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Construction and manipulation of a full-length infectious bacterial artificial chromosome clone of equine herpesvirus type 3 (EHV-3)

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ABSTRACT

Equine herpesvirus type 3 (EHV-3) is the causal agent of equine coital exanthema, a disease characterized by pox-like lesions on the penis of stallions and the vulva of mares. Although the complete genomic sequence of EHV-3 has been recently made available, its genomic content remains poorly characterized and the molecular mechanisms of disease development not yet elucidated. In an attempt to facilitate genetic manipulation of EHV-3, we describe here the construction of a full-length infectious bacterial artificial chromosome (BAC) clone of EHV-3. Mini-F vector sequences were inserted into the intergenic region between ORF19 and ORF20 (UL41 and UL40, respectively) of EHV-3 strain C175 by homologous recombination in equine dermal cells (NBL-6). DNA of the resulting recombinant virus was electroporated into *E. coli* and a full-length EHV-3 BAC clone was recovered. Virus reconstituted after transfection of the EHV-3 BAC into NBL-6 cells showed growth properties *in vitro* that were indistinguishable from those of the parental virus. To assess the feasibility of mutagenesis of the cloned EHV-3 genome, recombinant viruses targeting the glycoprotein E (gE) gene were generated using Red recombination in *E. coli* and *in vitro* growth properties of the recombinant viruses were evaluated. We first repaired the gE (ORF74) coding region, since the parental virus used for BAC cloning specifies a truncated version of the gene, and then created gE-tagged and gE-null versions of the virus. Our results demonstrated that: (i) EHV-3 can be efficiently cloned as a BAC allowing easy manipulation of its genome; (ii) gE is dispensable for EHV-3 growth *in vitro* and is expressed as a product of approximately 110-kDa in infected cells; (iii) viruses having a deletion compromising gE expression or with a truncation of the cytoplasmic and transmembrane domains are significantly compromised with regard cell-to-cell spread. The cloning of EHV-3 as a BAC simplifies future studies to identify the role of its coding genes in viral pathogenesis and host immune responses.

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1. Introduction

Equine herpesvirus type 3 (EHV-3) is the causative agent of equine coital exanthema (ECE), a sexually transmitted disease with worldwide distribution that is characterized by pox-like lesions on the penis of stallions and the vulva of mares (Barrandeguy and Thiry, 2012). EHV-3 is endemic in the horse population and recent outbreaks have been reported in France (Pronost et al., 2012), Argentina (Barrandeguy et al., 2010), the USA (Equine disease quarterly, 2015) and UK (ICC, 2015). The disease is often mild and self-limiting, with treatment consisting of enforced sexual rest and possibly topical antibiotics to reduce secondary bacterial infections (Barrandeguy and Thiry, 2012). Neither infertility nor abortion occur as sequelae of the infection. EHV-3 belongs to the genus *Varicellovirus* of the subfamily *Alphaherpesvirinae*, has a class D genome consisting of a long and a short unique region (UL and US), both flanked by inverted repeats (TRL/IRL and IRS/TRS) (Sijmons et al., 2014).

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Up to date, EHV-3 remains a relatively poorly characterized virus. No detailed molecular studies on the function of its viral genes and gene products have been performed and most of EHV-3 research has focused in the understanding of disease transmission and pathogenesis in the natural host (Barrandeguy et al., 2008, 2012). A big contribution in the study of EHV-3 has been recently made by the release of the complete genome sequence of EHV-3 (Sijmons et al., 2014). Its 150-kb genome is predicted to encode 76 distinct genes, which is similar to what has been reported for other equine alphaherpesviruses, but EHV-3 has a significant degree of nucleotide divergence when compared to its closest relatives.

The introduction of bacterial artificial chromosome (BAC) technology has facilitated the construction of recombinant viruses and subsequent studies of the biology and pathogenesis of herpesviruses (Warden et al., 2011). BACs have been established for several human and animal herpesviruses including important pathogens of horses, poultry, cattle and swine (reviewed in Tischer and Kaufer, 2012). The generated BACs can be then stably maintained in *E. coli*, in which genetic mutations such as point mutations, deletions and insertions can be easily introduced by different mutagenesis methods including RecA- and Red/ET-mediated recombinations (Tischer and Kaufer, 2012). Upon viral reconstitution in cells, characterization of the recombinant virus can be easily achieved.

