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In Vitro And in Vivo Translational Efficiencies of the 5′ Untranslated Region from Eight Genotype 2 Bovine Viral Diarrhea Virus Field Isolates

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Abstract
We determined the in vitro and in vivo translational efficiency mediated by the internal ribosomal entry site (IRES) from eight BVDV2 field isolates varying in virulence using a bicistronic reporter vector in rabbit reticulocyte lysates (RRL), and in primate and bovine cell lines. Using a T7-promoter system, the high virulence isolates had greater translational efficiencies in bovine lymphocytes (BL-3 cells), than did the low virulence isolates. The low virulence isolates translated with greater efficiencies than the high virulence isolates in RRL, African green monkey kidney (CV-1) and bovine turbinate (BT) cells. Our results demonstrate that despite a high degree of sequence identity in the 5′ untranslated region (UTR), subtle differences in the primary and secondary structures, as well as differences in cell lines, influence translational efficiencies.

Keywords: Bovine viral diarrhea virus, 5′ untranslated region, Translational efficiency

Introduction

Bovine viral diarrhea virus (BVDV) is a member of the family *Flaviviridae* within the genus *Pestivirus.* The viral genome is a single-stranded, positive-polarity, 12.5 kb RNA that can be divided into three distinct regions: a non-capped 5′ untranslated region (UTR), a single large open reading frame encoding a single polyprotein and a 3′ UTR lacking a polyadenylated tail (Murphy *et al.*, 1995). The 5′ UTR is highly conserved and comprised of approximately 385 bases (Qi *et al.*, 1992 and Ridpath *et al.*, 1994) with multiple AUG codons upstream of the authentic initiation codon (Collett *et al.*, 1988). Deng and Brock (1993) reported a secondary structure model for the pestivirus 5′ UTR consisting of a series of stem-loop structures divided into four domains designated A, B, C and D. The domains encompassed about 70% of the 5′ UTR nucleotides participating in base pairing to conform to the secondary structure. The most significant domain was domain D, a highly conserved, stable, multiple stem-loop structure (D1, D2, D3, and D4) spanning two-thirds of the 5′ UTR sequence from nucleotides 139 to 361. BVDV 5′ UTR features are similar to the 5′ noncoding region of picornaviruses and hepatitis C virus (HCV) in which translational initiation occurs through direct cap-independent internal binding of ribosomes to a distinct structural element, referred to as the internal ribosomal entry site (IRES) (Jackson *et al.*, 1990 and Tsukiyama-Kohara *et al.*, 1992). Translational initiation of BVDV involves internal ribosomal accession utilizing the IRES element located within domain D of the 5′ UTR of the BVDV genome (Deng and Brock, 1993 and Pestova and Hellen, 1999). The 5′ UTR of BVDV is capable of driving translational initiation from an internal RNA element in the context of a bicistronic reporter transcript (Chon *et al.*, 1998). Pellerin *et al.* (1994) concluded

that a 5′ UTR-dependent translation efficiency, due to either the stability of the secondary structure or particular sequences, could directly reflect on the virulence of the strains. This conclusion was based on the observation that the 5′ UTR of genotype 2 isolates had a significantly more stable secondary folding (domain D) than genotype 1 viruses. Domain D represents the majority of the 5′ UTR, therefore, its stability may represent the stability of the entire 5′ UTR. Our hypothesis was that the differences in virulence of BVDV2 viruses is influenced by the primary and secondary structure of the 5′ UTR affecting the translational efficiency of the IRES element. In the present study, we characterized the in vitro and in vivo efficiency of translational initiation mediated by the IRES element from eight BVDV2 field isolates varying in virulence using a bicistronic reporter vector in rabbit reticulocyte lysates (RRL), and in primate and bovine cell lines. Our results using individual cDNA plasmid constructs of the 5′ UTR of these eight BVDV2 isolates indicate that these isolates do not translate with equal efficiency within cell lines. The genetic diversity of pestiviruses within genotypes contributes to the differences in translational efficiency. The results in BL-3 cells suggest the interaction of cellular factors with the 5′ UTR, influencing cellular tropism and viral pathogenicity.

