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Use of homologous recombination in yeast to create chimeric bovine viral diarrhea virus cDNA clones

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ABSTRACT

The open reading frame of a Brazilian bovine viral diarrhea virus (BVDV) strain, IBSP4ncp, was recombined with the untranslated regions of the reference NADL strain by homologous recombination in *Saccharomyces cerevisiae*, resulting in chimeric full-length cDNA clones of BVDV (chi-NADL/IBSP4ncp#2 and chi-NADL/IBSP4ncp#3). The recombinant clones were successfully recovered, resulting in viable viruses, having the kinetics of replication, focus size, and morphology similar to those of the parental virus, IBSP4ncp. In addition, the chimeric viruses remained stable for at least 10 passages in cell culture, maintaining their replication efficiency unaltered. Nucleotide sequencing revealed a few point mutations; nevertheless, the phenotype of the rescued viruses was nearly identical to that of the parental virus in all experiments. Thus, genetic stability of the chimeric clones and their phenotypic similarity to the parental virus confirm the ability of the yeast-based homologous recombination to maintain characteristics of the parental virus from which the recombinant viruses were derived. The data also support possible use of the yeast system for the manipulation of the BVDV genome.

Introduction

Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle, belonging to the genus *Pestivirus* in the family *Flaviviridae*. Pestiviruses are small (40–60 nm) enveloped viruses containing a single-stranded, positive-sense RNA genome of approximately 12.3 kb. The BVDV RNA genome contains a single, long open reading frame (ORF) encoding a polyprotein of approximately 4000 amino acids.[1] The

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ORF is flanked by 5′ and 3′ untranslated regions (UTRs) of approximately 385 and 229 nucleotides (nt), respectively.[2] The viral RNA genome is uncapped at its 5′ end, and translation by host-cell ribosomes occurs through recognition of the internal ribosomal entry site (IRES), a tertiary structure located within the 5′ UTR. Direct translation of the viral genome gives rise to a polyprotein that is cleaved by cellular and viral-encoded proteases, resulting in following proteins: NH_2–(N^{pro}–C–E^{rns}–E1–E2–p7–NS2/3–NS4A–NS4B–NS5A–NS5B)–COOH.[1]

Field BVDV isolates are classified into two genotypes, BVDV-1 and BVDV-2, based on the differences in the 5′ UTR and on the diversity of glycoprotein E2.[3] In addition to the recognized species, an additional *Pestivirus* species have been proposed: Hobi-like or BVDV-3.[4] Most field isolates of both genotypes are non-cytopathic (ncp) for cultured cells. Non-cytopathic isolates are responsible for most infections in the field and are associated with the main clinical and reproductive consequences of BVDV infection.[5]

The development of reverse genetics tools for pestiviruses in the late 1990s facilitated studies concerning many aspects of BVDV biology[6] and its interactions with the host,[7] as well as the development of vaccine strategies.[8] After the pioneer description of a recombinant cDNA clone of the pestivirus classical swine fever virus by Moormann et al.[9] and the first BVDV clone (CP7) by Meyers et al.[10] in 1996, a number of BVDV cDNA clones have been constructed, including NADL, Oregon, SD-1, NY'93, Pec515, and 890,[11–13] among others. Molecular strategies to generate infectious BVDV from cDNA were mainly derived from the classical method described for flaviviruses by Rice et al.,[14] based on the assembly of full-genome-length cDNA in bacterial plasmid vectors. Instability of full-length pestiviruses cDNA in bacterial hosts has been partially overcome by the use of low-copy-number plasmids, but not yet solved.[13,15,16] To improve this system, a bacterial artificial chromosome strategy (BAC) was used to generate full-length cDNA copy of pestiviruses, which seemed to be more stable during passages in *Escherichia coli*.[17–19]

This study describes the construction of chimeric BVDV cDNA clones by homologous recombination in yeast (*Saccharomyces cerevisiae*). This strategy has several applications and has been used, in particular, to overcome the problem of instability of some flavivirus cDNA clones in *E. coli*.[20] Using this easy and simple method, chimeric full-length cDNA clones containing the 5′ and 3′ UTRs of the BVDV-1 reference strain NADL and the entire ORF of a representative Brazilian BVDV-1 strain, IBSP4ncp,[21] were successfully constructed through just one-step assembly.

Materials and methods

Cells and viruses

Pestivirus-free Madin-Darby bovine kidney (MDBK) cells (ATCC CCL-22) were used for all viral manipulation procedures. Cells were maintained in minimal essential medium (MEM, Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% equine serum, 1% penicillin (10,000 UI/mL stock), and streptomycin (10,000 μg/mL stock) (Gibco, Langley, OK, USA) at 37 °C, 5% CO_2. MDBK cells were monitored for pestiviruses contamination by indirect fluorescent antibody assay (as described in Section 'Fluorescent antibody and peroxidase assays') before and during all the experiments. The virus used for the construction was a Brazilian non-cytopathic BVDV-1b strain, IBSP4ncp (GenBank accession number KJ620017).[22]

Oligonucleotides and plasmid vector

Oligonucleotides for amplification and homologous recombination (Table 1) were initially designed based on the sequences of conserved regions of reference BVDV-1 strains, available in the GenBank. The sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Additionally, some oligonucleotides were designed to add 25 nt to the termini of amplicons for homologous recombination in yeast (Table 1). The yeast/*E. coli* shuttle vector pBSC_NADL_HDR containing the whole genome of the BVDV reference strain NADL was kindly provided by Dr. Ruben O. Donis (CDC Influenza Division, Atlanta, GA, USA).

Fluorescent antibody and peroxidase assays

An indirect fluorescent antibody (IFA) assay was performed using MDBK cells deposited over glass coverslips. Acetone-fixed cells were incubated for 1 h at 37 °C with a pool of monoclonal antibodies (MAbs) to BVDV (15c5, 12g4, and 20.10.6),[23] followed by washing in phosphate-buffered saline

Table 1 – Oligonucleotides used on the construction of the recombinant cDNA clone of BVDV strain IBSP4ncp by homologous recombination.

Oligonucleotide	Sequence
1 – BVDVqm 5′UTR_NADL_IBSP4-F[a]	**CTAAAAATCTCTGCTGTACATGGCAC**ATGGAGTTGATTGCAAATGAAC
2 – BVDV-Osloss-4458R[b]	TGAGGGGCAAGAGTATGCTGAC
3 – BVDV1-4121F	ACYATMCCRAACTGGAGRCCAC
4 – BVDV1-8893R	CATCTCATAGCCACATGGGCAC
5 – BVDV-Osloss-8520F	TTGAAGCAGTYCAGACAATTGG
6 – BVDVqm 3′UTR_NADL_IBSP4-R	**ATTTATTTACAATATATACATTTTGTCT**CAACTGCTGGCACCGACAG

Oligonucleotides are identified according to the sequences they amplify/possess. The homologous sequences for recombination are in bold.

[a] F –sense.

[b] R – antisense.