (B) Bar diagram summarizing the results of three independent experiments. The translation efficiency of the mutant RNAs was quantified with respect to that of the wild-type DI9c RNA (100%) measuring the incorporation of [³⁵S] methionine into N^{pro} or other viral proteins (not shown).

we tested the wild-type and mutant 7 (-stop) RNAs side by side with regard to replication capacity and, as mini-RNA versions, for translation termination. It was again assured that the (-stop) mutations as well as all subsequently described mutations had unexpected effects neither on the stability nor on the structure of the RNA (data not shown).

Thus, in striking accordance with the above working hypothesis we observed that the replication capacity of the wt(-stop) RNA was reduced but clearly not blocked (it corresponded to ~50% of wild-type DI9c level; Fig. 6B) and that translation of the corresponding mini-RNA terminated efficiently despite of the missing ORF stop-codon. However, the resulting protein product of the wt(-stop) mini-RNA was found to be slightly larger than NS_{fus} (Fig. 6C). Therefore, we deduced that translation most probably terminated at the next downstream pseudo stop-codon (U₂₈-A₃₀) of 3'V, that is, prior to entering the conserved 3' terminus of the 3'NTR (see Fig. 7A and Discussion). In contrast, the mutant 7(-stop) RNA, which lacked the ORF stop-codon as well as all pseudo stop-codons, was found to be entirely replication defective and translation of the mini-RNA construct yielded exclusively the complete NS_{fus} + 3' nonsense readthrough product (see Fig. 6B,C).

With these experiments it became evident that an important function of the viral 3'V region concerns termination

and/or displacement of ribosomes that continue translation beyond the ORF stop-codon. Accordingly, these data reinforced the notion that inefficient translation termination at the ORF stop-codon was a potential reason for the replication defect of 3'V variants such as mutants 6 and 7 (see Discussion).

To further validate these assumptions we decided to perform the inverse experiment, that is, to increase the translation termination capability of the wild-type and of the mutant 7 RNA. For this purpose, we replaced the original UGA ORF stop-codon by a stronger UAA stop-codon or by two adjacent UAA stop-codons (Fig. 6A; for a review dealing with the strength of translation termination codons, see Poole and Tate 2000). Interestingly, each of these mutations was found to increase the replication efficiency, not only of the mutant RNA (from ~10% to ~30% of the wild-type level) but also of the wild-type RNA (by ~20%–30%). In the case of mutant 7, we observed rather efficient termination of translation if the RNA contained a UAA or UAAUAA (double) stop-codon instead of the natural UGA stop-codon (Fig. 6B,C).

Hence, in sum, these experiments gave rise to a model according to which the genomic BVDV 3'V region represents an important functional determinant of the coordination of the viral translation and RNA replication process (see Fig. 7 and Discussion).

FIGURE 5. Mutations in the BVDV 3'V region affect translation termination. The efficiency of translation termination was assayed with BVDV mini-RNA constructs and the translation system that was also used in Figure 4. (Top) Organization of the BVDV mini-RNA. Δ indicates an in-frame deletion of most of the NS-coding region of BVDV DI9c (see Materials and Methods). The vertical arrow indicates the N^{pro} cleavage. The horizontal lines schematize the translation products N^{pro} , NS_{fus} , and $NS_{fus} + 3'$, the latter resulting from translation into the 3'NTR (see also Fig. 6). (Bottom) Representative SDS-PAGE of translation termination assays carried out with mini-RNA constructs of the wild-type RNA and mutants 4, 6, and 7. The translation products N^{pro} , NS_{fus} , and $NS_{fus} + 3'$ are indicated. Note that with mutant 4, the amount of translational readthrough product ($NS_{fus} + 3'$) was at the detection limit.

DISCUSSION

Studying pestiviruses such as BVDV is attractive for several reasons. First, pestiviruses are readily manipulative positive-strand RNA viruses that replicate at high efficiency in a broad spectrum of cultured cells and do not infect humans. Moreover, information on molecular determinants of the life cycle of BVDV is of value for the understanding of the life cycle of the closely related human pathogen, HCV. Toward the long-term goal of elucidating the biochemical mechanisms that coordinate the intracellular viral replication pathway, this work was aimed at unraveling the role of the 3'NTR of the BVDV genome as a junction between protein and RNA synthesis.