Results

BVDV2 IRES translational efficiencies

The in vitro and in vivo translational efficiency mediated by the 5′ UTR IRES from eight BVDV2 isolates varying in virulence was investigated using transfection assays with a bicistronic reporter vector in RRL, and in primate and bovine cell lines. These BVDV2 isolates have a wide range of virulence. Based on experimental infection of calves, the isolates can be separated into two groups, high and low virulence (Bolin and Ridpath, 1992, Kelling *et al.*, 1990, Kelling *et al.*, 2002, Odeón *et al.*, 1999 and Topliff and Kelling, 1998).

The 5′ UTRs were inserted into the intercistronic space between two reporter genes, chloramphenicol acetyltransferase (CAT) and luciferase (LUC) of the plasmid, pBiSpe (Chon *et al.*, 1998) and then subcloned into the mammalian expression vector, pcDNA3.1 (Promega, Madison, WI) (Figure 1). In this orientation, the CAT gene is expressed in a cap-dependent mechanism, while the LUC gene is translated in a cap-independent fashion based on internal ribosomal entry into the BVDV2 IRES. Bicistronic messages were transcribed from the T7 promoter in vitro using T7 polymerase or in vivo using a recombinant vaccinia virus expressing T7 RNA polymerase (a kind gift provided by Dr. Bernard Moss, National Institutes of Health, Bethesda, MD). The CAT and LUC activities were measured from the cell lysates. CAT activity may be used as an indicator of the steady-state level of bicistronic transcript in cells since the rate of 5′

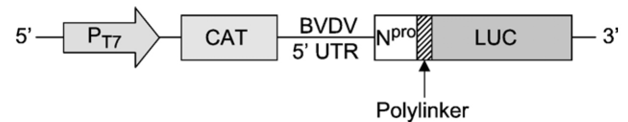

Figure 1. Schematic representation of the bicistronic reporter plasmid used to examine the in vitro and in vivo efficiency of BVDV2 IRES-driven translation. The 5′ UTR of each BVDV2 isolate was inserted into the intercistronic region between the upstream CAT (chloramphenicol acetyltransferase) and the downstream LUC (luciferase) reporter genes.

cap-dependent scanning translation initiation for the 5′ cistron is expected to be identical for each BVDV2 isolate. This steady-state level of CAT expression may be used to normalize LUC activity and allow a comparison of each BVDV2 isolate with the reference BVDV1 5′ UTR region. For comparison of each BVDV2 isolate, a relative IRES activity was calculated by determining the ratio of LUC to CAT activity using the formula: R = (LUC activity from BVDV2 IRES / LUC activity from BVDV1 IRES) / (CAT activity from BVDV2 IRES / CAT activity from BVDV1 IRES). The ratios calculated for the IRES elements of the BVDV2 isolates were normalized using the ratio of BVDV1/NADL IRES as 1 (or 100%). Normalizing the ratios in this manner allowed for comparison of translational efficiencies between isolates in the different cell types.

In vitro transfection assays

The translational efficiency of the 5′ UTR IRES from each of the eight BVDV2 isolates was measured in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega) (Figure 2A). The lowest relative translational efficiency was that of BVDV 890 with a ratio of 1.8, while BVDV 17011 translated with the greatest efficiency having a ratio of 6.1. The translational efficiencies of the low virulence isolates (7937, 17011, 5521, 713) ranged from 4.3 to 6.1, while the ratios of the high virulence isolates (890, NY93, 17583, 23025) ranged from 1.8 to 4.6. As a group, there was a significant difference ($P < 0.0001$) in translational efficiencies between the low and high virulence isolates. Western blot analyses were performed on the in vitro RRLs to detect the CAT (Figure 2B) and LUC (Figure 2C) proteins. Both proteins were detected by the appropriate secondary antibodies and migrated with similar molecular weights as purified protein.

