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Insertion and stable expression of *Gaussia* luciferase gene by the genome of bovine viral diarrhea virus

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ABSTRACT

As a tool to address selected issues of virus biology, we constructed a recombinant cDNA clone of bovine viral diarrhea virus (BVDV) expressing *Gaussia* luciferase (Gluc) reporter gene. A full-length genomic cDNA clone of a non-cytopathic BVDV isolate was assembled by recombination in yeast *Saccharomyces cerevisiae*. The Gluc gene was inserted between the N^{pro} and Core protein coding regions by recombination. The cDNA transcribed *in vitro* was infectious upon transfection of MDBK cells, resulting in reporter gene expression and productive virus replication. The rescued viruses were stable for 15 passages in cell culture, maintaining the replication kinetics, focus size and morphology similar to those of the parental virus. Expression and correct processing of the reporter protein were also maintained, as demonstrated by Gluc activity. These results demonstrate that genes up to 555 bp are simply assembled by a single step in yeast recombination and are stably expressed by this cDNA clone.

1. Introduction

Bovine viral diarrhea virus (BVDV) is a small, enveloped, positive sense RNA virus belonging to the genus *Pestivirus*, family *Flaviviridae* (Lindenbach and Rice, 2001). The BVDV genome is a linear single stranded RNA molecule of approximately 12.3 kilobases. The genome contains a single long open reading frame (ORF) that encodes a polyprotein of approximately 4000 amino acids (Donis, 1995). The viral RNA genome is directly translated by host cell ribosomes in a *cap* independent fashion, dependent on a 5′-UTR internal ribosomal entry site (IRES). Translation of the genomic RNA produces a polyprotein that is co- and post-translationally cleaved by cellular and viral encoded proteases, giving rise to eleven mature proteins: NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2/3-NS4A-NS4B-NS5A-NS5B-COOH (Collett et al., 1988).

BVDV is an important pathogen of cattle and produces important losses to the livestock industry worldwide (Ridpath, 2010). Infection of seronegative cattle with BVDV may result in a variety of clinical manifestations ranging from subclinical infection to a fatal disease called mucosal disease (Baker, 1995). Respiratory or gastroenteric disease, thrombocytopenia, hemorrhagic disease and immunosuppression-associated syndromes are commonly associated with BVDV infection (Bolin and Grooms, 2004). Infection of pregnant cows may result in embryonic or fetal death, abortions, congenital malformations and the birth of weak and unthrifty calves (Bolin and Grooms, 2004). Fetal infection between days 40 and 120 of gestation frequently results in the birth of immunotolerant, persistently infected (PI) calves. PI animals are the main carriers and shedders of BVDV in nature and represent the key point in the epidemiology of the infection (McClurkin et al., 1984). Field BVDV isolates present high genetic and antigenic variability and are classified into two species, BVDV-1 and BVDV-2. The two species are distinguished mainly by differences in the 5′-UTR of the genome and on the diversity in the major envelope glycoprotein E2 (Kümmerer and Meyers, 2000). BVDV isolates may also be divided into two biotypes: cytopathic (cp) and non-cytopathic (ncp) viruses, based on the effects of virus replication in cell cultures (Gillespie et al., 1960).

Ncp-BVDV is responsible for most infections in the field and is the only biotype to establish persistent fetal infection (Bolin and Grooms, 2004). A small proportion of field isolates contain a mixture of ncp and cp viruses, the latter being generated from the ncp virus by diverse genetic mechanisms, all leading to the expression of NS3 as a separate polypeptide. In contrast, ncp viruses express NS3 as a COOH-terminal third of the NS2/3 protein (Kümmerer and Meyers, 2000; Ridpath, 2005; Tautz et al., 1994). Thus, ncp isolates are considered the *"true"* BVDV since they are responsible for most infections and associated with the main clinical and reproductive consequences of BVDV infection (Bolin and Grooms, 2004).

The biology of persistent fetal infection remains a major issue on BVDV biology and has been subject of extensive investigations

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in recent decades. In this sense, the development of reverse genetics for pestiviruses (Moormann et al., 1996) and for BVDV (Meyers et al., 1996) paved the way for a number of studies concerning different aspects of pestivirus biology. A number of BVDV cDNA clones have been constructed and used to investigate several aspects of virus replication, pathogenesis and interactions with the host immune system (Dehan et al., 2005; Fan and Bird, 2008b; Gil et al., 2006; Harding et al., 2002; Henningson et al., 2009; Kümmerer and Meyers, 2000; Meyer et al., 2002; Meyers et al., 2007; Reimann et al., 2003; Vassilev et al., 1997).

Most recombinant BVDV clones were constructed by the assembly of full genome length cDNA in bacterial plasmid vectors. The bacterial artificial chromosome (BAC) strategy was recently used to generate a full-length cDNA copy of the BVDV SD-1 strain, which seemed to be more stable in *Escherichia coli* (Fan and Bird, 2008a, 2008b). We recently described the construction of a chimeric BVDV cDNA clone by homologous recombination in yeast method (*Saccharomyces cerevisiae*) (Arenhart et al., submitted for publication). This strategy has been shown to overcome the problem of instability of some flavivirus genomes in *E. coli* (Polo et al., 1997; Puri et al., 2000). Using this method, a chimeric full-length cDNA clone containing the entire ORF of a representative Brazilian BVDV-1 strain (IBSP4ncp) flanked by the 5′ and 3′-UTRs of the reference BVDV strain NADL was assembled. In the present article we demonstrate that this clone can be manipulated easily and that foreign genes up to 555 bp may be inserted and expressed by the recombinant BVDV genome, thus representing a powerful tool to address many aspects of virus biology.