During previous work with the BVDV replicon RNA DI9c, we found that the SLI and SS motifs in the conserved downstream (3'C) region of the 3'NTR encode sequence and structure signals, which are essential for the initiation of negative-strand RNA synthesis (Yu et al. 1999; Fig. 1). In contrast, the role of the upstream portion of the 3'NTR, the so-called variable region 3'V, remained elusive. This was related to the surprising inconsistency of 3'V among different virus strains and to findings in the HCV system, which showed that genomic RNAs with incomplete 3'V re-

gions nevertheless caused infections in chimpanzees (Yanagi et al. 1999; for a further discussion of these data see below). However, the fact that all *Flaviviridae* genomes encode variable sequence stretches downstream of the ORF (Lindenbach and Rice 2001) implied an important function of this domain and encouraged investigations aimed at defining it.

The BVDV 3'V region is a complex RNA motif

Sequence alignments and RNA structure probing revealed that the BVDV 3'V region encodes a series of features, namely the UGA box motifs, pseudo stop-codons, and the weak stem-loop structures SL_{stop} and SLII that are common to all pestiviruses (Fig. 1B; Isken et al. 2003). Irrespective of their conservation, these motifs turned out to be surprisingly tolerant to mutational changes. That is, modification of single UGA box motifs, single pseudo stop-codons or individual stem loop structures reduced but did not ablate the replication rate of the viral RNA (Figs. 2, 3; data not shown). Moderate negative effects on viral replication were also observed when we changed, for example, the spacing between SL_{stop} and SLII (not shown). These findings explained the evolutionary drift of 3'V, and they were in striking contrast to observations with *cis*-acting replication signals in the conserved genomic 5' and 3' termini, where analogous interventions completely inhibited replication (Yu et al. 1999, 2000; for a further Discussion see below).

The situation was different, that is, RNA replication was severely impaired, when we altered several of the conserved 3'V sequence and structure elements together (Figs. 2, 3). Combining defined point mutations, we observed stepwise accumulating effects. For example, mutant 6 RNA, which carried three inactivated pseudo stop elements and a modified SL_{stop} , replicated at a significantly lower level than mutant 4 RNA (two inactivated pseudo stop-codons and a modified SL_{stop}) or an RNA variant with three inactivated pseudo stop-codons and an intact SL_{stop} (Fig. 3; data not shown). Conversely, the replication capacity of mutant 7, which, in comparison to mutant 6 encoded an additionally modified SLII, was found to be further reduced with respect to mutant 6.

These data implied that the UGA box motifs, pseudo stop-codons, SL_{stop} , and SLII form a higher order RNA motif of cooperating sequence and structure signals, which, in sum, are crucial for the multiplication of the viral RNA.

What is the function of 3'V?

There are two scenarios conceivable to explain the crucial role of 3'V during viral replication. The first scenario posits that the 3'V region, in addition to the previously characterized signals in the 3'C portion of the 3'NTR, represents another, particularly complex-structured *cis*-acting replication element that participates directly in the assembly of the catalytic replication complex. Along this line, one of the

FIGURE 6. Efficient termination of translation is crucial for efficient viral RNA replication. (A) Scheme of mutations affecting the ORF stop-codon (for further details, see Table 1 and Materials and Methods). (B) Replication capacity of stop-codon mutants. (Above) Representative RNase protection experiment detecting newly synthesized positive-strand RNA molecules at 11 h post-transfection (GDD, negative control). (Below) Bar diagram summarizing the results of three independent transfection/replication experiments. Note that similar to the experiments shown in Figure 3 with all replicative RNAs, the ratio of positive- and negative-strand RNA replication products was found to be unchanged with respect to that of the wild type, and that negative-strand RNA was undetectable with mutant 7(-stop) and the GDD mutant (not shown). All controls were performed as described in Figure 3. (C) Translation termination of stop-codon mutants. The experiment was performed as described in Figure 5. The figure shows two exposures of the same gel; read-through products are indicated by dots. The low amount of detectable NS_{fus} + 3' as compared to N^{pro} and NS_{fus} (mutant 7 and mutant 7(-stop)) was explained by a low stability of this product (see text).