In vivo transfection assays in African green monkey kidney (CV-1), bovine turbinate (BT), bovine testicular (RD420) cells and bovine lymphocytes (BL-3)

Using the T7-promoter system, the relative translational efficiencies in CV-1 cells were similar to those observed in vitro (Figure 3A). As a group, the BVDV2 isolates with the highest virulence had significantly ($P <$ 0.0001) lower translational efficiencies than the isolates

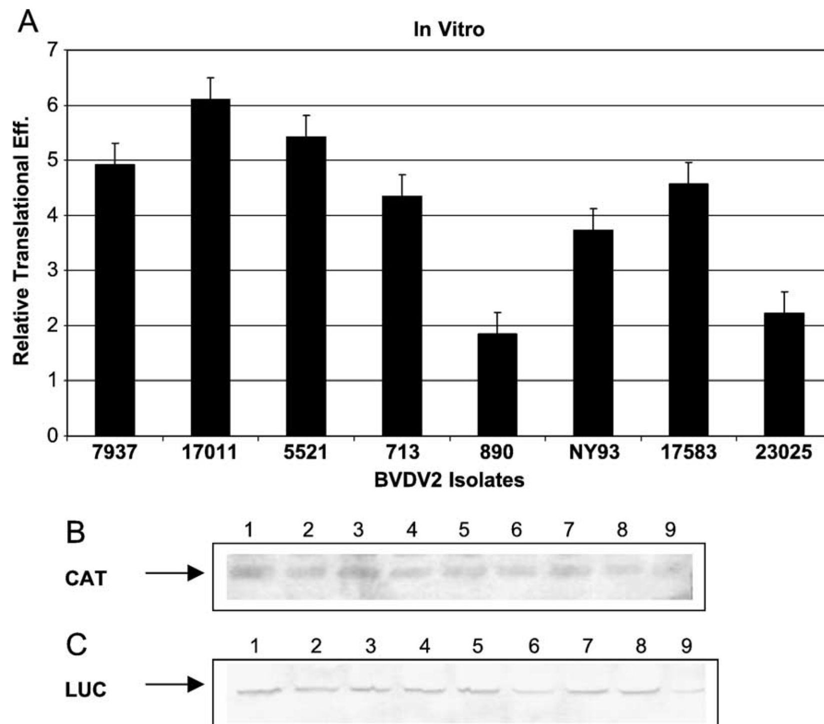

Figure 2. In vitro T7 coupled reticulocyte lysate translation. (A) Relative translational efficiency of the 5′ UTR IRES from each of the BVDV2 isolates measured in vitro using T7 RNA polymerase. The ratios calculated for the IRES elements of the BVDV2 isolates were normalized using the BVDV1-NADL IRES as 1 (or 100%). (B) Expression of the CAT protein from the bicistronic reporter plasmid in rabbit reticulocyte lysate. Lane 1: 3.1/CAT. Lane 2: 3.1/CAT7937LUC. Lane 3: 3.1/CAT17011LUC. Lane 4: 3.1/CAT5521LUC. Lane 5: 3.1/CAT713LUC. Lane 6: 3.1/CAT890LUC. Lane 7: 3.1/CATNY93LUC. Lane 8: 3.1/CAT17583LUC. Lane 9: 3.1/CAT23025LUC. (C) Expression of the LUC protein from the bicistronic reporter plasmid in rabbit reticulocyte lysate. Lane 1: LUC T7 Control DNA. Lane 2: 3.1/CAT7937LUC. Lane 3: 3.1/CAT17011LUC. Lane 4: 3.1/CAT5521LUC. Lane 5: 3.1/CAT-713LUC. Lane 6: 3.1/CAT890LUC. Lane 7: 3.1/CATNY93LUC. Lane 8: 3.1/CAT17583LUC. Lane 9: 3.1/CAT23025LUC.

considered to be low virulence. The low virulence isolates ranged between 0.63 and 0.87, while the high virulence isolates ranged between 0.44 and 0.62 with the order of translational efficiency 7937 > 17011 > 5521 > 713 > 17583 > NY93 > 23025 > 890 (Figure 3A).

Translational efficiency ratios in BT cells ranged between 0.89 and 1.37, with the low virulence isolates ranging between 1.07 and 1.37 and the high virulence isolates ranging between 0.89 and 1.35 (Figure 3B). As a group, the ratios of the low virulence isolates were significantly greater ($P < 0.05$) from the high virulence isolates. BVDV2-5521 had the greatest translational efficiency, while BVDV2-23025 and 890 were the least efficient.

