

Construction and Manipulation of an Infectious Clone of the Bovine Herpesvirus 1 Genome Maintained as a Bacterial Artificial Chromosome

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The complete genome of bovine herpesvirus 1 (BoHV-1) strain V155 has been cloned as a bacterial artificial chromosome (BAC). Following electroporation into *Escherichia coli* strain DH10B, the BoHV-1 BAC was stably propagated over multiple generations of its host. BAC DNA recovered from DH10B cells and transfected into bovine cells produced a cytopathic effect which was indistinguishable from that of the parent virus. Analysis of the replication kinetics of the viral progeny indicated that insertion of the BAC vector into the thymidine kinase gene did not affect viral replication. Specific manipulation of the BAC was demonstrated by deleting the gene encoding glycoprotein E by homologous recombination in DH10B cells facilitated by GET recombination. These studies illustrate that the propagation and manipulation of herpesviruses in bacterial systems will allow for rapid and accurate characterization of BoHV-1 genes. In turn, this will allow for the full utilization of BoHV-1 as a vaccine vector.

Bovine herpesvirus 1 (BoHV-1) is a member of the family *Herpesviridae* and has been placed within the subfamily *Alpha-herpesvirinae*. The herpesviruses are characterized by large double-stranded DNA genomes ranging in size from 80 to 250 kbp (21). The genome of the Cooper strain of BoHV-1 has been completely sequenced. The genome is 135,301 bp in length and contains an estimated 73 genes (GenBank accession number AJ004801).

BoHV-1 is a major pathogen of cattle throughout the world. BoHV-1 is responsible for severe respiratory, reproductive, neonatal, and dermal disease in cattle. One of the most important clinical syndromes associated with BoHV-1 infection is the multifactorial disease bovine respiratory disease (BRD). BRD-related economic losses to the cattle industries of North America have been estimated at \$1 billion (U.S.) (32). It has been demonstrated that BoHV-1 vaccination can prevent the development of BRD.

On the basis of clinical signs and restriction endonuclease profiles of genomic DNA, BoHV-1 has been divided into three subtypes: 1.1, 1.2a, and 1.2b (6, 14). Subtypes 1.1 and 1.2a have been associated with severe disease and with infections of the fetus resulting in abortion. Subtype 1.2b does not cause abortion and is the only subtype found in Australia (25).

To date there has been no genetic information to support the subtyping of BoHV-1. Rijsewijk et al. (19) reported two monoclonal antibodies which reacted only with glycoprotein C (gC) from type 1.1 viruses. The importance of antigenic differences in gC to the BoHV-1-related clinical syndromes has not been determined. Earlier, Nadin-Davis et al. (15) reported sequence variations at the 3' end of glycoprotein B gene, as

subtype 1.1 viruses had fewer G/C repeats than did type 1.2 viruses in this region.

There are many reports in the literature describing the development of recombinant BoHV-1 (rBoHV-1) as a vaccine vector (2, 33). All of these reports have utilized conventional techniques based on eukaryotic homologous recombination, and while these techniques have been successful generally, only poor efficiencies have been achieved. These approaches have utilized homologous recombination of BoHV-1 genes in bovine cells for reduced virulence, such as deletion of the gene encoding thymidine kinase (TK) (11, 26), or for the construction of marker vaccines through deletion of antigenic markers (10, 30). Homologous recombination has also been used to insert gene expression cassettes into the genome of BoHV-1 to deliver antigens from other viruses and, more recently, immunomodulating molecules (12, 17, 18, 22, 29). None of these approaches has fully utilized the potential of rBoHV-1 as a vaccine vector. The large size of the BoHV-1 genome should enable the delivery of multiple genes in a single rBoHV-1; however, current technologies do not facilitate the efficient construction of these viruses.

Recently there have been a number of reports describing the propagation of infectious herpesvirus genomes in bacteria as bacterial artificial chromosomes (BACs). The BAC is stably propagated in the bacterial host, and subsequent transfection of a BAC DNA into virus-permissive cells results in the reconstitution of infectious virus. To date, the genomes of murine cytomegalovirus (13), human cytomegalovirus (3), herpes simplex virus type 1 (9, 28), pseudorabies virus (24), Epstein-Barr virus (5), and Marek's disease virus serotype 1 (MDV-1) (23) have been successfully propagated as infectious BAC clones. The manipulation of herpesvirus genomes is more efficient when using the BAC technology, as it is no longer dependent on either the growth of viruses in eukaryotic cells or homologous recombination in eukaryotic cells. Further, any manipu-

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lations made can be fully characterized prior to the reconstitution of infectious virus, and as a result the reconstituted virus requires no further purification. Finally, there is also the advantage of making genetic changes that inhibit viral replication. Previously, these types of modifications which resulted in either partial or complete inhibition of viral replication could not be characterized, as the virus could not be isolated or grown in culture without complementing cell lines.

Due to the large size of the BACs, it is not possible to manipulate these clones with standard cloning techniques. However, increased utilization of BACs to generate accurate disease models in animal transgenics has resulted in the development of methods for efficient and specific BAC manipulation. One such mutagenesis system is the GET system, which utilizes transient expression of *recE* and *recT* to enable homologous recombination in the commonly used laboratory *Escherichia coli* strains. Recently the GET system has been modified to allow for BAC modification in the BAC host *E. coli* strain DH10B (16). GET recombination has also proven suitable for the manipulation of a BAC which contains the MDV-1 genome (23).

To enable the efficient evaluation of BoHV-1 as a vaccine vector, a BAC which contains the complete genome of BoHV-1 strain V155 has been constructed. Infectious BoHV-1 virions were recovered following transfection of this BAC into bovine cells. Further, the BAC was stable after multiple generations in the bacterial host and passage in bovine cells. The GET recombination system was also utilized to demonstrate that the infectious BoHV-1 BAC could be manipulated via homologous recombination in the bacterial host. The advantages of the BAC approach for construction of recombinant herpesviruses were demonstrated by deleting the gene for gE. Previous attempts to isolate a gE gene-negative mutant of the BoHV-1 strain V155 using conventional approaches were not successful. However, by using the BoHV-1 BAC and GET recombination the gE gene was successfully deleted. These studies confirm the advantages of using BAC technology for the development of herpesvirus-vector vaccines. The utilization of BAC technology to help define the genetic elements responsible for the different clinical signs observed between proposed BoHV-1 subtypes is also discussed.