major functions of 3'V may involve recruiting the NFAR host factors to the viral replication machinery. Nevertheless, it is surprising that in contrast to the situation with the 3'C region, even drastic mutational changes of 3'V did not completely inhibit replication (mutant 7 still replicated at ~ 10% the wild-type level). Thus, 3'C may be considered as a component of an invariant core of the nascent replication

complex, whereas 3'V may take on the role of a flexible spacer between the ORF and the RNA 3' terminus (for a further discussion, see below).

The second scenario relates to the surprising finding that 3'V has a significant impact on the efficiency of the translation termination process at the ORF stop-codon. This observation raised the important question about the

FIGURE 7. Postulated role of the viral 3'NTR during translation and RNA replication. (A) Model of the roles of the BVDV 3'V and 3'C domains during translation and RNA replication. Although formation of the replication complex (light shaded circle) involves 3'C (SLI and SS), 3'V (SL_{stop} and SLII) is important for efficient termination and displacement of ribosomes at the ORF stop-codon. As indicated by the translation data, 3'V somehow contacts the 5'NTR. This may be mediated by NFAR proteins (dark shaded circles), which associate with BVDV 3'V as well as with the 5'NTR (Isken et al. 2003; see also text). (B) Model of a 5'/3' protein-mediated communication of both ends of the viral genome that may be essential for regulating translation and replication. (Top) The termini of the viral genome act synergistically to promote efficient translation of the viral polypeptide. Nascent viral proteins and cellular factors form the replication complex (shaded circles) with both NTRs. 3'V precludes interferences of the translation and replication machineries. (Middle) At a yet undefined stage, initiation of translation is inhibited. (Bottom) Clearance of translating ribosomes from the viral genome enables initiation of the replication cycle (for further details, see text).

mechanism by which a defect in translation termination may lead to a lower replication efficiency of the viral RNA, as this is clearly implied by the experimental data shown in Figures 5 and 6. Obviously, translational readthrough of the ORF stop-codon is expected to generate NS5B RNA-dependent RNA polymerase molecules that are C-terminally extended by the cotranslated 3'NTR (see Fig. 1A). Our experiments with the BVDV DI9c RNA that encoded an inactivated stop-codon (wt(-stop)) demonstrated that a short C-terminal extension of NS5B had only minor consequences for the activity of the viral replication complex, because this mutant still replicated at 50% wild-type level (see Fig. 6B,C). In comparison, the NS_{fus} + 3' products that were synthesized by the mini-RNA constructs of mutant 7 or mutant 7(-stop) were often unstable (see Fig. 6C), which may suggest that further elongated NS5B molecules are similarly unstable. However, except for the unlikely possibility that C-terminally extended NS5B molecules have a

dominant negative effect, their impact on the overall replication process of the viral RNA should be in any case negligible. This becomes apparent by the fact that even with the least replicative mutant 7 RNA, the amount of C-terminally extended protein came to a maximum of 5% of total translation products (see Figs. 5, 6). Considering again that reductions in the rate of translation of the viral proteins by 50%–70% still allowed viral propagation at the wild-type level (Yu et al. 2000), an amount of 5% C-terminally extended and potentially inactive NS5B should be irrelevant for the overall viral replication process. Therefore, we favor a second explanation of the replication defect of mutants that revealed a defect in translation termination. It postulates that ribosomes entering the viral 3'NTR somehow interfere with the assembly of the replication complex at the genomic 3' terminus (see model in Fig. 7A). This notion becomes particularly evident considering that with cellular mRNAs the average accuracy of translation termination

comes to just 0.0001% readthrough (Kirkwood et al. 1984). Thus, in spite of the low amount of C-terminally extended protein (see Figs. 5, 6, and above), 1%–5% nonsense readthrough as measured here with the viral RNAs **6** and **7** nevertheless indicates considerable numbers of ribosomes encountering the 3'NTR. In line with the idea that collisions of the translation and replication machineries explain the replication defect of the 3'V mutants RNAs, it is important to note that with the variant (mutant 7(-stop)) that revealed the highest degree of translational readthrough, negative-strand RNA synthesis was definitely undetectable (data not shown).