A statistically significant difference between the BVDV2 virulence groups was not observed in the RD420 cells (Figure 3C). Six of the BVDV2 IRESs had equivalent translational efficiencies (7937, 17011, 5521, NY93, 17583, 23025), while the remaining two isolates (713, 890) had the lowest translational efficiencies.

The ratios observed in BL-3 cells ranged between 0.82 and 3.65 (Figure 3D). In this cell line, the isolates grouped according to virulence were significantly ($P < 0.05$) different from one another, as was observed in CV-1 and BT cells. However, unlike in the other cell lines, the high virulence isolates translated with greater efficiency than did the low virulence isolates. Similar to the other cell lines evaluated, BVDV2-713 was the least efficient of the low virulence group, while BVDV2-890 translated with the least efficiency of the high virulence isolates.

BVDV2 IRES secondary structure

The secondary structures of the eight BVDV2 field isolate 5′ UTRs was predicted using the MFOLD algorithm (Zuker, 1989) of the Wisconsin Package version 10.1, Genetics Computer Group (GCG, Madison, WI). The predicted free energy values of the eight BVDV2 isolates ranged between −126.5 (low virulence isolate, 17011) and −145.6 (high virulence isolate, 890) kcal/mole. The remaining low virulence isolates, 7937, 5521 and 713 had free energy values of −135.7, −129.7, and −128.9 kcal/mole, respectively, while the remaining high virulence isolates NY93, 17583, and 23025 had free energy values of −132.8, −133.0, and −133.9 kcal/mole, respectively. All isolates demonstrated a well-conserved IRES element between bases 139 and 361, based on the nomenclature of Deng and Brock, 1993 (data not shown).

The Phylogenetic Inference Package (PHYLIP), version 3.6a3 (J. Felsenstein, University of Washington, Seattle, WA) and TreeView, version 1.6 (R. D. M. Page, Uni-