In an attempt to facilitate the genetic manipulation of EHV-3, we describe here the construction of a full-length infectious BAC clone of EHV-3. Mini-F vector sequences were inserted into the intergenic region between ORF19 and ORF20, the homologues of UL41 and UL40 of herpes simplex virus type 1 (HSV-1). We used EHV-3 strain C175 and recombination in equine dermal cells (NBL-6). DNA of the resulting recombinant virus was electroporated into *E. coli* and a full-length EHV-3 BAC clone was recovered. The transfection of the EHV-3 BAC into equine cells resulted in the reconstitution of infectious virus which showed similar growth properties *in vitro* when compared to the parental virus. The power of BAC mutagenesis was demonstrated by the generation of recombinant viruses targeting the glycoprotein E (gE) gene. Our results show that the BAC system applied to EHV-3 provides a reliable and efficient method to introduce genetic alterations into the viral genome in *E. coli* and subsequently analyze their effects on virus biology and pathogenesis.

2. Materials and methods

2.1. Virus strain, cells and transfection

Strain C175, isolated from a perineal-vaginal swab of a mare with signs of coital exanthema (Meyer et al., 1992), was used for the generation of the full-length infectious BAC clone of EHV-3. NBL-6 cells were used throughout the study. Cells were propagated in supplemented Iscove's modified Dulbecco medium (IMDM) (ThermoFisher Scientific) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 1 mM sodium pyruvate and 1× non-essential amino acids. All transfections described in the study were performed with the Amaxa Nucleofector II device (program T-030) using the Cell Line Nucleofector Kit V (Lonza) according to the manufacturer's instructions with 2×10^6 NBL-6 cells and 3–5 μg of purified plasmids or BAC DNA. After transfection, cells were plated in 2 wells of a 6-well plate.

2.2. Viral and BAC DNA extraction, PCR and sequencing

Viral DNA for PCR was extracted using the RTP DNA/RNA Virus Mini Kit (Stratek Molecular GmbH) from 200 μl of cell culture supernatants of infected cells according to the manufacturer's instructions. Primers were designed based on the published data for EHV-3 strain AR/2007/C3A (GenBank Acc. No KM051845). All PCRs reported in the study were carried out in a 25 μl reaction mixtures containing 5 μl 5× Phusion High Fidelity buffer, 0.2 μl of Phusion DNA polymerase (both from ThermoFisher Scientific), 2 μl of 2.5 mM each dNTPs (Bioline), 5 μl of 5× Q-solution (Qiagen), 1 μl each of 10 μM forward and reverse primers, 5.8 μl of H_2O and 5 μl of purified DNA. The temperature profile consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of DNA denaturation at 94 °C for 15 s, primer annealing for 0.5 min at 53 °C and an elongation step at 72 °C for 1 min. The final extension step was carried out at 72 °C for 5 min. For sequencing of PCR products, amplified DNA was purified with GeneMATRIX Basic DNA purification kit (Roboklon GmbH). Sanger sequencing was performed in both directions and carried out by LGC Genomics. BAC DNA was extracted by a column-based Nucleobond BAC 100 kit (Clontech Laboratories) following the manufacturer's instructions. Viral DNA extraction for restriction fragment length polymorphism (RFLP) analysis was done as follow. NBL-6 cells grown in 150 cm^2 dishes were infected with appropriate viruses. After total cytopathic effect (CPE) was evident, cellular debris were removed by centrifugation at 3000 × *g* for 15 min and supernatants were layered onto a 3 ml cushion of 30% (w/v) sucrose in PBS and centrifuged at 130,000 × *g* for 90 min at 4 °C. Viral pellets were resuspended in 1 ml of DNA extraction buffer (10 mM Tris [pH 8], 10 mM EDTA, 0.6% SDS) supplemented with 0.2 mg proteinase K/ml, 100 μg/ml of RNAse and incubated overnight at 56 °C. DNA was purified by phenol-chloroform separation, precipitated from the aqueous phase with isopropanol, and the final pellet resuspended in 100 μl of H_20. Two micrograms were digested with a chosen restriction enzyme for 3 h, applied to a 20 cm long 0.8% agarose gel and DNA fragments separated by electrophoresis at 65 V for 18 h.