2. Materials and methods

A recombinant BVDV cDNA clone expressing the *Gaussia* luciferase (Gluc) reporter gene was constructed by homologous recombination in yeast, using the previously constructed cDNA clone IC-pBSC_IBSP4ncp#2 as the parental vector. The Gluc gene (555 bp) was introduced between the genes encoding the N^{pro} and Core viral proteins, along with a linker and the foot and mouth disease virus protease 2A gene ($FMDV2A^{pro}$). The viruses rescued from the recombinant cDNA clones – after *in vitro* transcription and RNA transfection into MDBK cells – were characterized concerning their biological properties and Gluc expression.

2.1. Cells, viruses and plasmid vectors

Pestivirus-free MDBK cells (Madin–Darby bovine kidney, ATCC CCL22) were used for all procedures of virus multiplication, characterization and quantification. Cells were maintained in MEM (minimal essential medium – Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% equine serum, penicillin (100 U/mL) and streptomycin (10 μg/mL, GIBCO, BRL). Cell cultures were maintained at 37 °C with 5% CO_2. The recombinant cDNA clone IC-pBSC_IBSP4ncp#2 (Arenhart et al., submitted for publication) was used as the parental clone. The plasmid pGluc-NS (WF10) containing the *Gaussia princeps* luciferase gene was kindly provided by Dr. Daniel R. Perez (Department of Veterinary Medicine, University of Maryland, USA). The virus chi-NADL/IBSP4ncp#2 rescued from the parental clone was used as the control in the characterization of viruses expressing the Gluc reporter gene.

2.2. Fluorescent antibody (IFA) and peroxidase (IPX) assays

Indirect fluorescent antibody assays (IFA) for BVDV antigens were performed on MDBK cells that had been deposited on to glass coverslips, left to attach and fixed in cold acetone. Fixed cells were incubated for 1 h at 37 °C with a pool of monoclonal antibodies (MAbs) to BVDV (MAb 15c5 against E0; MAb 12g4 to E2; MAb 20.10.6 to NS3) (Corapi et al., 1989) as primary antibodies, followed by washing in PBS and incubation with an anti-mouse IgG FITC-conjugated secondary antibody (1:100 in PBS; Sigma-Aldrich). Slides were examined under UV light in an epifluorescence microscope (Zeiss).

Immunoperoxidase (IPX) staining for BVDV antigens was performed in cell monolayers grown in 6-well plates and inoculated with dilutions of the respective viruses. Acetone-fixed cell monolayers were incubated with the same MAbs described above (1 h at 37 °C), washed and incubated with an anti-mouse HRPO-conjugated antibody (1:1000 in PBS; Sigma-Aldrich). After washing, HRPO substrate (AEC, aminoethylcarbazole in acetate buffer 50 mM, pH 5.0) was added to cell monolayers and incubated for an additional hour at 37 °C. Viral foci were visualized under light microscopy.

2.3. Assay for Gluc activity

The expression and activity of the Gluc reporter gene was detected through a *Gaussia* luciferase assay performed on the supernatants of MDBK cells transfected with RNA obtained by *in vitro* transcription of recombinant clones and from cells inoculated with the progeny viruses at different passages, as described below. Gluc activity was measured using a BioLux *Gaussia* luciferase Assay Kit (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocol. Briefly, 10 μL of supernatant was mixed with 50 μL of 1× Biolux buffer and read in a luminometer Mithras LB 940 (Berthold). The results were expressed as RLU (Relative Light Units) and mock-infected MDBK cells were used as negative control.

2.4. Construction of a BVDV cDNA clone expressing the Gaussia luciferase gene

The recombinant BVDV cDNA clone IC-pBSC_IBSP4ncp#2 was used as platform to construct the recombinant genome expressing the *Gaussia* luciferase (Gluc) gene. The cDNA clone IC-pBSC_IBSP4ncp#2 contains the entire ORF of a Brazilian ncp BVDV isolate (IBSP4ncp, GenBank accession number KJ620017) flanked by the 5′ and 3′-UTRs of NADL strain (Arenhart et al., submitted for publication). The recombinant BVDV IBSP-4ncp cDNA was assembled according to a strategy described by Fan et al. (2008), who introduced the gene eGFP2A between the genes N^{pro} and Core of the BVDV SD-1. All manipulations of the plasmid IC-pBSC_IBSP4ncp#2 were performed by homologous recombination in yeast. The strategy of construction is depicted in Fig. 1.

2.5. Preparation of the vector for recombination

Plasmid IC-pBSC_IBSP4ncp#2 was digested with SacI (New England BioLabs, Ipswich, MA, USA) removing part of the N^{pro} and NS2 genes and intervening sequences. Four μg of plasmid DNA was digested at 37 °C during 3 h followed by dephosphorylation with 5 U of the enzyme CIAP (New England Laboratories) for 45 min at 37 °C. The final product was resolved in an ethidium bromide stained 1% agarose gel followed by excision of the band and purification with QIAquick Gel Extraction kit (Qiagen).

2.6. PCR reaction for construction of the recombinant

The recombinant clone IC-pBSC_IBSP4ncpGluc was assembled by yeast recombination of three PCR products containing homologous ends, and the vector IC-pBSC_IBSP4ncp#2 digested with SacI. The first PCR fragment (561 bp) was amplified with oligonucleotides BVDVqm 5′-UTR_NADL_IBSP4-F and IBSP-4/Npro_linker_Gluc-R, from the plasmid IC-pBSC_IBSP4ncp#2, which contains part of N^{pro} and a linker sequence of 21 nt N-terminal of Core protein gene modified and optimized. The second fragment (632 bp) was