Both discussed scenarios are not mutually exclusive. In fact, the observation that a strong UAA stop-codon could only partially compensate for the replication defect of mutant **7** RNA (Fig. 6) suggests that the 3'V region holds a double function, namely, as a signal that increases the efficiency of translation termination and also as an important determinant of the assembling replication complex (see Fig. 7B and below). In any case, the bipartite composition of the pestiviral 3'NTR that was originally proposed by sequence alignments (Deng and Brock 1993) was shown here to have an evident functional impact. Whereas 3'C acts as a crucial determinant of the assembling replication complex, 3'V takes on the role as a modulator of the translation and RNA replication process (Fig. 7).

Translation termination may regulate the level of viral replication

The observation that complex RNA motifs in the BVDV 3'NTR control translation termination is reminiscent of other viruses that exploit genomic RNA sequence and/or structure motifs for a recoding of translation. For example, RNA plant viruses and retroviruses encode pseudo-knot or stem-loop structures downstream from ORF-internal stop-codons, which increase the leakiness (i.e., the binding affinity for suppressor t-RNAs) of these stops to express additional viral proteins (for reviews, see Herr et al. 2000; Beier and Grimm 2001). However, in contrast to these examples, the BVDV genome is considered to be monocistronic, which leads to the important question of why the viral RNA encodes an additional translation termination signal downstream from the ORF stop. Putting all the experimental data together, the most obvious model posits that 3'V may act as a sort of translation safety valve, the stringency of which is determined by the presence and/or consistency of its diverse composing RNA motifs. In other words, the observed synergistic interplay of the conserved features of 3'V may determine the extent to which ribosomes finally dissociate at the ORF stop (Fig. 7A). This idea is strongly supported by the observation that the replication capacity of the wt(-stop) mutant was still high (Fig. 6), and it fits well with the fact that the genomic 3'V regions of all *Flaviviridae* members encode pseudo stop-codons (data not shown). Finally, it explains the aforementioned HCV infec-

tion data of Yanagi et al. (1999), because these authors observed the occurrence of a pseudorevertant (at day 5 of the infection, this revertant came to 95% of total HCV RNA), where an incomplete 3'V was compensated by a UGA-to-UAA mutation of the ORF stop-codon.

In line with this model, it is worth discussing that all known pestiviral (and HCV) genomes encode weak UGA rather than strong UAA stop-codons (data not shown). The suspicion that the natural stop-codon is indeed slightly leaky was supported by our experimental data showing that the replication efficiency of the wild-type BVDV RNA was clearly increased just by changing UGA into UAA (Fig. 6). Recalling again the variability of 3'V and its striking tolerance to nucleotide exchanges, it is tempting to assume that 3'V variants account for a major portion of the pool of replication-competent RNA quasi-species that occur in the course of a viral infection. Down- or up-regulation of the viral load may therefore occur by selection of differently replicating RNAs encoding a more or less leaky stop-codon. The variability of 3'V among different virus strains would thus reflect their adaptation to different environments.

A speculative model on the initiation of viral replication

The final interesting result of this study concerned the correlation that was observed between the formation of the viral RNA (3'V)/NFAR protein complex and the replication capacity of the RNA (Fig. 3). Though not formally proven, these data substantiate previous suppositions that the NFAR proteins are recruited to induce or to stabilize the fold of the 3'V region as a prerequisite for its function. Moreover, the RNA/protein interaction data are particularly intriguing because a complex consisting of RHA, NF90/NFAR-1, and NF45 was shown to support RNA–RNA interactions between the BVDV 3' and 5'NTR (Isken et al. 2003). It is important to note that binding of the NFAR proteins to the 5'NTR involves also the regulatory hairpin Ia motif at the immediate genomic 5' terminus, some elements of which were shown to modulate the efficiency of the IRES whereas others are essential for the initiation of the replication cycle (Yu et al. 2000). Taking these data and findings with other RNA virus systems (e.g., poliovirus) together, we postulate the following order of events at the early stage of the BVDV replication cycle (Fig. 7B). During the mRNA phase, the 3'NTR may contact the 5' end of the viral genome by RNA–RNA interactions and the activity of NFAR proteins (Isken et al. 2003). As indicated by the translation data shown in Figure 4 of this study, the 3'V region may operate synergistically with signals in the 5'NTR to support efficient IRES-mediated translation initiation. Along the same line, a loop-conformation of the RNA may favor translation reinitiation of ribosomes after a completed round of polypeptide synthesis. Concomitant with the maturation of the viral proteins, the replication complex starts to emerge at the 3'C