MATERIALS AND METHODS

Virus and cells. BoHV-1 strain V155 (27) was propagated in CRIB-1 cells (ATCC CRL-11883), a pestivirus-resistant derivative of Madin-Darby bovine kidney cells (7). The CRIB-1 cells were maintained in Hanks' minimal essential medium containing antibiotics and antimycotics, nonessential amino acids, glutamax, 25 mM HEPES, and 5% donor calf serum at 37°C. All reagents utilized for cell and virus propagation were obtained from Invitrogen Australia unless otherwise stated.

Transfection of CRIB-1 cells. CRIB-1 cells were seeded in six-well plates (Corning) at 5×10^5 cells/well 24 h prior to transfection and incubated at 37°C in an atmosphere of 5% CO₂. The CRIB-1 monolayers were transfected with Lipofectamine (Invitrogen Australia) as described below. For each transfection, 1 to 2 µg of DNA was diluted to 100 µl in OptiMEM and mixed with 8 µl of Lipofectamine diluted to 100 µl in OptiMEM. This reaction mixture was incubated at room temperature for 45 min to allow lipid-DNA complexes to form. The reaction volume was increased to 1 ml with OptiMEM, and the mixture was added to monolayers which had been washed twice with OptiMEM. The transfected monolayers were incubated at 37°C with 5% CO₂ for 16 to 18 h prior to the addition of 1 ml of OptiMEM containing 10% donor calf serum. At 24 h, the transfection liquid was removed and replaced with maintenance medium. Mono-

layers were monitored for the development of cytopathic effect (CPE) for up to 7 days posttransfection.

Construction of BoHV-1 infectious clone BAC. Unless otherwise stated, all enzymes and reagents used for DNA cloning were obtained from Roche Molecular Biosystems.

BoHV-1 genomic DNA was purified from viral particles as follows. CRIB-1 cells were infected with BoHV-1 strain V155 at a multiplicity of infection (MOI) of 5, and the infection was allowed to proceed to completion. The cell culture supernatant was clarified by centrifugation at $5,000 \times g$ for 10 min. The mature BoHV-1 virions were pelleted by centrifugation at $120,000 \times g$ for 2 h, and the supernatant was discarded. BoHV-1 genomic DNA was recovered from the pelleted virus by using the Qiagen genomic DNA extraction kit essentially as described by the manufacturer. Viral pellets were resuspended in genomic DNA extraction buffer at a ratio of 1 to 65 of the starting supernatant volume. Following elution from the column, the BoHV-1 DNA was stored in aliquots at -20°C until required.

To facilitate the insertion of transgenes into the TK gene of BoHV-1 by homologous recombination, a deletion-and-insertion vector was constructed. This vector was constructed by using two segments of the TK gene, TKleft and TKright, as recombination arms. TKleft and TKright were amplified from purified BoHV-1 genomic DNA by PCR using standard procedures except for the inclusion of 5% (vol/vol) dimethyl sulfoxide and 10% (vol/vol) glycerol in the reaction mixture. Following amplification, the TKleft PCR product was digested with *KpnI* and *SalI*, gel purified, and ligated into pBluescript SK+ (Stratagene), which had also been digested with *KpnI* and *SalI*. The TKright PCR product was cloned into the plasmid containing the TKleft product following *EcoRI* and *SpeI* digestion by similar cloning procedures. The resultant plasmid was called pTKdel. The primers used for the PCR amplification of the TK targeting regions are shown in Table 1. *NsiI* sites were incorporated into the Tklft5' and Tkrigt3' primers to allow the excision of the transgene product from the pTKdel vector for recombination experiments. Four unique restriction endonuclease sites are present between the two TK crossover regions to allow the insertion of transgenes for transfer to the BoHV-1 genome. These sites are *SalI*, *HindIII*, *EcoRV*, and *EcoRI*.

The BAC vector pBello-BAC II (31) (kindly provided by Robert Henry, Southern Cross University, Lismore, New South Wales, Australia) was digested with *HindIII* and gel purified. It was ligated into pTKdel which had been digested with *HindIII* and dephosphorylated. Following transformation into *E. coli* strain XL1-Blue cells, transformants were plated on selective agar containing 12.5 µg of chloramphenicol (CAP)/ml and 100 µg of ampicillin/ml. Insertion of the BAC vector was confirmed by excision with *HindIII*. The TK deletion fragment (TK-BAC), containing the BAC vector flanked by TKleft and TKright, was excised from pTKdel-BAC by digestion with *NsiI* and gel purified.

To promote homologous recombination between the BAC-TK fragment and BoHV-1 genomic DNA, purified BoHV-1 DNA was digested with *NsiI* and dephosphorylated with bacterial alkaline phosphatase (Pharmacia). The BAC-TK fragment and *NsiI*-digested BoHV-1 genomic DNA were cotransfected into CRIB-1 cells as described above. After 18 to 24 h, the transfection mixture was removed and replaced with complete Hanks' minimal essential medium containing 2 mM *N,N'*-hexamethylene-bis-acetamide (ICN Pharmaceuticals) to promote viral gene transcription (28).

The resultant viral supernatants were passaged once in CRIB-1 cells. Insertion of the BAC vector into the BoHV-1 genome was confirmed by a PCR assay specific for the CAP resistance gene using the primer pair ChloramF and ChloramR (Table 1). PCR templates were prepared by incubation of 10 µl of viral supernatant with 10 µl of lysis buffer (10 mM Tris-HCl [pH 8.0] containing 0.45% [vol/vol] Triton X-100 and 0.45% [vol/vol] Tween 20) with 2 µl of 10-mg/ml proteinase K followed by incubation at 60°C for 2 h. The proteinase K was inactivated at 95°C for 15 min. PCRs were performed with 1 to 2 µl of this preparation as a template. Following PCR detection of the CAP resistance gene within the BoHV-1 genome, bulk genomic DNA was recovered from virus particles as described above.

To facilitate transformation and growth in a bacterial host, the purified BoHV-1 genomic DNA was circularized by standard ligation procedures. Aliquots of the ligation mixtures were electroporated into *E. coli* DH10B cells (1.5 kV, 100 Ω, 25 µF; Electroporator II; Invitrogen, San Diego, Calif.). Following electroporation, DH10B cells were recovered in 960 µl of SOC broth (Invitrogen) and incubated at 37°C for 5 to 6 h with gentle shaking. Aliquots of the electroporation mix were plated onto Luria-Bertani (LB) plates containing 12.5 µg of CAP per ml. Colonies were allowed to develop for 24 to 48 h at 37°C.

