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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.

(PBS) and incubation with an anti-mouse IgG fluorescein isothiocyanate-conjugated secondary antibody (1:100 in PBS; Sigma–Aldrich). Slides were examined under ultraviolet light in a DMI 4000B fluorescence microscope (Leica, Wetzlar, Germany). Immunoperoxidase (IPX) staining for BVDV antigens was performed using cell monolayers grown in six-well plates and inoculated the respective viruses. Cell monolayers were fixed in 30% cold acetone by 13 min and incubated with the same MAbs for IFA (1 h at 37 °C), washed, and incubated with an anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:1000 in PBS; Sigma–Aldrich). After washing, an HRP substrate (aminoethylcarbazole – AEC, in acetate buffer 50 mM – pH 5.0 and hydrogen peroxide 0.2%) was added and incubated for approximately 30 min at 37 °C.

Construction of BVDV cDNA clones by homologous recombination in yeast

Recombinant cDNA clones, pBSC_IBSP4ncp, were constructed by homologous recombination in yeast using three reverse transcription-polymerase chain reaction (RT-PCR) products encompassing the IBSP4ncp ORF and a *Bam*HI-digested pBSC_NADL_HDR vector. After the digestion, the NADL 5′ and 3′ UTRs in the vector remained intact. The strategy of cloning is depicted in Fig. 1.

Amplification of the IBSP4ncp ORF

Viral RNA was extracted from the supernatant of MDBK cells infected with the IBSP4ncp BVDV strain, using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). RT was performed in a 20-μL reaction using 50 ng of the RNA template and 200 U of Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified with KlenTaq-LA polymerase (Clontech, Mountain View, CA, USA). The entire IBSP4ncp ORF was PCR-amplified in three fragments. The first PCR fragment (4081 bp) was amplified with oligonucleotides 1 and 2, the second fragment (4466 bp) was amplified with oligos 3 and 4, and the third fragment (3360 bp) was amplified with oligos 5 and 6 (Table 1). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles (denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min for each 1000 bp), and final extension at 72 °C for 10 min.

Yeast transformation and identification of recombinant clones

The plasmid pBSC_NADL_HDR was digested with *Bam*HI (New England BioLabs, Ipswich, MA, USA) to remove the NADL ORF, leaving intact the 5′ and 3′ UTRs (Fig. 1), followed by dephosphorylation with 5 U of calf-intestinal alkaline phosphatase (New England BioLabs). The three PCR products corresponding to the whole IBSP4ncp ORF plus the *Bam*HI-digested vector pBSC-NADL-HDR were introduced simultaneously into the yeast *S. cerevisiae* strain RFY206 using the lithium acetate method.[24] After transformation, yeast cells were inoculated in YNB solid medium (Yeast Nitrogen Base – Sigma–Aldrich) without tryptophan and maintained at 30 °C for up to three days. Five recombinant yeast colonies were chosen, picked and amplified in YNB medium. Plasmid DNA was extracted from yeast spheroplast pellets using QIAprep Spin MiniPrep kit (Qiagen). The presence of cloned fragments was confirmed by PCR, followed by nucleotide sequencing. After the confirmation, two in three recombinant clones (pBSC_IBSP4ncp#2 and pBSC_IBSP4ncp#3) were further amplified and characterized.

Nucleotide sequencing

The constructed cDNA clones, pBSC_IBSP4ncp#2 and #3, and full-length PCR products obtained from both clones were submitted for complete nucleotide sequencing. The parental virus IBSP4ncp and a chimeric virus, chi-NADL/IBSP4ncp#2, derived from the corresponding pBSC_IBSP4ncp#2 cDNA clone and recovered at passages 0 (p0, rescued virus) and p5 were submitted for nucleotide sequencing of the whole genomes. All viral sequences were amplified from supernatant of infected cells. Sequencing reactions were performed using the mix BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer. Products of sequencing were resolved in an ABI 3100 Genetic Analyzer sequencer (Applied Biosystems). Sequence analyses were conducted by using the package Lasergene® (DNAstar Inc.) and a consensus sequence of each virus was generated by the program SeqMan II (Lasergene®, DNAstar Inc.).