portion of the 3'NTR while a major task of 3'V is to efficiently terminate the translation machinery at the ORF stop (Fig. 7A). As a possible scenario for the switch from translation to replication, the replication complex may at a certain stage of the assembly process change the conformation of 3'V such that this affects also the 5'NTR leading to translation inhibition. De novo synthesized viral proteins may trigger this event in a feedback-like manner (Kolakowsky and Weissmann 1971; Gamarnik and Andino 1998). Clearance of ribosomes from the viral RNA would then allow initiation of replication involving again signals at both termini of the genome such as hairpin Ia and SLI (Barton et al. 1999, 2001; Yu et al. 1999, 2000; Fig. 7B).

Further studies are required to fully understand the biochemical mechanisms that control the replication of positive-strand RNA viruses. The findings reported here on the organization and functions of the BVDV 3'V region represent an important step toward this goal.

MATERIALS AND METHODS

Cells

All types of cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acids.

Construction of recombinant plasmids

Mutagenesis of the BVDV 3'NTR (for details, see Table 1) was performed with the "Quickchange" procedure (Stratagene) and a plasmid, pCAS, encoding the 3'-terminal portion of the BVDV "DI9c" cDNA (Yu et al. 1999). Each mutation was confirmed by sequencing and cloned via the ClaI and AatII restriction sites into the plasmid pA/BVDV/D9, which encodes the full-length BVDV DI9c replicon RNA (Meyers et al. 1996). The mutant viral RNAs were generated by in vitro transcription with T7 RNA polymerase (Stratagene) using the pA/BVDV/D9 derivatives linearized with SmaI.

The plasmids (termed pBRT) used to generate mini-genome RNAs for the translation termination assays were engineered from the pA/BVDV/D9 3'V derivatives by removal of a PvuII fragment that encodes a major portion of the nonstructural ORF (nucleotides 3222–12070 according to the nomenclature of Meyers et al. 1996; see also Fig. 5).

The plasmids used to generate the probes for the UV-cross-linking/label transfer experiments were generated by cutting the diverse 3'V pCAS derivatives with PvuII (cuts at position 12070 in the BVDV sequence) and HincII (cuts within the multiple cloning site of the parental pBluescript KS (+) vector) and religation of the vector fragments. The resulting plasmids, termed pUV 1–12, encoded the BVDV 3'NTR and a small portion of the BVDV NS5B-coding region. Transcription of the RNA probes was performed with T3 RNA polymerase in the presence of [³²P] UTP following linearization of the plasmids with SmaI.

The plasmid constructs applied for transcription of the probes for RPA detection of the BVDV replication products and of the

cotransfected control plasmid (see below) were described previously (Behrens et al. 1998; Grassmann et al. 1999).

Transcription and transfection of RNA

Transcription of the diverse RNA molecules was performed with either T3 or T7 polymerase using standard transcription procedures. Transfection of viral RNA transcripts into BHK-21 and MDBK was performed by electroporation (BioRad gene pulser II) following protocols that were previously developed for these cells (Behrens et al. 1998; Tautz et al. 1999). At the indicated time points, the cells were harvested and the cytoplasmic RNA was prepared (see below).

Preparation of cytoplasmic extracts

Ca. 10⁶ cells were harvested by centrifugation at 1000g for 2 min and washed in cold phosphate buffered saline (PBS). The cell pellet was resuspended in 400 µL of lysis buffer (50 mM Tris/HCl at pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 0.5% NP40) and the nuclei removed by centrifugation for 2 min at 1000g. To inhibit proteases, PMSF (phenylmethylsulfonyl-fluoride) was added at 1 mM.