CAP-resistant colonies were inoculated into 5 ml of LB broth containing 12.5 µg of CAP per ml and grown at 37°C for 16 h. BAC DNA was recovered by a standard alkaline lysis method (1). The *HindIII* digestion profiles of these BAC

TABLE 1. Oligonucleotides used to generate the PCR products described in this study

Primer	Primer sequence ^a	Product (size in bp) and plasmid
TKleft5'	5'-GT <u>GGTACC</u> <u>ATGCAT</u> CTGATACCCCTTCGCCGCTACTG-3'	Tkleft (301); pTKdel
	<i>KpnI</i> <i>NsiI</i>	
TKleft3'	5'-TTTGC <u>GTCGAC</u> CCACTCCAGCGCGTCCCAG-3'	
	<i>SalI</i>	
TKright5'	5'-AT <u>GAATTC</u> GCCGCGCTCGCAGACCCCA-3'	TKright (337); pTKdel, TK-probe
	<i>EcoRI</i>	
TKright3'	5'-GGACTAGTCATGCAT CTCTAGCGCGAACTGACG-3'	
	<i>SpeI</i> <i>NsiI</i>	
ChloramF	5'-TCACTGGATATACCACCGTTGA-3'	CAP ^r gene (402);
ChloramR	5'-TCACCGTAACACGCCACATCTT-3'	CAP ^r -probe
gE-KanF	5'-GGGGAACGGCGCACGCGAGAGGGTTCGAAAAGGGCATTGGCAATGCAAC- <u>ATTTAAAT</u> -ccacgtgtgtctcaaatctctgatg-3'	gE-Kan ^r (1,237)
	<i>SwaI</i>	
gE-KanR	5'-TCGCGCTGCTACCACGGTGTAACTCTGGTGCGGCCGGGTCCGCGCTGGCG- <u>ATTTAAAT</u> -cgggtgatgagacgtttagtg-3'	
	<i>SwaI</i>	
BHV1.3	5'-GGG CAT TTG GCA ATG CAA C-3'	gE-probe (845)
BHV1.6	5'-CGT CTC GTA TAT GCG GAT G-3'	
Kan ^r fwd	5'-GGT ATT AGA AGA ATA TCC TGA TTC-3'	Kan ^r -probe (483)
Kan ^r rev	5'-CTC ATC GAG CAT CAA ATG AAA CT-3'	

^a Restriction enzyme sites are underlined in the oligonucleotide sequence. Sequences in lower case for gE-KanF and gE-KanR are specific for the kanamycin resistance cassette of EZ::TN (Kan-1) (Epicentre Technologies).

clones were compared with the *HindIII* profile of BoHV-1 genomic DNA. BAC clones with *HindIII* profiles similar to those of genomic DNA were transfected into CRIB-1 cells as described above. The transfections were monitored daily for the development of CPE considered typical of BoHV-1.

Stable propagation of the BoHV-1 BAC clone in DH10B cells. To determine the stability of the BAC clone in the bacterial host, DH10B cells containing a BAC carrying the BoHV-1 genome were grown in serial culture for 1, 5, 10, and 20 days. BAC DNAs were isolated as previously described and characterized by restriction endonuclease digestion and field inversion gel electrophoresis (FIGE). DNA samples from each time point were also transfected into CRIB-1 cells to confirm that the BAC DNA was still infectious.

Recombination of BoHV-1 BAC in DH10B. Modifications of the BACs containing the complete BoHV-1 genome were performed using GET recombination in DH10B cells. GET cloning facilitates homologous recombination reactions between the BAC and linear transgenes (16). The plasmid pGETrec (kindly provided by Panos Ioannou, Murdoch Institute, Melbourne, Australia) was transformed into DH10B cells harboring pBACBHV37. DH10B cells containing both plasmids were selected on agar containing 12.5 µg of CAP per ml and 100 µg of ampicillin per ml. Electrocompetent cells of double-resistant DH10B cells were prepared using 0.2% (wt/vol) arabinose induction to enable homologous recombination as previously described (16).

To demonstrate that GET recombination could be utilized to modify the BoHV-1 genome, the gene encoding gE was targeted for deletion. A minimal kanamycin resistance cassette (Kan^r) was amplified by PCR from the transposon EZ::TN<KAN-1> (Epicentre Technologies). The PCR primers gE-KanF and gE-KanR utilized included regions of 50-bp homology to the 5' (base numbers 121671 to 121720 of AJ004801) and 3' (base numbers 123371 to 123420 of AJ004801) ends of the gE gene and are shown in Table 1. The resultant product, gE-Kan^r, was 1,300 bp in length and was recovered following agarose gel separation by using a gel extraction kit according to the manufacturer's instructions (Qiagen). Approximately 200 ng of the gel-purified PCR product was electroporated into the electrocompetent DH10B(pBACBHV37, pGETrec) as previously described. Recombinant colonies were identified by plating on LB plates containing 12.5 µg of CAP per ml and 50 µg of kanamycin per ml.

Southern blotting. Viral genomic DNA for Southern blotting was purified as described below. Cell monolayers were infected with BoHV-1 strain V155 at an MOI of 5, and the infection was allowed to proceed until approximately 40% of

the cells showed a "rounding up" type of morphology. Following removal of the growth medium, the monolayer was gently washed twice with phosphate-buffered saline at 0°C. Cell lysis buffer at 0°C (10 mM sodium phosphate, pH 7.3, containing 1% [vol/vol] Nonidet P-40) was added to each flask (4 ml per 175 cm²), and the flasks were rocked so that the lysis buffer contacted the entire monolayer. The lysis buffer was removed and placed on ice, and another 4 ml was added to each flask with gentle rocking; then it was removed and added to the initial lysis solution. The lysates were clarified at 4,300 × g for 10 min at 4°C. The supernatant was collected and centrifuged at 112,700 × g for 100 min at 5°C. The viral pellet was resuspended in 500 µl of G2 buffer (Qiagen). Proteinase K (25 µl at a concentration of 10 mg/ml) was added to the resuspended viral pellet followed by incubation at 50°C for 1 h. Genomic viral DNA was recovered by using genomic tip 20/G (Qiagen) as follows. The genomic tip 20/G was equilibrated with 1 ml of QBT buffer. The material treated with proteinase K was diluted with an equal volume of QBT buffer and loaded onto the genomic tip (660 µl per tip). Tips were washed twice with 7.5 ml of QC buffer. Viral DNA was eluted from the tip with 2 × 1.5 ml of QF buffer at 50°C. The DNA was precipitated, washed once with 75% ethanol, and resuspended in 50 µl of 10 mM Tris-HCl, pH 8.5.