2.3. Generation of an EHV-3 recombinant containing mini-F sequences

Two pairs of primers, P1/P2 and P3/P4 (Table 1), were designed to amplify the regions of the EHV-3 genome flanking the intergenic region between ORF19 and ORF20 (Fig. 1). PCR products of 1416- and 1137-bp in size were cloned into pUC18 using restriction enzyme sites present in the primers and the resulting recombinant plasmid was termed pUC_ORF19/20. A DNA cassette containing the mini-F sequence, the enhanced green fluorescent gene (GFP) under the control of the immediate early promoter of human cytomegalovirus (HCMV-IE), the *E. coli* xanthine-guanosine phosphoribosyl transferase (xgpt) and the chloramphenicol (CAM) resistance marker (cat) was released as a *Pac*I fragment from plasmid pHAII (a generous gift from Prof. Martin Messerle, Hannover, Germany) and cloned into the *Pac*I site present in pUC_ORF19/20 to construct transfer vector plasmid pMiniF_ORF19/20. The structure of the created plasmid was verified by restriction enzyme digestion and sequencing using primers P5-P11 (Table 1).

The transfer vector pMiniF_ORF19/20 was purified according to the protocol of the Qiagen plasmid Mini kit (Qiagen) and used for nucleofection as described above. The following day, cell culture supernatant was removed, and transfected cells were infected with EHV-3 strain C175 at a multiplicity of infection (moi) of 0.1 and harvested with a cell scraper when CPE was advanced. After one cycle of freezing and thawing, cellular debris was removed by low-speed centrifugation and the supernatant kept for further use. Recombinant viruses expressing the GFP were selected by plaque assay as follows: Appropriately diluted virus stocks (approximately 100 plaques per well) were inoculated onto NBL-6 cells grown in 6-well plates, overlaid with supplemented IMDM containing 3.2% low melting carboxy-methylcellulose (CMC, Sigma-Aldrich) and incubated for 3 days until virus plaques were clearly devel-

Table 1
Primers used in the study.

	Primer	Sequence 5′–3′
P1	19-1FW	gcag*aattc*agaagtttgcctccgatgaa
P2	19-1RV	gatg*gatcc*ctt*taattaa*gcgattaaaaatacagcgtt
P3	20-2FW	aca*ctgca*gct*ttaattaa*aacccggctcgagagtgttt
P4	20-2RV	cga*aagctt*atggctaggcgtttgtcccc
P5	M13 RV	agcggataacaatttcacacagg
P6	M13 RV	cccagtcacgacgttgtaaaacg
P7	miniF FW	ttgtagaggttttacttgct
P8	miniF RV	atatgcgaagtggacctgggaccg
P9	ORF19 RV	agcctcgtcgccctcgccgg
P10	ORF20 FW	agcatgtgactgccgcttgg
P11	ORF19 FW	agacgcaacgcccctgtttc
P12	join19_FW	ctggttatggttttccgttt
P13	join19_RV	aggttgacgcataggcgata
P14	join20_FW	ccgcagcgtcgtttctaaacag
P15	join20_RV	ccagagccaatcaatgtcgc
P16	gErestFW	tcccgccgaggttccgccagctcccggacgcggctgggctcggcgcta tctcgcgttcctgatcatagggataacagggtaatcg
P17	gErestRV	agcgaaccgccaagggaggcgaagatgatcaggaacgcgaga tagcgccgagcccagccgcgtccgccagtgttacaaccaattaacc
P18	gEnegFW	gagtgataggacgctccttcgcgggtttcggagaccaacca tgagccgccgcgccgtagggataacagggtaatcg
P19	gEnegRV	ttgtcgagcgccgcttcgataccctcggcgcggcggctcatg gttggtctccgaaagccagtgttacaaccaattaacc
P20	gEHAFW	ctacagcagcgtcgtctgtaaaattaagtccatcctaaaa**tacccatacgatgttccagattacgct** tgagccgccgcgccta*gggataacagggtaatcg*
P21	gEHARV	tgtcgagcgccgcttcgataccctcggcgcggcggctca**agcgtaatctggaacatcgtatgggta** ttttaggatggacttgccagtgttacaaccaattaacc
P22	gE FW	aacgcagcgttgtacagagt
P23	gE RV	cctcggtctccgattcaaag
P24	gE FW4	gacagggctgtatgtgtttatg

Restriction enzyme sites are given in italics. Underlined sequences indicate the template-binding region of the primers for PCR amplification with pLAY2. Sequence coding for HA tags are indicated in bold.