UV cross-linking assay

Fifteen µL of cytoplasmic extract were mixed with an equal volume of 10 mM HEPES (pH 7.6), 0.3 mM MgCl₂, 40 mM KCl, 1 mM DTT, 5% glycerol. Twenty µg of tRNA and 40 U RNasin were included and the mixture was incubated for 10 min at 30°C. Then 500,000 cpm (~ 10 ng) of [³²P]-labeled RNA transcript were added and the incubation continued for 30 min. The samples were exposed to UV light for 15 min at 4°C followed by treatment with 7 µg RNase A and 20 U RNase T1 for 1 h at 37°C. The proteins were separated by SDS-PAGE and radiolabeled bands identified by autoradiography. In competition experiments, a 50–100 molar excess of unlabeled competitor RNA was included in the reaction prior to the treatment with UV.

RNase protection assay

Transfected cells (~ 3 × 10⁶) were grown for the indicated time points, washed once with PBS, harvested and lysed in lysis buffer (50 mM Tris/HCl at pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40) at 0°C. The nuclei were removed by centrifugation for 2 min at 1000g. The cytoplasmic supernatant was adjusted to 0.5% SDS and proteins removed by digestion with proteinase K (Roche). After phenol-chloroform extraction, the nucleic acids were precipitated with ethanol and the washed pellet dissolved in 200 µL hybridization buffer (80% [v/v] formamide, 40 mM PIPES-HCl at pH 6.4, 400 mM NaCl, 1 mM EDTA). Dissolved RNA (12–30 µL) was denatured for 2.5 min at 85°C and subjected to hybridization overnight at 45°C with radiolabeled DI9c sense or antisense probes, respectively (1 × 10⁵ cpm, ~ 10⁹ cpm/µg). Subsequently, 350 µL of RNase digestion buffer (10 mM Tris/HCl at pH 7.5, 1 M NaCl, 5 mM EDTA) as well as 25 U of RNase T1 and 3.5 µg of RNase A were added, and digestion was performed for at least 1 h at 37°C. To obtain efficient negative-strand detection, the excess of positive-strand RNA was removed, performing a prior

cycle of hybridization and RNase treatment before applying the radiolabeled probe in a second hybridization and protection procedure (Behrens et al. 1998). After proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation, the protected fragments were analyzed electrophoretically on 5% 7 M urea polyacrylamid gels (see above). Quantification of the protected RNA fragments was performed in comparison to a fixed amount of cotransfected plasmid that was also visualized by an RNA probe (data not shown; Grassmann et al. 1999). Quantification was carried out with a Fuji Bio Imaging Analyzer and the corresponding software.

RT-PCR

RT-PCR was performed essentially as described previously by Yu et al. (1999).

Probing of the RNA secondary structure

Chemical modification of RNA transcripts to determine the RNA secondary structures of the viral 3'NTRs was performed as described by Yu et al. (1999). The applied in vitro transcripts were generated from plasmids encoding the individual 3'NTR or the full-length replicon. Predictions of RNA secondary structures were performed with *mfold* (<http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi>).

In vitro translation/translation termination assay

The preparation of BHK-21 S10 extract and BHK-21 cell translation initiation factors (eIFs) was carried out following the protocol of Barton and Flanagan (1993). In vitro translation reactions (50 μ L total volume) were performed at 30°C for the indicated time intervals, in the presence of 1 μ g of RNA template, 30% (v/v) BHK-21 S10 extract, 10% (v/v) BHK-21 cell eIFs, 40 U RNaseOut (Gibco BRL), and 10–15 μ Ci of [³⁵S] methionine. Translation products were analyzed by 10% SDS-PAGE and monitored by autoradiography. Quantification was performed with a Fuji Bio Imaging Analyzer measuring the amounts of NP^{pro} or NS3. For translation termination analysis, 2 μ g of BVDV mini-RNA transcript were applied in 20 μ L of an in vitro translation reaction. Following a reaction period of 2 h at 30°C in the presence of [³⁵S] methionine, the translation products were separated by 10% SDS-PAGE and analyzed by autoradiography.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 535, Graduiertenkolleg "Biochemie von Nukleoproteinkomplexen") at the J-L-U Giessen and by grants of Glaxo-SmithKline and the "Kompetenznetz Hepatitis" (Bundesministerium für Bildung und Forschung). At Fox Chase Cancer Center (FCCC), S.E.B. was supported by start-up funds and a Tobacco Formula Grant of the Commonwealth of Pennsylvania.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby

marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

Received March 3, 2004; accepted July 2, 2004.

REFERENCES

- Agol, V.I., Paul, A.V., and Wimmer, E. 1999. Paradoxes of the replication of picornaviral genomes. *Virus Res.* **62**: 129–147.
- Barton, D.J. and Flanagan, J.B. 1993. Coupled translation and replication of poliovirus RNA *in vitro*: Synthesis of functional 3D polymerase and infectious virus. *J. Virol.* **67**: 822–831.
- Barton, D.J., Morasco, B.J., and Flanagan, J.B. 1999. Translating ribosomes inhibit poliovirus negative-strand RNA synthesis. *J. Virol.* **73**: 10104–10112.
- Barton, D.J., O'Donell, B., and Flanagan, J.B. 2001. 5' cloverleaf in poliovirus RNA is a *cis*-acting replication element required for negative-strand synthesis. *EMBO J.* **20**: 1439–1448.
- Behrens, S.E., Grassmann, C.W., Thiel, H.J., Meyers, G., and Tautz, N. 1998. Characterization of an autonomous subgenomic pestivirus RNA replicon. *J. Virol.* **72**: 2364–2372.
- Beier, H. and Grimm, M. 2001. Misreading of termination codons in eukaryotes by natural nonsense suppressor tRNAs. *Nucleic Acids Res.* **29**: 4767–4782.
- Bergami, G., Preiss, T., and Hentze, M.W. 2000. Picornavirus IRESes and the poly(A) tail jointly promote cap-independent translation in a mammalian cell-free system. *RNA* **6**: 1781–1790.
- Black, D.L. and Pinto, A.L. 1989. U5 small nuclear ribonucleoprotein: RNA structure analysis and ATP-dependent interaction with U4/U6. *Mol. Cell Biol.* **9**: 3350–3359.
- Claude, A., Arenas, J., and Hurwitz, J. 1991. The isolation and characterization of an RNA helicase from nuclear extracts of HeLa cells. *J. Biol. Chem.* **266**: 10358–10367.
- Deng, R. and Brock, K.V. 1993. 5' and 3' untranslated regions of pestivirus genome: Primary and secondary structure analyses. *Nucleic Acids Res.* **21**: 1949–1957.
- Garmarnik, A. and Andino, R. 1998. Switch from translation to replication in a positive-stranded RNA virus. *Genes & Dev.* **12**: 2293–2304.
- Grassmann, C.W., Isken, O., and Behrens, S.E. 1999. Assignment of the multifunctional NS3 protein of bovine viral diarrhea virus during RNA replication: An in vivo and in vitro study. *J. Virol.* **73**: 9196–9205.
- Grassmann, C.W., Isken, O., Tautz, N., and Behrens, S.E. 2001. Genetic analysis of the pestivirus nonstructural coding region: Defects in the NS5A unit can be complemented in trans. *J. Virol.* **75**: 7791–7802.
- Herr, A.J., Atkins, J.F., and Gesteland, R.F. 2000. Coupling of open reading frames by translational bypassing. *Annu. Rev. Biochem.* **69**: 343–372.
- Isken, O., Grassmann, C.W., Sarisky, R.T., Kann, M., Zhang, S., Grosse, F., Kao, P.N., and Behrens, S.E. 2003. Members of the NF90/NFAR protein group are involved in the life cycle of a positive-strand RNA virus. *EMBO J.* **22**: 5655–5665.
- Ito, T., Tahara, S.M., and Lai, M.M.C. 1998. The 3'-untranslated region of hepatitis C virus RNA enhances translation from an internal ribosomal entry site. *J. Virol.* **72**: 8789–8796.
- Kao, P.N., Chen, L., Brock, G., Ng, J., Kenny, J., Smith, A.J., and Corthesy, B. 1994. Cloning and expression of cyclosporin A- and FK506-sensitive nuclear factor of activated T-cells: NF45 and NF90. *J. Biol. Chem.* **269**: 20691–20699.
- Kirkwood, T.B., Holliday, R., and Rosenberger, R.F. 1984. Stability of the cellular translation process. *Int. Rev. Cytol.* **92**: 93–132.
- Kolakowsky, D. and Weissmann, C. 1971. Possible mechanism for transition of viral RNA from polysome to replication complex. *Nat. New Biol.* **231**: 42–46.