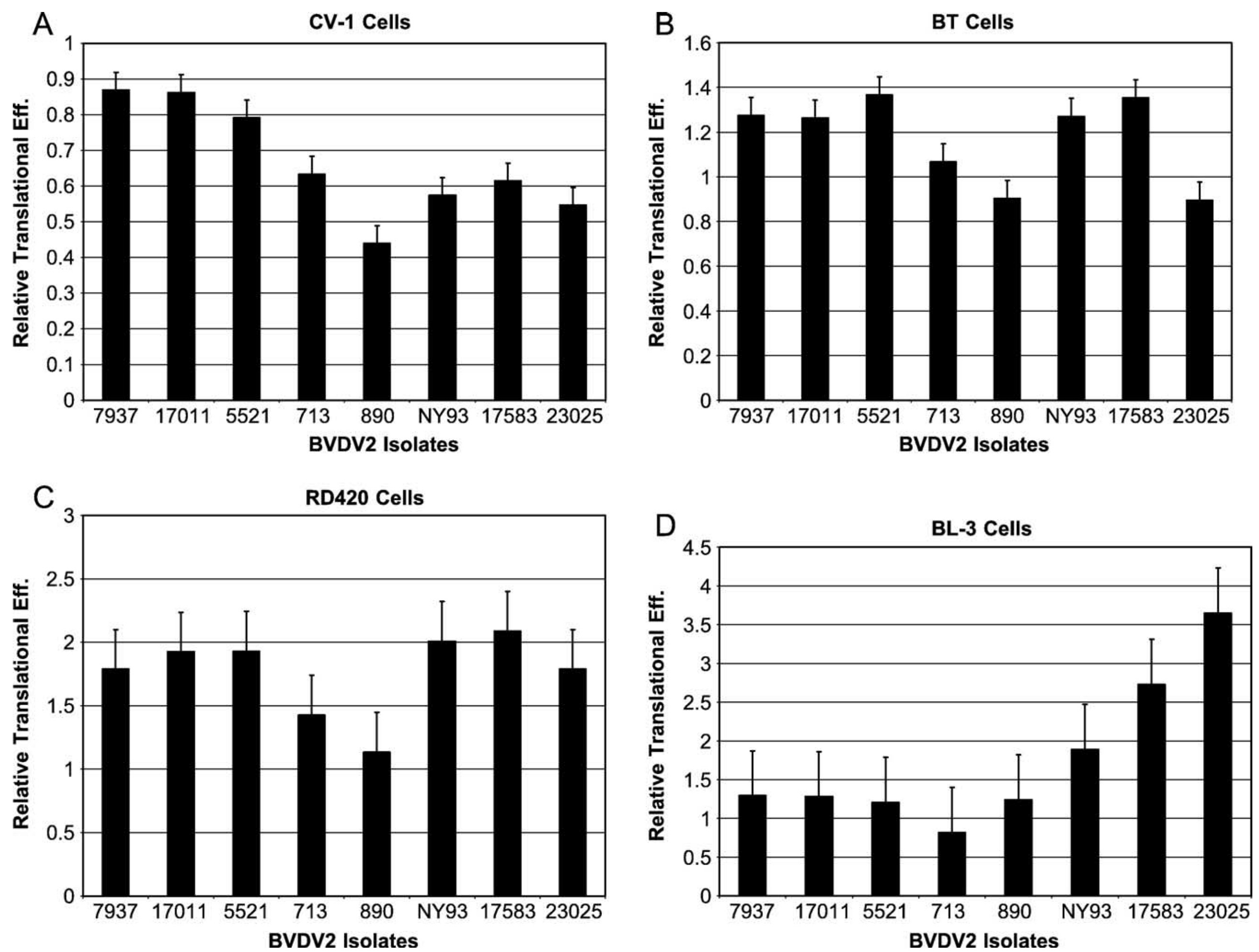

Figure 3. Relative translational efficiency of the 5′ UTR IRES from each BVDV2 isolate measured in vivo from the T7-promoter in different cell lines. The ratios calculated for the IRES elements of the BVDV2 isolates were normalized using the BVDV1-NADL IRES as 1 (or 100%). (A) CV-1 (African green monkey kidney) cells, (B) BT (bovine turbinate) cells, (C) RD420 (bovine testicular) cells and (D) BL-3 (bovine lymphocyte) cells.

versity of Glasgow, Glasgow, Scotland, UK) were used to create a phenogram showing the relationships of the 5′ UTRs of the eight BVDV2 isolates. Three distinct clustering relationships were identified. One cluster consisted of the high virulence isolates 890, NY93, 23025 and 17583, while a second cluster encompassed three of the four low virulence isolates (17011, 5521, 713). BVDV2 7937 is distantly related to each of the other two clusters.

Discussion

Using a bicistronic reporter expression system, we evaluated the in vitro and in vivo translational efficiency of eight BVDV2 5′ UTRs varying in virulence in RRL, and in primate and bovine cell lines. These BVDV2 isolates have been previously grouped into low and high virulence based upon clinical histories and experimental infections (Bolin and Ridpath, 1992, Kelling *et al.*, 1990, Kelling *et al.*, 2002 and Odeón *et al.*, 1999). The 5′ UTR of each of the eight BVDV2 isolates evaluated in this study has been previously characterized by sequence analysis and found to have greater than 92.5% sequence identity (Topliff and Kelling, 1998). Despite this high degree of identity, significant differences in translational efficiencies between isolates were observed in the different cell lines examined indicating the importance of subtle changes in the primary and secondary structures.

The high virulence isolates had a significantly ($P < 0.05$) increased translational efficiency compared to the low virulence isolates in the BL-3 cells, a bovine lymphocyte cell line. In BL-3 cells, three out of four high virulence isolates translated with greater efficiency than did the low virulence isolates. The BVDV2 isolates in our study, grouped as high virulence, were all recovered from clinical cases of fatal, peracute infections and have been shown experimentally to induce severe disease (Bolin and Ridpath, 1992, Kelling *et al.*, 2002 and Odeón *et al.*, 1999). BVDV is a lymphotropic virus having an affinity for leukocytes (Truitt and Schechmeister, 1973). The virus targets lymphoid organs throughout the body resulting in variable degrees of lymphocytolysis and lymphoid depletion. Kelling *et al.* (2002) com-