Restriction enzyme-digested DNA samples were electrophoresed on a 1% gel in 0.5× Tris-borate-EDTA buffer for 13.5 h by use of a FIGE apparatus (Bio-Rad) at 5°C. The switch time ramp was 0.1 to 2 s (linear shape) with a forward voltage of 180 V and a reverse voltage of 120 V. The DNA fragments were transferred to a Hybond-N noncharged membrane (Amersham) by capillary action, and the DNA was fixed to the membrane by application of UV light (1). Probes were labeled with digoxigenin-11-dUTP (Roche Molecular Biosystems). Probes were synthesized by PCR using a reaction mix containing the following: *Taq* polymerase buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl [pH 8.5]); 1.25 mM each dATP, dCTP, dGTP, and dTTP; 1.25 µM DIG-II-dUTP; 1 U of *Taq* polymerase (Roche Molecular Biosystems); and 5 ng of template DNA in a final volume of 20 µl. PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by final incubation at 72°C for 7 min. Following gel purification, PCR probes were hybridized to membranes for 12 to 16 h at 55°C (DIG System User's Guide for Filter Hybridization; Roche Molecular Biosystems). Hybridizations were carried out in rotating bottles in a hybridization oven (Hybrid). Membranes were washed in dishes on a shaking platform at room temperature.

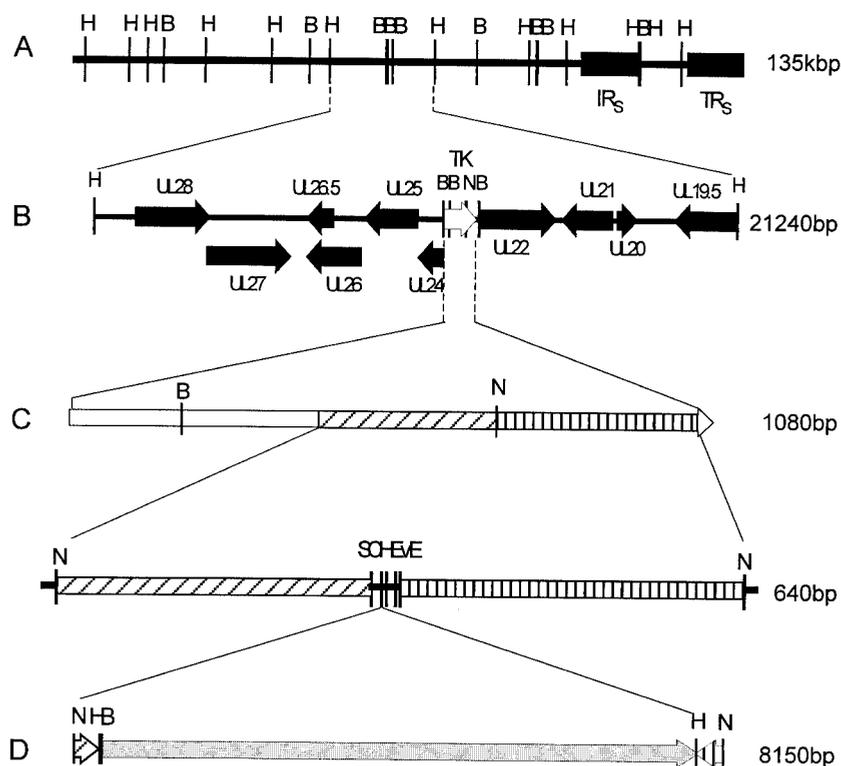


FIG. 1. Schematic representation of the construction of an infectious clone for BoHV-1. (A) Combined *Hind*III and *Bam*HI restriction map of the bovine herpesvirus genome (135,307 bp; GenBank accession number AJ004801) derived from the complete genome sequence of the BoHV-1 Cooper strain. The internal repeat sequence (IR_s) and terminal repeat sequence (TR_s) are illustrated. (B) BoHV-1 *Hind*III genomic fragment containing the TK gene (white arrow). Open reading frames (black arrows) surrounding the TK gene are illustrated. The unique *Nsi*I (N) site of the BoHV-1 genome used to promote homologous recombination is illustrated. (C) TK gene of BoHV-1. TK gene-specific crossover regions immediately upstream (301 bp, forward hatch) and downstream (337 bp, vertical hatch) of the unique *Nsi*I site were amplified by PCR and cloned to the recombination transfer vector pTKdel. The transfer vector contains five unique restriction enzyme sites (including *Cla*I and *Eco*RV) for insertion of transgenes to be transferred to the BoHV-1 genome. The completed transgenes can be excised from the transfer vector by *Nsi*I digestion or amplified by PCR. (D) The BAC vector, pBelloBACII, was cloned into the *Hind*III site of pTKdel. The resultant transgene was excised with *Nsi*I prior to transfer into the BoHV-1 genome by homologous recombination. Abbreviations for the illustrated restriction enzyme sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N, *Nsi*I; S, *Sal*I.

Replication kinetics. The replication kinetics of the various rBoHV-1 strains were determined by standard virologic techniques. Briefly, one 50% tissue culture infective dose ($TCID_{50}$) of virus was allowed to infect 10^5 CRIB-1 cells plated in 24-well plates for 90 min at 37°C in a 5% CO_2 atmosphere. Any extracellular virus was then inactivated by addition of sodium citrate solution (40 mM sodium citrate, 10 mM KCl, 135 mM NaCl [pH 3.0]), the cell layers were then washed twice with phosphate-buffered saline, 1 ml of maintenance medium was added, and the mixture was incubated at 37°C in a 5% CO_2 atmosphere. Viral supernatants and cell pellets were collected at 2, 4, 6, 12, 24, 48, and 72 h postinfection (p.i.) and frozen at $-70^\circ C$ until required. The $TCID_{50}$ of each supernatant from each time point was then determined in triplicate. Following one freeze-thaw cycle, the $TCID_{50}$ of intracellular virus was also determined for each time point in triplicate.