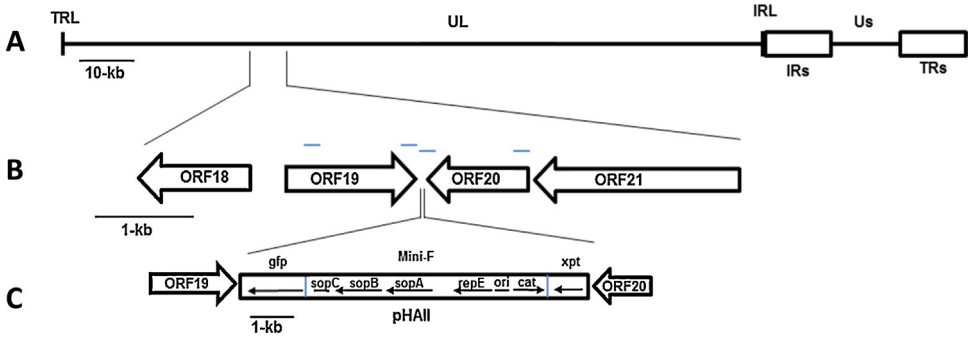

Fig. 1. Strategy for generation of the EHV-3 BAC. (A) The structure of the EHV-3 genome with the UL and US components is shown at the top. Terminal and internal repeat sequences are indicated (B) Genomic organization from ORF18 to ORF21 of the UL region of the EHV-3 genome. Representative positions of the primers used for amplification and subsequent cloning of homologous arms are shown. (C) The mini-F vector (enclosing sequences for F-plasmid replication and the cat gene), the eukaryotic selection marker xgpt and the gfp gene were integrated between the ORF19 and ORF 20 ORFs in the UL region of the viral genome by homologous recombination in Nbl-6 cells.

oped. Recombinant viruses expressing GFP were identified under the fluorescence microscope. Fluorescent plaques were picked and transferred to 1 ml of IMDM. After two more rounds of the plaque purification procedure, a stock of a plaque-purified GFP-expressing virus was prepared. The recombinant virus was designated rEHV-3 and its genomic structure verified using PCR followed by sequencing with primers P7-P15 described in Table 1.

2.4. Construction of the BAC clone of EHV-3

For extracting extrachromosomal circular forms of viral DNA, a modified Hirt's procedure was used (Hirt, 1967). Briefly, NBL-6 cells grown in a 10-cm dish were infected with the rEHV-3 virus at a moi of 3 for 8 h. Cells were washed on the tissue culture dish once with PBS, and then 1 ml of DNA extraction buffer (described above) was added for 5 min. The sample was transferred to a microcentrifuge tube followed by addition of 10 μl of proteinase K (10 mg/ml). The sample was incubated at 55 °C for 60 min before 250 μl of 5 M NaCl was added to each tube. After overnight incubation at 4 °C, the supernatant was collected by 30 min of centrifugation at 18,000 × *g*. Supernatants were extracted with phenol-chloroform twice before DNA was precipitated with 2.5 vol. of ethanol. The DNA pellets were dissolved in a volume of 50 μl of H_20. DNA (500 ng) was electroporated into MegaX DH10B *E. coli* (ThermoFisher Scientific) using 1-mm cuvettes with the following settings: 1800 V, capacitance 25 μF and resistance 200 Ω (Genepulser Xcell electroporation system – Biorad). The transformed bacteria were incubated in 1 ml of recovery medium for 1 h and then plated on Luria-Bertani (LB) agar containing 30 μg/ml CAM. Two days after, single colonies were picked and cultured in LB broth supplemented with 30 μg/ml CAM and BAC DNA was isolated by alkaline lysis. EHV-3 BAC DNA (named pEHV-3) was screened by RFLPs. Column-purified pEHV-3 BAC DNA

was used to electroporate *E. coli* GS1783 (a kind gift from Gregory A. Smith, Northwestern University, USA) using the procedure described above.