pared the virulence of 17011, 713, 5521, 23025, and 17583 in 6- to 9-month-old calves. The calves inoculated with 23025 and 17583 (high virulence isolates) developed more severe clinical disease characterized by fever, diarrhea, more severe lymphopenia and more severe lymphoid depletion and atrophy of the thymus, than calves inoculated with the low virulence isolates (17011, 713, 5521). The lesions reported by Kelling *et al.* (2002) are consistent with the results of other studies involving the high virulence isolates 890 (Bolin and Ridpath, 1992) and NY93 (Odeón *et al.*, 1999). Consistent with the behavior of the low virulence isolates, isolate 7937 caused only mild disease in experimentally infected gnotobiotic calves (Marshall *et al.*, 1996). Thus, the lymphotropism of BVDV and the observed higher translational efficiencies in bovine lymphocytes in the present study are consistent with the more severe disease induced by the high virulence BVDV2 isolates.

There was a significant difference ($P > 0.05$) in translational efficiencies between the low and high virulence isolates in all assays, with the exception of RD420 cells. In RRL, CV-1, and BT cells, where statistically significant differences between low and high virulence groups exist, the BVDV2 isolate with the greatest translational efficiency varied, but consistently belonged to the low virulence group. The least efficient isolate in RRL, CV-1, BT, and RD420 cells, was either 890 or 23025, members of the high virulence group. In the BL-3 cells, the high virulence isolate 23025 translated with the greatest efficiency, while the low virulence isolate 713 translated with the least efficiency.

BVDV infection and replication occurs in the cytoplasm of the target cell. In the T7-promoter system, transcription and translation occur totally within the cytoplasm, similar to that which occurs within the host cell during infection with BVDV. Thus, the results obtained using the T7-promoter system mimic translation in cells infected with BVDV.

The secondary structural analysis of our BVDV2 isolates demonstrated a well-conserved IRES element between the isolates, but minor differences in the secondary structure upstream from the IRES element. Our results and the reports in other viral systems (HCV and picornaviruses) support our hypothesis that relatively subtle differences in primary and secondary structures may have profound effects on translational efficiency (Buratti *et al.*, 1997, Collier *et al.*, 1998, Laporte *et al.*, 2003 and Martínez-Salas *et al.*, 1993).

Similar to what has been reported in HCV and picornaviruses, the difference in translation efficiencies of our BVDV2 isolates in different cells lines and in vitro strongly suggests that IRES activity is influenced by the interaction of the IRES element with eukaryotic initiation factors (eIFs) and IRES-specific cellular trans-acting factors (ITAFs) which may be distributed differently in different cell types (Ali *et al.*, 2000, Buratti *et al.*, 1998 and Pilipenko *et al.*, 2000). The observation that the translational efficiencies of the high virulence isolates were greater than the low virulence isolates in BL-3 cells, a bovine lymphocyte cell line, compared to that observed in the other cell lines, suggests that there may be eIFs and ITAFs unique to BL-3 cells that influence tissue tropism, thus affecting disease pathogenesis.

The effect of viral-specific proteins and/or the 3′ UTR on IRES-dependent translation efficiency was not evaluated in this study, but has been evaluated in other viral systems. Imbert *et al.* (2003) showed that HCV IRES-mediated translation was not altered by the genomic 3′ UTR alone or in the presence of HCV structural or nonstructural proteins. However, De Quinto *et al.* (2002) and Edgil *et al.* (2003) showed that the 3′ UTR of foot-and-mouth disease virus and dengue virus, respectively, did influence viral translation; therefore, we cannot rule out the potential for other genomic regions influencing the BVDV IRES function during the course of an infection.

The IRES element of BVDV2 isolates varying in virulence has variable translational activity depending on the cell line used. The results suggest that the difference in translational efficiency is dependent on the primary and secondary structure of the 5′ UTR, specifically the IRES element, acting alone and/or interacting with factors distributed differently in cell lines. The replication cycle of BVDV, like HCV (Kamashita *et al.*, 1997), is complex with translational efficiency being only one part of the complete replication cycle. As indicated by the studies mentioned above, numerous factors interact with one another and the IRES element to influence the translational efficiency of a virus. Individual cell lines containing different assortments and quantities of factors may influence BVDV IRES function, thus influencing tissue tropism and disease pathogenesis. Our data are in agreement with previous reports on HCV and picornaviruses demonstrating different translational activities in different cell lines. Evaluating the translational efficiencies of BVDV2 isolates varying in virulence provides a basis for understanding the pathogenesis of BVDV2 infection in cattle.