RESULTS

Construction and analysis of a BAC containing the BoHV-1 genome. To promote the insertion of foreign DNA into the BoHV-1 genome, we have used a method called restriction enzyme-mediated recombination (REMR), which will be reported in detail elsewhere. Briefly, REMR utilizes a unique *Nsi*I restriction site (nucleotide position 63980 of the Cooper strain genomic sequence) to promote homologous recombination between BoHV-1 DNA and transgene DNA

molecules. The *Nsi*I site is located within the TK gene, which has previously been shown to be nonessential for in vitro and in vivo growth of the BoHV-1 strain V155 used in this study (26). Following *Nsi*I digestion and dephosphorylation, if the BoHV-1 DNA is transfected into CRIB-1 cells, the two genomic segments do not produce infectious BoHV-1. To exploit this, a transfer vector, pTK-del, which contains the BoHV-1 genomic sequences immediately upstream and downstream of the *Nsi*I site was constructed (Fig. 1). Following excision, the insert of pTK-del was co-transfected into CRIB-1 cells with *Nsi*I-dephosphorylated genomic DNA; homologous recombination between the three molecules facilitated the reconstitution of infectious BoHV-1 (data not shown). After cloning into pTK-del the BAC vector was excised by *Nsi*I digestion and gel purified (Fig. 1). The BAC vector was transfected into CRIB-1 cells along with *Nsi*I-digested and -dephosphorylated BoHV-1 genomic DNA. Typically, between 5 and 7 days following transfection, a CPE considered typical of BoHV-1 was evident. Following one passage of the resultant virus, PCR amplification of the CAP resistance gene from pBelloBacII

confirmed that the BAC vector had been inserted into the BoHV-1 genome (data not shown).

To facilitate propagation in *E. coli* strain DH10B, viral genomic DNA was purified from virions and circularized by standard ligation protocols. This ligation process utilizes single-base 3' overhangs on the termini of the virus genome which are required for virus circularization prior to replication in vivo (8). The circularized genomes were electroporated into DH10B cells and plated onto CAP-selective agar plates. CAP-resistant colonies were screened by a gE-specific PCR, which identified six clones as putatively positive BAC clones (data not shown). Plasmid DNAs were prepared from overnight cultures of the six colonies. The *Hind*III digestion profiles of these six BAC clones identified two of them, pBACBHV27 and pBACBHV37, with profiles that could not be distinguished from that of native BoHV-1 DNA. Plasmid DNA from both of these clones was transfected into CRIB-1 cells. A CPE considered typical of BoHV-1 was observed in the cells transfected with both of these clones after 4 days (data not shown). Neither the plaque size nor the morphology produced by virus derived from the BAC clones differed from those of the wild-type virus. All subsequent experiments were conducted with the BAC clone pBACBHV37.

The insertion of the BAC vector into the BoHV-1 genome was demonstrated by Southern blotting (Fig. 2). The CAP-specific PCR product was digoxigenin (DIG) labeled and hybridized to pBACBHV37 DNA and BoHV-1 genomic DNA which had been digested with *Hind*III and *Bam*HI. Figure 2B illustrates specific hybridization of the CAP probe to the positive control BAC vector, pBac108L, which also carries the CAP resistance gene. The CAP probe also binds specifically to a 7.5-kbp band in the *Hind*III-digested pBACBHV37 DNA which corresponds to the excised pBeloBACII vector (Fig. 2B). The hybridizing fragment in the *Bam*HI digest is slightly larger than the *Hind*III fragment, as the BAC vector is inserted into a 969-bp *Bam*HI genomic fragment. As the vector contains a *Bam*HI site, digestion with this enzyme releases the BAC vector plus 500 bp of BoHV-1 genomic DNA resulting in the hybridization pattern observed. As expected, there was no hybridization of the CAP probe to DNA isolated from wild-type BoHV-1 virions (Fig. 2B). The slight variation in the sizes of the hybridized bands is due to the difference in size of the control vector pBac108L (6,812 bp) and the BAC vector inserted into the BoHV-1 genome pBeloBacII (7,507 bp).

The specific insertion of the BAC vector into the *Nsi*I site was demonstrated by stripping the membrane and reprobing it with a TK gene-specific probe (TKright) (Table 1). The TK probe did not bind to the BAC vector DNA on the membrane (Fig. 2A). The TK gene is located on a 21.2-kbp *Hind*III genomic fragment (Fig. 1B). This fragment is evident in the *Hind*III-digested BoHV-1 DNA (Fig. 2A). The relatively low intensity of this band is probably due to inefficient transfer of such a high-molecular-weight fragment. As expected, in BoHV-1 DNA digested with *Bam*HI, the TK probe bound specifically to a 1,000-bp band (Fig. 1 and 2A). Insertion of the BAC vector (7,507 bp) into the 21.2-kbp *Hind*III genomic fragment at the *Nsi*I site increases the size to 28.2 kbp and introduces two *Hind*III sites and one *Bam*HI site (Fig. 1). As a result, the TK-specific band detected by the probe increased in size to 9 kbp, due to the incorporation of the BAC vector in

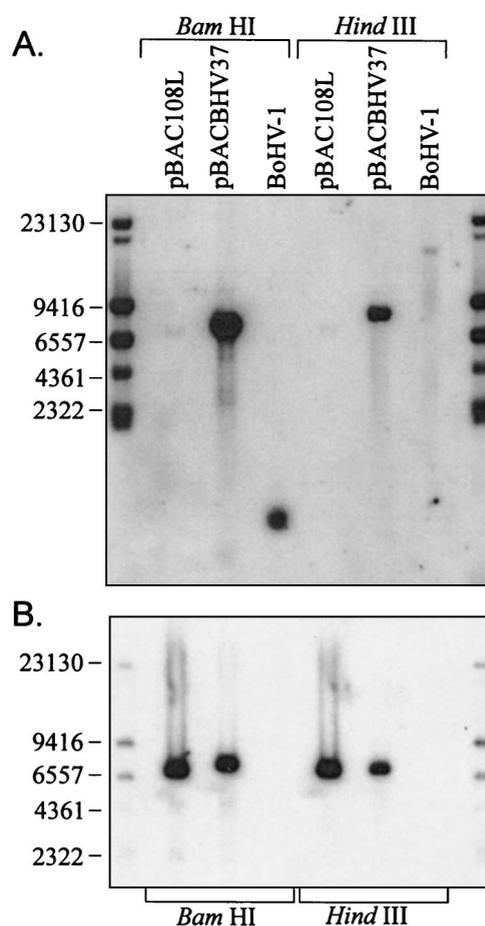


FIG. 2. Southern blotting analysis of the clone pBACBHV37 illustrating the insertion of the BAC vector, pBeloBACII, into the genome of the BoHV-1 strain V155. DNA was digested with either *Bam*HI or *Hind*III and separated by FIGE in a 1% agarose gel. DNA fragments were transferred onto nylon membranes by Southern blotting. Duplicate membranes were hybridized with DIG-labeled probes specific for either TK of BoHV-1 or the CAP resistance gene (CAP^r) of the BAC vector. (A) Southern blot hybridized with a DIG-labeled probe specific for the TK gene of BoHV-1. (B) Southern blot hybridized with a DIG-labeled probe specific for the CAP^r gene. Standard molecular sizes (in base pairs) are given.