Full-genome PCR amplification and in vitro transcription

A PCR was performed to amplify and linearize the entire genomes of the chimeric viruses including the

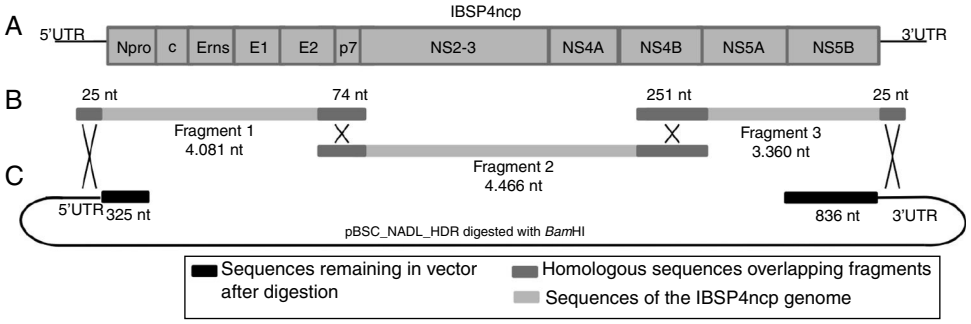

Fig. 1 – Representation of the strategy of construction of the chimeric cDNA clones of BVDV strain IBSP4ncp. (A). Genome organization of BVDV IBSP4ncp. (B). Homologous recombination in yeast of three PCR products (fragments 1, 2 and 3) and (C). *Bam*HI digested pBSC_NADL_HDR vector.

sequence of the bacteriophage T7 RNA polymerase promoter for *in vitro* transcription. For PCR amplification, the reaction mixture contained 100 ng of DNA of a recombinant clone (pBSC_IBSP4ncp#2 or #3), 1 U of KlenTaq-LA DNA polymerase mix (Clontech), 0.5 U of Vent DNA polymerase (Sigma–Aldrich), and oligonucleotides (pBSC-T7-NADL-F and NADL-3′UTR-R, Table 1). The PCR products were phenol-chloroform-extracted and ethanol-precipitated prior to performing *in vitro* transcription using the MEGAscript® T7 kit (Ambion, Foster City, CA, USA).

Transfection and monitoring of infectivity of in vitro-transcribed RNAs

For transfection, MDBK cells were trypsinized, washed with serum-free MEM, then washed twice with PBS, pH 7.2/diethylpyrocarbonate at 4 °C, and resuspended at 8×10^6 cells/mL in 400 μL of Cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES, 2 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP, 5 mM glutathione, and pH adjusted to 7.6 with KOH). Electroporation was performed using 5 μg of transcribed RNA as described by Qu et al.[25] Cell cultures were maintained for three days and monitored daily for BVDV antigens by the IFA assay. The presence of infectious virus in the supernatants of the transfected MDBK cultures was confirmed by inoculating fresh MDBK cell cultures with aliquots of the supernatants, followed by the IFA assay 72 h later. Viruses recovered from the supernatants of the transfected cells were designated chi-NADL/IBSP4ncp#2 and chi-NADL/IBSP4ncp#3, passage 0 (p0).

Characterization of viruses derived from cDNA clones

Infectivity and stability in cell culture

The chimeric viruses (chi-NADL/IBSP4ncp#2 and chi-NADL/IBSP4ncp#3) were subjected to 10 passages in MDBK cells. Cells were inoculated with the viruses at a multiplicity of infection (MOI) of 0.5 for each passage and incubated at 37 °C, 5% CO_2 for 72 h. At the end of each passage, the supernatants were tested by the IFA assay, and virus quantification was performed by a plaque assay.

Kinetics of virus replication and focus assays

The viruses chi-NADL/IBSP4ncp#2 and #3 at p5 and the parental virus IBSP4ncp were individually inoculated into MDBK cell cultures at an MOI of 0.3 each. The inoculum was removed, and the cell monolayers were washed with MEM, followed by the addition of fresh culture medium and incubation at 37 °C, 5% CO_2. Aliquots of the culture supernatants were harvested at different times (0, 8, 16, 24, 32, 40, 48, 56, 64, and 72 h), and viruses were quantified by limiting dilution. To examine the size and morphology of infectious foci produced by the recombinant viruses at p5 and by the parental virus IBSP4ncp, 90% confluent MDBK cell monolayers grown in six-well plates were inoculated with viral dilutions (10^{-1} to 10^{-7}). After 1 h adsorption at 37 °C, the viral inoculum was removed, and the cell monolayers were washed, overlaid with 1% agarose in MEM plus 5% equine serum, and incubated at 37 °C, 5% CO_2. After 72 h, the monolayers were fixed and subjected to IPX staining as described above.