Materials and methods

Reporter plasmid construction

The pBiSpe plasmid was created by the insertion of a *Spe*I linker into the *Bam*HI site of pBi, a bicistronic reporter plasmid (Chon *et al.*, 1998). Each of the BVDV2 5′ UTRs was amplified and cloned into pCR2.1 as previously described (Topliff and Kelling, 1998). The 5′UTR of each BVDV2 isolate consisted of nucleotides 30 to 386, the authentic initiation codon. Nucleotides 10–29 of the 5′ UTR correspond to primer P5 based on the BVDV1-NADL sequence with a *Kpn*I restriction site incorporated five bases from the 5′ end. The 3′ end of the fragment consists of the primer P6 designed to include the first six amino acids of the BVDV1-NADL N^{pro} region as well as an *Xba*I restriction site incorporated six bases

from the 5′ end. Each of the BVDV2 5′ UTRs was excised from pCR2.1 using *Apa*I and *Spe*I and inserted into the *Apa*I–*Spe*I site of pBiSpe. The pBiSpe/BVDV2 plasmids have the 5′ UTR IRES inserted in the intercistronic region between the CAT and LUC coding regions. The authentic 5′ UTR initiation codon and the start codon of the LUC gene are separated by 27 amino acids, the first six amino acids contributing to the N^{pro} coding region of the BVDV1-NADL genome. The CAT-5′UTR-LUC region was then excised using *Hin*dIII, *Stu*I, and *Nde*I. The *Hin*dIII–*Stu*I fragment was then subcloned into pcDNA3.1 at the *Hin*dIII–*Eco*RV site. The resulting plasmids are referred to as pcDNA3.1/CATBVDV2LUC. A control plasmid, pcDNA3.1/Bi5′BVD was constructed by digesting pBi5′BVD (Chon *et al.*, 1998) with *Hin*dIII, *Sal*I, and *Nde*I and inserting the *Hin*dIII–*Sal*I fragment into pcDNA3.1 digested with *Hin*dIII–*Xho*I. The plasmid pcDNA3.1/Bi5′BVD contains the BVDV1 NADL-5′ UTR and N^{pro} region (Chon *et al.*, 1998).

DNA sequencing

The 5′UTR junction with the LUC coding region of pcDNA3.1/CATBVDV2LUC plasmids were sequenced using the dideoxy-chain termination method (Sequenase Version 2.0 DNA Sequencing Kit, USB Corporation, Cleveland, OH) and the primer LUC REV (5′-AGCTTCTGCCAACCGAACGGACA-3′), complementary to the LUC coding region 1554–1579 of pGEM-LUC (Promega). All plasmids were in the correct open reading frame with the LUC gene.

Cells and viruses

BT cells (bovine turbinate) were obtained from the National Veterinary Services Laboratory, USDA, Ames, IA. CV-1(African green monkey kidney, CCL-70), RD 420 (a bovine testicular cell line transformed with SV40) and BL-3 (bovine lymphocytes, CRL-8037) cells were provided by Dr. Ruben Donis, University of Nebraska, Lincoln, Nebraska. The BT, CV-1 and RD420 cell lines were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% equine serum. BL-3 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 50 μg/ml Gentamicin (Sigma Chemical Co., St. Louis, MO). All cell lines were incubated at 37 °C in a humidified incubator with 5% CO_2.

The BVDV2 isolates have been previously described (Topliff and Kelling, 1998). Recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3) was kindly provided by Dr. Bernard Moss, National Institutes of Health, Bethesda, MD.