*Bam*HI-digested pBACBHV37 DNA (Fig. 1 and 2A). In *Hind*III-digested pBACBHV37 DNA, the TK-specific band decreases to approximately 9 kbp due to the *Hind*III sites introduced during insertion of the BAC vector (Fig. 1 and 2A).

Stable propagation of the BoHV-1 genome as a BAC. The stable propagation of pBACBHV37 in *E. coli* DH10B was demonstrated by serial culture for 5, 10, and 20 days. As *E. coli* has a generation time of approximately 20 min, and given that the stationary growth phase is reached at 12 h, each day represents about 36 generations. Following culture, the restriction endonuclease pattern of BAC DNA purified from cells at each time point was examined by FIGE. The restriction endonuclease patterns following *Hind*III and *Bam*HI digestion of the purified DNA are illustrated in Fig. 3. There were no distinguishable differences in the profiles of the infectious clone pBACBHV37 after multiple passages in the bacterial host (Fig. 3). Further, BAC DNA isolated at each time point was

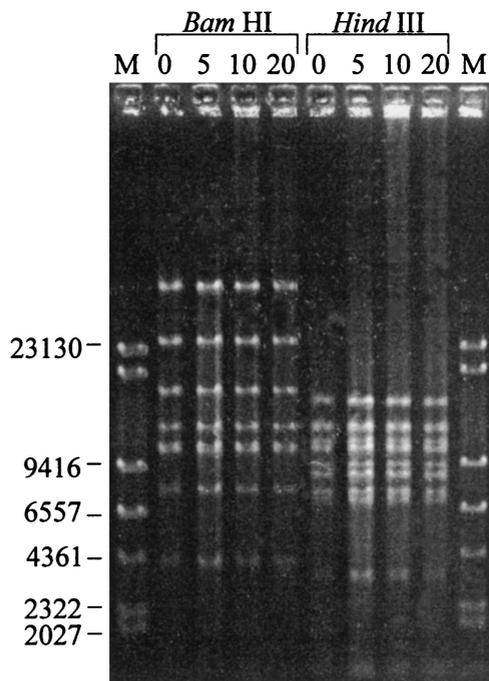


FIG. 3. Stability of the BAC pBACBHV37 over multiple generations in *E. coli* DH10B. DH10B cells containing pBACBHV37 were serially grown for 5, 10, and 20 days. BAC DNA was prepared from these cultures, and the restriction enzyme profiles were compared by FIGE with 1% agarose. DNA fragments were stained with ethidium bromide and photographed. Standard molecular sizes (in base pairs) are given.

infectious following transfection into CRIB-1 cells. The recovered virus produced a CPE which was typical of BoHV-1 and pBACBHV37 (data not shown).

Replication kinetics of BoHV-1 rescued from a BAC. Replication kinetics of the infectious clone was compared to that of wild-type BoHV-1 to determine if incorporating the BAC vector into the BoHV-1 genome has any effect on viral replication in vitro, as the insertion of the vector essentially deletes TK activity. CRIB-1 cells were infected with BoHV-1 and with pBACBHV37-derived virus at equivalent MOIs. The titers of viral supernatant were determined at 2, 4, 6, 12, 24, 48, and 72 h p.i. The results of these titrations are illustrated in Fig. 4. On the basis of these titrations, the insertion of the BAC vector into the BoHV-1 genome did not have any effect on the replication kinetics of BoHV-1. There was no difference in the amount of virus recovered following infection with either pBACBHV37 or BoHV-1 (Fig. 4).

Specific recombination of the BoHV-1 infectious clone. The efficient and specific modification of BAC in DH10B cells has recently become possible through the development of GET cloning (16). To determine if this procedure was adaptable to the plasmid pBACBHV37, the gene encoding gE was targeted for deletion. A kanamycin resistance cassette (Kan^r) was amplified by PCR. To facilitate the specific deletion of the gE gene, 50-bp sequences of homology to the gE gene 5' and 3' regions were incorporated into the Kan^r PCR product. The purified Kan^r PCR product was electroporated into DH10B cells containing pBACBHV37 and pGETrec, which had been induced with arabinose.

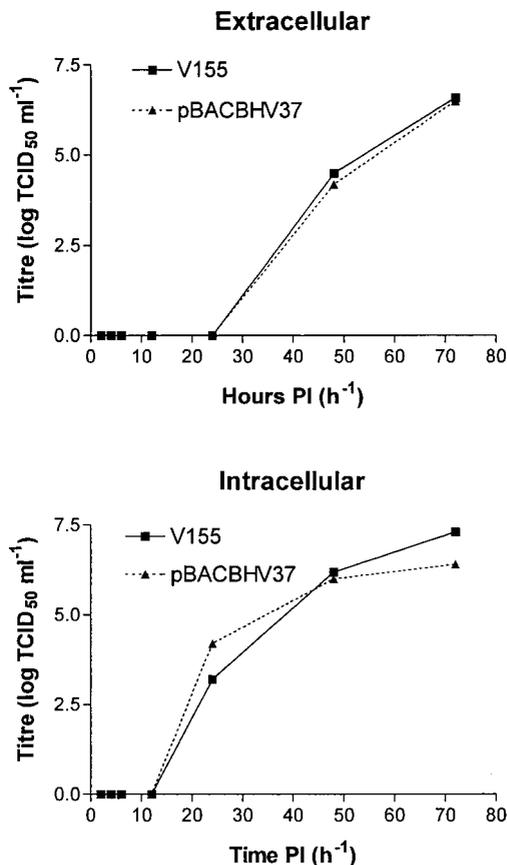


FIG. 4. Replication kinetics of infectious BoHV-1 rescued from pBACBHV37 compared to wild-type BoHV-1 strain V155. Following recovery of infectious BoHV-1 from pBACBHV37, DNA and viral stock was amplified and titrated. CRIB-1 monolayers were infected with one TCID₅₀ of either the BoHV-1 strain V155 or pBACBHV37. The culture supernatants of each infection were recovered at 2, 4, 6, 12, 24, 48, and 72 h p.i. (PI), and the TCID₅₀ was determined for each. The titrations were done in triplicate, and averaged titers are illustrated. TCID₅₀s were determined for both extracellular and intracellular viruses.