Stability of recombinant yeast cDNA clones in E. coli

The recombinant plasmids pBSC_IBSP4ncp#2 and #3 were electroporated into *E. coli* DH10B (Invitrogen) using an ECM 830 BTX electroporator. The electroporation conditions were as follows: 2.75 kV, 99 μs, 5 pulses, and 1 s between pulses. Colonies were grown at 37 °C in Luria-Bertani medium with chloramphenicol (20 μg/mL) at 37 °C for 18–20 h. Plasmid DNA was then purified with the QIAGEN Plasmid Midi Kit (Qiagen) and used as a template for *in vitro* transcription. MDBK cells were electroporated with the RNA as described above. Infectivity of the RNA was accessed by the IFA assay performed at 24, 48, and 72 h after the transfection. Progeny viruses were harvested and subjected to five passages in MDBK cells, with the infectivity monitored at each passage by the IFA assay.

Results

Construction of chimeric NADL-IBSP4ncp cDNA clones

Recombinant cDNA clones containing the entire ORF of the Brazilian BVDV strain IBSP4ncp and the UTRs of the NADL strain were successfully constructed by homologous recombination in yeast. The correct recombination of the NADL UTRs and the IBSP4ncp ORF was confirmed by nucleotide sequencing of the UTR-ORF junctions. Two recombinant viruses were recovered for further characterization (chi-NADL/IBSP4ncp#2 and chi-NADL/IBSP4ncp#3).

Infectivity and stability of RNA transcribed in vitro from recombinant cDNA clones

To access the infectivity of RNA derived from the cloned BVDV genome, the plasmid DNA of the clones pBSC_IBSP4ncp#2 and #3, isolated from yeast cells, was initially subjected to a PCR encompassing the entire viral genome. The amplicons were used as templates for *in vitro* transcription, and the transcribed RNAs were then used for transfection of MDBK cells. Viral antigens were detected in the MDBK cells transfected with the transcribed RNA of both clones at 24 h post-transfection by the IFA assay. The transfection resulted in efficient virus recovery for both clones, with a mean P0 titer of $10^{4.5}$ plaque-forming units (PFU)/mL at 72 h after the transfection (data not shown). The viruses recovered from the transfected cell cultures were named chi-NADL/IBSP4ncp#2 and chi-NADL/IBSP4ncp#3. To confirm the presence of infectious progeny viruses, the supernatants collected from these cultures were inoculated to fresh MDBK cells, followed by the IFA assay for viral antigens 72 h later (data not shown). The stability of the chi-NADL/IBSP4ncp#2 and #3 viruses in MDBK cells was confirmed during 10 passages (Fig. 2A). At all tested passages, the viruses reached titers of approximately $10^{5.5–6.0}$ PFU/mL at 72 h post-infection (data not shown).

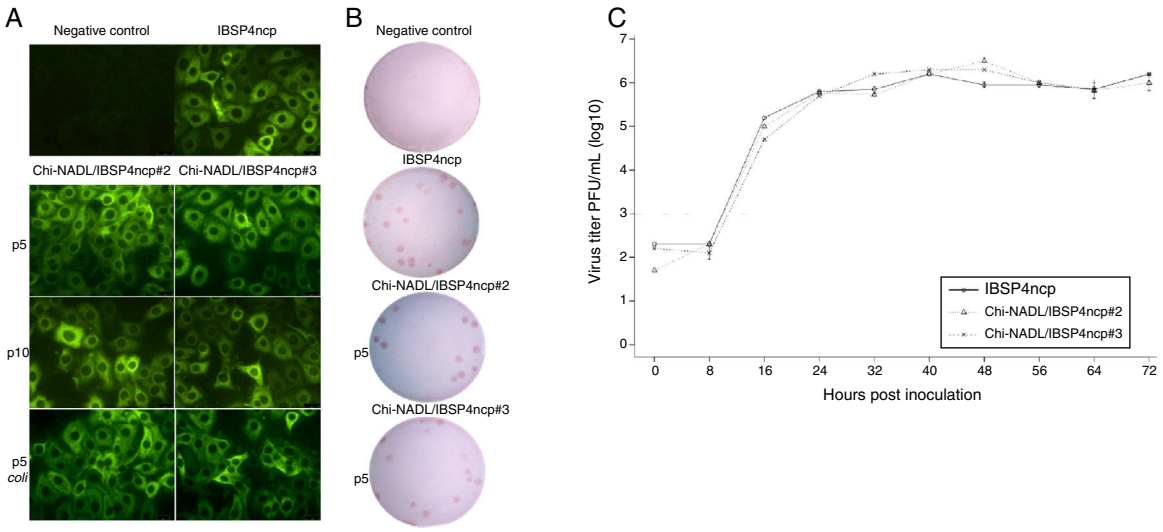

Fig. 2 – Infectivity of recombinant viruses. (A) IFA for viral antigens. Negative control: mock-infected MDBK cells; positive control: MDBK cells inoculated with the parental strain; chi-NADL/IBSP4ncp #2 and #3 virus. And the same viruses (# 2 and 3) recovered from plasmid DNA amplified in *E. coli* (p5). (B) IPX for viral antigens. Morphology of infectious foci produced in MDBK cells by viruses rescued at p5. (C) Replication curve of viruses recovered at p5 and parental virus. MDBK cells were inoculated with the respective viruses at an MOI of 0.3 and supernatants collected at different intervals were submitted to virus quantification by IPX. Each value represents the mean of two independent experiments.