In vitro transfection assay

In vitro transcription/translation was performed using the TNT T7 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's directions. Briefly, a reaction mixture consisting of: 1 μg of plasmid DNA, 25 μ1 TNT Rabbit Reticulocyte Lysate, 2 μ1 TNT Reaction Buffer, 1 μl TNT T7 RNA polymerase, 0.5 μl Amino Acid Mixture minus Leucine, 0.5 μl Amino Acid Mixture minus Methionine (1 mM), 1 μl RNasin Ribonuclease Inhibitor (40 U/μl) and nuclease-free water to a final volume of 50 μl was incubated at 30 °C for 90 min. Following incubation, the lysate was split into protein, LUC and CAT aliquots and stored at −80 °C. The protein concentration of the reactions was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). LUC and CAT assays were performed as described elsewhere (see Reporter Enzyme Analysis).

In vivo transfection assay

Cells were seeded onto 12-well tissue culture plates 24 h before transfection so as to reach 90–95% confluency at the time of transfection. Cells were infected with the recombinant vaccinia virus, vTF7-3, at an input multiplicity of 5, and incubated for 1 h. Following infection, the cells were then washed with DMEM without serum and transfected with 0.5 μg of each of the bicistronic plasmids using a cationic-liposome reagent (LipofectAMINE PLUS, Invitrogen, Carlsbad, CA) according to the manufacturer's suggestions and incubated at 37 °C and 5% CO_2 for 2 h. Following transfection, the medium containing the DNA–lipid complexes was removed and the cells supplemented with 1 ml DMEM and 10% equine or fetal bovine serum and incubated for an additional 18 h. Each transfection experiment was repeated a minimum of three times.

Reporter enzyme analysis

Cell extracts were prepared using a 1× reporter lysis buffer (Promega) according to the manufacturer's directions. Protein concentration of duplicate cell lysate samples was determined using the BCA Protein Assay Kit (Pierce) according to the manufacturer's directions. Samples for CAT and LUC activities were normalized to the amount of protein measured in the cell extracts. CAT activity was determined by the liquid scintillation assay as directed by the manufacturer in duplicate reaction mixtures (Promega). Briefly, duplicate reaction mixtures containing cell extract, ^{14}C-chloramphenicol, N-butyryl-Coenzyme A in a total volume of 125 μl was incubated at 37 °C for 90 min. The reactions were terminated by the addition of 300 μl of mixed xylenes (Aldrich Chemical Co., Milwaukee, WI). Following centrifugation, the entire upper phase (xylene) was transferred to a fresh tube and back extracted again using 100 μl of 0.25M Tris–HCl, pH 8.0. Following the second back extraction, 200 μl of the upper xylene phase was transferred to a scintillation vial containing 5 ml of scintillation fluid and the counts per minute were measured in

the duplicate samples. Luciferase enzyme activity was measured using a Luciferase Assay System (Promega) according to manufacturer's directions. Light emission of duplicate samples was determined with a TOP Count plate luminometer.

Western blot analyses

A portion of the in vitro transcription/translation samples were separated by discontinuous SDS-PAGE electrophoresis, transferred onto a nitrocellulose membrane and proteins labeled with rabbit anti-CAT antiserum (Invitrogen) or goat anti-LUC antibody (Promega), followed by alkaline phosphatase-labeled goat anti-rabbit IgG (H&L) or rabbit anti-goat IgG (H and L) (Vector Laboratories, Burlingame, CA), respectively, as the secondary antibody. Antibody binding was detected using 5-bromo-4-chloro-3-indolyle-phosphate/nitroblue tetrazolium substrate (KPL, Gaithersburg, MD).

Computer and statistical analyses

A folding algorithm, MFOLD (GCG Software Package, Version 10.2, Genetics Computer Group) (Zuker, 1989) was used to predict the optimal secondary structure of the eight BVDV2 5′ UTRs. The evolutionary tree was predicted using PHYLIP (Phylogeny Inference Package, Version 3.6a3, J. Felenstein, University of Washington) and viewed using TREEVIEW (TreeView, Version 1.6, R. D. M. Page, University of Glasgow).

Relative translational efficiencies of in vitro and in vivo IRES activities of each isolate was tested for statistical significance ($P \leq 0.05$) across the isolates using analysis of variance (ANOVA) for a randomized complete block experimental design, mean comparisons and contrasts.

Acknowledgments

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