Following electroporation, the cells were allowed to recover and were plated onto CAP- and kanamycin-selective agar plates. BAC DNA was recovered from six of the double-resistant colonies and transfected into CRIB-1 cells. Five of the six colonies transfected produced a CPE considered typical of BoHV-1. The restriction enzyme profiles of these clones were examined following *Hind*III and *Bam*HI digestion and FIGE. To determine if Kan^r had replaced the gE gene, Southern blotting was performed. The membrane was first probed with a gE gene-specific probe. It was evident that the fragment which normally contains the gE gene had been significantly altered (Fig. 5A). It was apparent that the gE gene had been deleted via GET recombination. Following removal of the gE probe, the blot was probed with a Kan^r -specific probe as illustrated in Fig. 5B. The Kan^r probe did not bind to any fragments in either BoHV-1 DNA or pBACBHV37. However, following GET-mediated recombination, the Kan^r probe specifically bound to two DNA fragments in both the *Bam*HI and *Hind*III digests (Fig. 5). The DNA utilized for the construction of this Southern blot was isolated from virus-infected cells. The

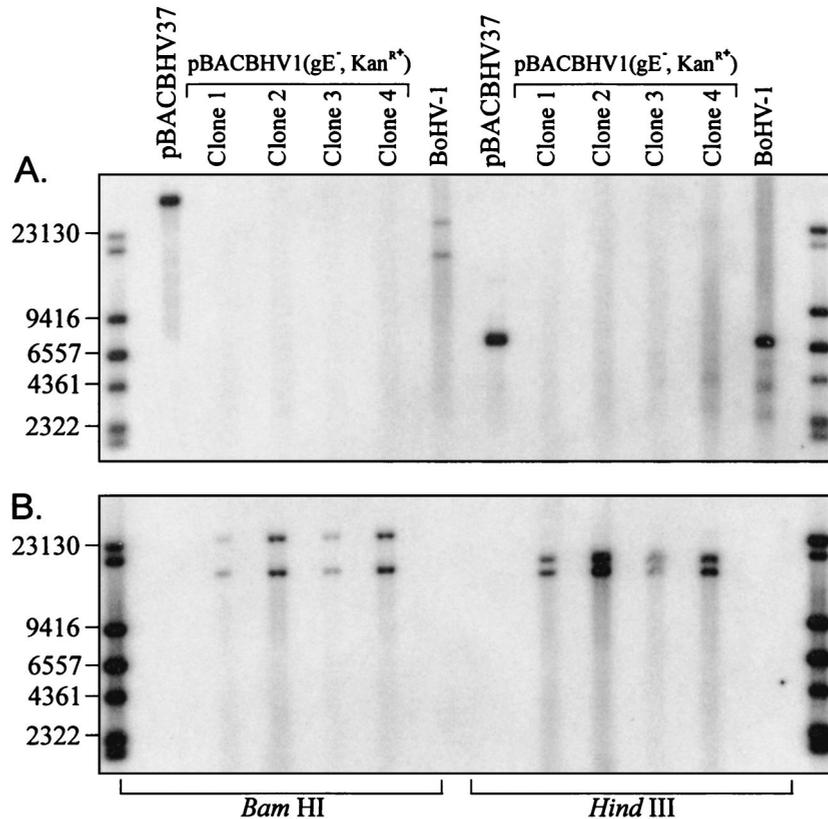


FIG. 5. Southern blot analysis of four BoHV-1 infectious BAC clones following the replacement of the gene coding for glycoprotein E (gE) with a kanamycin resistance gene (Kan^r). Viral genomic DNA was isolated from cells infected with modified virus. DNA was digested with either *Bam*HI or *Hind*III, resolved on a 1% agarose gel by FIGE, and transferred onto nylon membranes. Blots were hybridized with a DIG-labeled probe specific for either gE or Kan^r . Standard molecular sizes (in base pairs) are given. (A) Southern blot hybridized with a probe specific for the gE gene. (B) Southern blot hybridized with a probe specific for the Kan^r gene.

gE gene is located within the unique short (US) region of the BoHV-1 genome, which is flanked by the internal and terminal genomic repeat sequences. As the US region may switch orientations during virus replication, the relative locations of the restriction enzyme sites change and hence the digestion pattern is also altered, giving rise to two genomic isomers which harbor the gE gene on fragments of different sizes.

To determine if the deletion of the gE gene from pBACBHV37 has any effect on the replication, the kinetics of virus rescued from pBACBHV1 ($gE^- Kan^{r+}$) was compared to that of virus rescued from pBACBHV37. Following infection at the same MOI, samples of the supernatants were collected at 2, 4, 6, 12, 24, 48, and 72 h p.i. and the viral titers were determined. The results of these titrations are illustrated in Fig. 6. On the basis of these titrations, there was no difference in the amounts of virus recovered following transfection of pBACBHV37 or pBACBHV1 ($gE^- Kan^{r+}$) after in vitro recovery of virus. The replication kinetics of the negative viruses were determined by using $TCID_{50}$ calculations, as the rBoHV-1 strains negative for the gE gene did not produce visible plaques under agarose overlays.

DISCUSSION

Here we have described the construction of the first infectious clone for BoHV-1. This infectious clone contains the

complete genome of BoHV-1 strain V155. We have demonstrated that when maintained as a BAC, the BoHV-1 clone is stable through multiple generations of the host strain *E. coli* DH10B. The stability was evaluated by restriction enzyme analysis and also through the reconstitution of infectious BoHV-1 following transfection into bovine cells.