Replication kinetics, focus size, and morphology

Confluent MDBK monolayers were inoculated with each virus at an MOI of 0.3, and the culture supernatants collected at different intervals were titrated for viruses. Both chi-NADL/IBSP4ncp#2 and #3 replicated to similar levels, and the kinetics was indistinguishable from that of the parental strain (Fig. 2C). Then, we investigated using IPX staining the morphology and size of the plaques/foci produced on MDBK cells by chi-NADL/IBSP4ncp#2 and #3 at p5 and compared the features with those of the parental strain. The size and morphology of the infectious foci produced by chi-NADL/IBSP4ncp#2 and #3 were indistinguishable from those of the foci produced by the parental virus (Fig. 2B).

Genome sequencing

We chose one clone (chi-NADL/IBSP4ncp#2) to investigate for possible mutations in the viral genome. At p0, one silent mutation was detected in the N^{pro} gene, and another mutation was detected in the E1 gene, but the latter reverted to the original nucleotide at p5. At p5, a few additional mutations were detected in the first ORF-encoded protein, N^{pro}. Among those, three single-nucleotide changes (nt 463, 499, and 514) did not result in amino acid changes, while one resulted in a substitution by an amino acid from the same chemical group (Glu550Asp). Besides, one mutation (Ser12079Asn) was detected in the NS5B gene, resulting in a substitution by an amino acid from a different chemical group. The mutations are shown in Table 2.

Stability of cDNA clones in E. coli

To access the stability of the recombinant cDNA clones in bacteria, the plasmids pBSC_IBSP4ncp#2 and #3 were amplified in *E. coli*. Plasmid DNA was extracted from the bacterium, amplified by PCR, and used as a template for *in vitro* transcription. The RNAs transcribed from these amplicons were

Table 2 – Analysis of sequences of the virus recovered from the cDNA clone and parental virus.

Localization	Nucleotide[a]	IBSP4ncp	Chi-NADL/IBSP4ncp#2 passage 0	Chi-NADL/IBSP4ncp#2 passage 5	Name or type of mutation
N^{pro}	439	T	C	C	Silent
N^{pro}	463	C	C	T	Silent
N^{pro}	499	G	G	A	Silent
N^{pro}	514	A	A	G	Silent
N^{pro}	550	A	A	T	Glu550Asp
E1	2295	A	C[b]	A	–
NS5B	12,079	G	G	A	Ser3898Asn

[a] Nucleotide and amino acids positions correspond to those of the published IBSP4ncp genome/polyprotein.
[b] This nucleotide change reverted to the original at passage 5.

infectious upon transfection into MDBK cells. The progeny viruses were subjected to five passages in MDBK cells and maintained their infectivity and replication efficiency (Fig. 2A). At all tested passages, the viruses reached titers of approximately $10^{5.5–6.0}$ PFU/mL at 72 h post-infection (data not shown). The stability of viral cDNA in prokaryote system is important due to higher amount and quality of recombinant DNA extracted from bacteria in relation to yeast, facilitating its future use in some experiments, as such genome sequencing and *in vitro* transcription.

Discussion

Full-length, chimeric cDNA clones of BVDV-1 were constructed using the strategy of homologous recombination in yeast. The assembled clones contained the NADL 5′ and 3′ UTRs flanking the ORF of a representative Brazilian BVDV-1 strain (IBSP4ncp). The viruses recovered from the cells transfected with *in vitro*-transcribed RNA were viable, maintained the main replicative properties of the parental virus, and were stable over 10 passages in cell culture. A few mutations in the viral genome were identified; nevertheless, there were no phenotypic changes in the infectious clone *in vitro*. Moreover, the yeast-assembled plasmids were amplified in *E. coli*, the RNA transcribed *in vitro* from the bacterial plasmid DNA generated infectious viruses upon transfection, and the resulting viruses were stable in cell culture. Thus, the strategy of homologous recombination in yeast seems suitable for the construction and manipulation of pestivirus recombinant cDNA clones.