- Lindenbach, B.D. and Rice, C.M. 2001. Flaviviridae: The viruses and their replication. In *Virology*, 4th ed. (ed. B.N. Fields), pp. 991–1042. Lippincott-Raven, Philadelphia, NY.
- Meyers, G., Tautz, N., Becher, P., Thiel, H.J., and Kümmerer, B.M. 1996. Recovery of cyto-pathogenic and noncytopathogenic bovine viral diarrhea viruses from cDNA constructs. *J. Virol.* **70**: 8606–8613.
- Michel, Y.M., Borman, A.M., Paulous, S., and Kean, K.M. 2001. Eukaryotic initiation factor 4G-poly(A) binding protein interaction is required for poly(A) tail-mediated stimulation of picornavirus internal ribosome entry segment-driven translation but not for X-mediated stimulation of hepatitis C virus translation. *Mol. Cell. Biol.* **21**: 4097–4109.
- Poole, E. and Tate, W. 2000. Release factors and their role as decoding proteins: Specificity and fidelity for termination of protein synthesis. *Biochim. Biophys. Acta* **1493**: 1–11.
- Saunders, L.R., Perkins, D.J., Balachandran, S., Michaels, R., Ford, R., Mayeda, A., and Barber, G.N. 2001. Characterization of two evolutionarily conserved, alternatively spliced nuclear phosphoproteins, NEAR-1 and -2, that function in mRNA processing and interact with the double-stranded RNA-dependent protein kinase, PKR. *J. Biol. Chem.* **276**: 32300–32312.
- Tautz, N., Thiel, H.J., Dubovi, E.J., and Meyers, G. 1994. Pathogenesis of mucosal disease: A cytopathogenic pestivirus generated by an internal deletion. *J. Virol.* **68**: 3289–3297.
- Tautz, N., Harada, T., Kaiser, A., Rinck, G., Behrens, S., and Thiel, H.J. 1999. Establishment and characterization of cytopathogenic and noncytopathogenic pestivirus replicons. *J. Virol.* **73**: 9422–9432.
- Yanagi, M., St. Claire, M., Emerson, S.U., Purcell, R.H., and Bukh, J. 1999. In vivo analysis of the 3' untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. *Proc. Natl. Acad. Sci.* **96**: 2291–2295.
- Yu, H., Grassmann, C.W., and Behrens, S.E. 1999. Sequence and structural elements at the 3' terminus of bovine viral diarrhea virus genomic RNA: Functional role during RNA replication. *J. Virol.* **73**: 3638–3648.
- Yu, H., Isken, O., Grassmann, C.W., and Behrens, S.E. 2000. A stem-loop motif formed by the immediate 5'-terminus of the bovine viral diarrhea virus genome modulates translation as well as replication of the viral RNA. *J. Virol.* **74**: 5825–5835.

RNA

A PUBLICATION OF THE RNA SOCIETY

Complex signals in the genomic 3' nontranslated region of bovine viral diarrhea virus coordinate translation and replication of the viral RNA

OLAF ISKEN, CLAUS W. GRASSMANN, HAIYING YU, et al.

RNA 2004 10: 1637-1652

References

This article cites 28 articles, 21 of which can be accessed free at:
<http://rnajournal.cshlp.org/content/10/10/1637.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to RNA go to:
<http://rnajournal.cshlp.org/subscriptions>