Further, the recently described GET mutagenesis system (16) has been successfully used to delete the gene coding for gE. While the gE gene has been deleted from other strains of BoHV-1 (20, 30), this is the first report describing the deletion of gE from the 1.2b subtype. Previous attempts in our laboratory using established homologous recombination protocols failed to identify any gE-negative mutants despite extensive screening (unpublished data). As a result, we had suspected that gE is an essential gene in BoHV-1 subtype 1.2b, which represents a significant divergence from conventional paradigms of herpesvirus biology.

In the study described here, the application of a mutagenesis system which was independent of viral replication allowed for the rapid production of a gE-negative mutant. The subsequent transfection into bovine cells confirmed that gE is a nonessential gene in the strain of BoHV-1 used. Experiments with BoHV-1 gE-negative mutants recovered from the mutated infectious clone demonstrated that the virus did not form visible plaques under agarose overlays. DNA sequence analysis across the junctions of recombination indicated that the recombina-

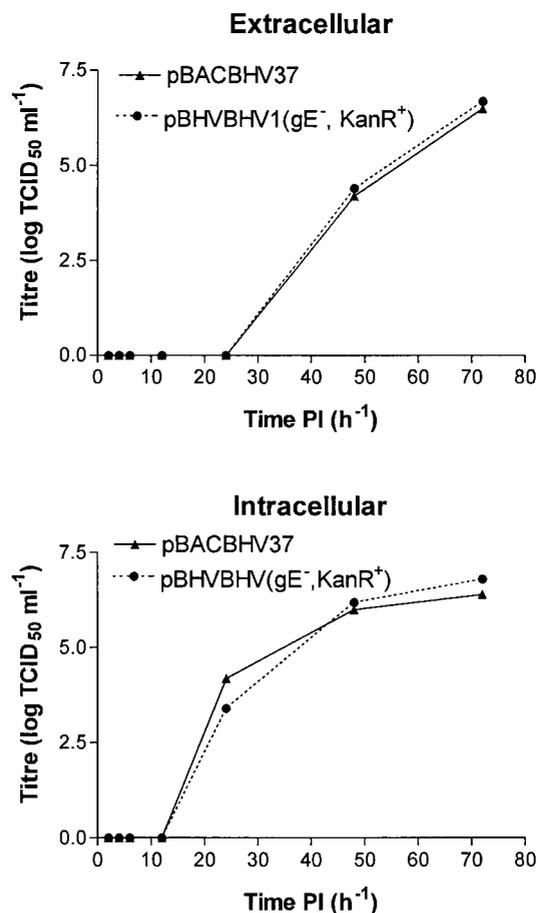


FIG. 6. Comparison of the replication kinetics of BoHV-1 virus rescued from pBACBHV37 with that of rescued virus following the deletion of the gE gene from the BAC clone. Following recovery of infectious BoHV-1 from pBACBHV37 or pBACBHV1 (gE^- , Kan^{R+}), the viral stock was amplified and titrated. CRIB-1 monolayers were infected with one $TCID_{50}$ of virus from either pBACBHV37 or pBACBHV1 (gE^- , Kan^{R+}). The viral supernatants were sampled at 2, 4, 6, 12, 24, 48, and 72 h p.i. (PI), and the $TCID_{50}$ was determined for each. These samples were titrated. The titrations were done in triplicate, and the averaged titers are illustrated. $TCID_{50}$ s were determined for both extracellular and intracellular viruses.

tion was specific and had not altered any of the control elements for the genes either upstream (glycoprotein I) or downstream (tegument protein) from gE (data not shown), indicating that the lack of plaque formation observed was due to deletion of gE. Our initial experiments using conventional homologous recombination techniques aimed to replace the gE gene with a cassette expressing green fluorescent protein with mutants to be identified by visualization of rBoHV-1 through fluorescent plaques. As the BAC-derived gE-negative mutant was unable to form plaques, this is the most likely explanation for the inability to identify any fluorescent plaques. This result was unexpected, as previously reported rBoHV-1 strains negative for gE were able to form plaques under overlays, although the plaques were smaller than those of the parent virus (20, 30). However, all of these rBoHV-1 were constructed from subtype 1.1 viruses, not the subtype 1.2 virus used in this study. The as-yet-undefined genetic differences

between the subtypes are the most likely causes for the lack of plaque formation observed.

Further application of the techniques described in this study will greatly aid the elucidation of the genetic elements responsible for the BoHV-1 subtypes. By specifically replacing or swapping genes and regulatory sequences from different subtypes, the roles that individual genes or genomic segments play in viral pathogenesis and/or biotypes could be specifically evaluated. To fulfill this goal, an infectious clone of a type 1.1 virus is currently under construction.

The example discussed above highlights the powerful approach of BAC-based methods for the manipulation and characterization of herpesvirus genomes. Schumacher et al. (23) have also demonstrated the power of these approaches through manipulation of MDV-1, which had previously proven very difficult to modify by conventional approaches. These studies also illustrated how BAC manipulation can be used to delete essential genes, which is not possible through conventional approaches without complementing cell lines or helper viruses.

An important and attractive feature of BAC-based manipulation of the BoHV-1 genome is the ease with which gene-targeting constructs can be generated. It has been demonstrated here and by Schumacher et al. (23) that successful recombination can occur with crossover regions of only 50 bp. Consequently, the crossover regions can be incorporated into PCR primers to allow rapid and simple generation of insertion and deletion constructs for any region of the genome for which the sequence is known. With an estimated 80 genes and a completed genome sequence available for BoHV-1, it is now possible to construct a full library of gene deletions for analysis.

Since gene deletions can now be constructed in a replication-independent environment, those genes which are essential for replication and those that severely limit replication can be differentiated, whereas conventional technologies do not permit this. The application of BAC technologies to herpesvirus biology (reviewed in reference 4) will enable a more complete understanding of these complex DNA viruses.

In conclusion, the complete genome of BoHV-1 has been cloned and can be stably propagated in *E. coli* strain DH10B as a BAC. In addition, the recently described GET recombination has been applied to demonstrate that specific changes can be made to the BoHV-1 genome in DH10B cells while maintaining the infectivity of the clone. Through application of this technology, significant advances in determining the roles that virus-encoded factors play in the different diseases involving BoHV-1 will be possible. Further, by simplifying the construction of recombinant BoHV-1, the more efficient utilization of BoHV-1 as a vaccine vector is now possible. The application of these technologies to BoHV-1 and other herpesviruses should lead to the development of safer and more efficient vaccines and potentially more-useful gene therapy vectors.

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