Homologous recombination in the yeast *S. cerevisiae* has arisen as molecular methodology for many applications, including the construction of cDNA clones of various viruses. In this strategy, DNA fragments containing nucleotide sequences homologous to the vector at their ends may be directly cloned by *in vivo* recombination in the host. In this way, multiple DNA fragments can be correctly assembled in a unique molecule.[26] This strategy has several applications and shows advantages over traditional cloning methods. In particular, it does not depend on multiple restriction sites or the use of many amplification products, unlike traditional cloning in bacteria, as reported by Park et al.[16] to construct a CSFV cDNA clone.[16] These properties make this approach easier, less laborious, and more efficient than traditional cloning strategies.[26] Along with other applications using viruses,[24] this methodology has successfully been used to overcome the problems of instability of some flavivirus recombinant cDNA genomes in *E. coli*.[20,27] A prerequisite for homologous recombination in yeast is the presence of nucleotide sequence homology (15–50 bp) at the ends of the DNA fragments and the vector to be recombined, necessary to recruit the yeast recombination machinery.[28,29] In our system, homologous sequences of 25 bp in both UTRs at the ends of fragments 1 and 3 were shown to suffice to direct recombination in the yeast. The flexibility in terms of the size of cloned fragments, the length of homologous sequences, and the use of intrinsic restriction sites presents obvious advantages of this cloning strategy, simplifying the procedures. Also, there is no need to purchase cloning kits used for conventional cloning strategies.

The rescued viruses were stable over 10 passages in cell culture, maintaining the replication efficiency and kinetics, focus size, and morphology of the parental virus. The stability in cell culture achieved by our system was better than BAC system for some pestiviruses, although this technique has been used to reduce the instability of cDNA clones.[17] Thus, neither the genetic manipulation nor the presence of the heterologous UTRs adversely affected the replication properties of the rescued viruses or altered their main phenotypic properties *in vitro*. The NADL UTR sequences were used in the construct since the corresponding IBSP4ncp sequences were unknown at the time when we assembled the cDNA clones. A similar strategy was described earlier, in one study parts of the CP7 5′ and 3′ UTRs were replaced by parts of the respective NADL UTRs[10] and Hoffmann[30] creates an interspecies cDNA clone with BVDV UTRs plus CSFV ORF, both viruses replicated efficiently.[10,30] The heterologous UTRs present in our construct may serve as genetic markers, confirming the recombinant origin and helping avoid potential contaminations with the parental virus. Anyway, the viability and replication efficiency of the rescued viruses show the promise of this methodology for generating recombinant BVDV.

Nucleotide sequencing of the rescued virus chi-NADL/IBSP4ncp#2 at p0, revealed one silent mutation in the N^{pro} gene, which was still detected at p5. Another mutation was detected in the E1 gene at p0 but reverted to the original nucleotide at p5 (Table 2). At p5, mutations were mainly found in the first ORF-encoded protein, N^{pro} (Table 2). Three nucleotide changes (nt 463, 499, and 514) did not result in amino acid substitutions. However, two nucleotides changes in the NS5B gene resulted in amino acids substitution: one by an amino acid from the same chemical group (Glu550Asp) and other by an amino acid from a different chemical group (Ser12079Asn) (Table 2). However, as mentioned above, no obvious phenotypic changes were observed in the rescued viruses in cell culture, compared to the parental strain. Park et al.[16] recently constructed, a cDNA clone using standard techniques and obtained similar mutations rates, nevertheless the phenotype of the rescued virus showed lower levels of replication. No mutation was found in the original plasmids or in the PCR products used as templates for *in vitro* transcription in our study. Since certain DNA manipulations would require higher amounts of DNA, we also investigated the stability of the yeast-assembled DNA plasmids in *E. coli*. The successful recovery of infectious viruses that were stable in cell culture over five passages from the RNA transcribed from the plasmids replicated in *E. coli* demonstrated that these plasmids were stable and, therefore, may be amplified and maintained in this host.

In summary, the recombinant viruses reported herein, chimeric in their UTRs, contain the entire ORF of the Brazilian BVDV strain IBSP4ncp and retain its protein and antigenic properties. IBSP4ncp was demonstrated to display a high degree of serological cross-reactivity with a wide range of Brazilian BVDV isolates[21] and, therefore, is considered to be a representative strain and a candidate for vaccine development. Thus, in the future, a recombinant derivative of IBSP4ncp can be used as a backbone to generate recombinant vaccine clones, due to its high antigenic similarity to Brazilian field isolates.

Conflicts of interest

The authors declare no conflicts of interest.

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