2.5. Reconstitution and characterization of BAC-derived EHV-3

Three micrograms of pEHV-3 was nucleofected into NBL-6 cells as described. When CPE was advanced, cells and supernatants were collected, freeze-thawed, and a virus stock named vEHV-3_BAC prepared and titrated by standard plaque assay. Viral DNA of the reconstituted virus was analyzed by RFLP. The *in vitro* growth characteristics of vEHV-3_BAC were evaluated as follows: For plaque size measurements, confluent monolayers of NBL-6 cells in 12-well plates were infected with 20 PFU/well of the respective virus. After 1 h of incubation at 37 °C, a semi-solid medium consisting of supplemented IMDM containing 3.2% low melting CMC was added. At 2 days post-infection (p.i.), plaques were either visualized under the fluorescence microscope or formalin-fixed and stained with 0.75% crystal violet. Fifty plaques were photographed and plaque diameters were determined using the ImageJ software (http://rsb.info.nih.gov/ij/). The Shapiro–Wilks test was used to assess for normality and the Student's *t*-test was employed to compare the mean diameters of the plaques of the examined viruses.

For one- and multi-step growth kinetics, NBL-6 cells grown in 24-well plates were infected respectively at a moi of 1 or 0.01. Virus was allowed to attach for 1 h after which two PBS washes were carried out to remove unbound virus. At indicated time points, cells were scraped into the culture medium, freeze-thawed twice and collected by centrifugation. Supernatants were aspirated and kept at −80 °C. Viral titers were calculated by standard plaque assays using NBL-6 cells. Growth curves were determined in three independent experiments and means and standard deviations were computed and plotted. Student's *t*-test was used to test the differences of viral growth kinetics of examined viruses.

2.6. BAC mutagenesis and virus reconstitution

For all genetic manipulations, *E. coli* strain GS1783 harboring a temperature-sensitive Red recombination system and a gene encoding the endonuclease I-*Sce*I in its genome was used (a kind gift from Dr. Greg Smith, Northwestern University, Chicago, IL, USA). GS1783 cells containing pEHV-3 were maintained in LB broth containing 30 μg/ml CAM. For the manipulation of the EHV-3 BAC, the two-step Red recombination protocol was followed as described by Tischer et al. (2006). First, we reconstituted the gE (ORF74) coding region by deleting two additional nucleotides (GC), that caused a change of reading frame, present after position 1205 in the gE gene as reported for strain AR/2007/C3A (position 139,250 in GenBank Acc. No KM051845) using primers 16 and 17. Then, gE-null and -tagged versions were constructed respectively by deleting completely ORF74 (positions 138,046–139,710 in KM051845) using primers P18 and P19 or adding an HA tag before the stop codon (located at position 139,711–139,713 in KM051845) with primers P20 and P21. PCR amplification generated a linear DNA fragment containing a kanamycin resistance expression cassette (Kan^r), an I-*Sce*I restriction enzyme site, and flanking sequences derived from EHV-3 genomic DNA. The recombination protocol was done as follows: GS1783 containing pEHV-3 BAC were cultured in LB medium containing 30 μg/ml CAM at 32 °C overnight. The overnight culture was then diluted at a 1:10 ratio with fresh LB CAM and continued to grow until and OD_{600nm} of 0.4–0.6 was reached. Then, the culture was transferred to a 42 °C water bath and shaken for 20 min which induced the expression of the Red recombination system. Finally, the bacteria were chilled in ice water and then harvested for the preparation of electrocompetent cells by successive washes in cold distilled water followed by centrifugation. Next, 100 ng of a *Dpn*I-digested and column-purified PCR product generated using plasmid pLAY2 (Tischer et al., 2006) and appropriate primers was transformed into GS1783 cells harboring the BAC construct. The bacteria were cultured at 32 °C for 1 h, and then plated onto LB agar plates containing 30 μg/ml CAM and 50 μg/ml kanamycin to select for *E. coli* clones harboring the Kan^r gene (named "intermediates"). The final constructs pEHV-3_gErest, pEHV-3_ΔgE and pEHV-3_gErestHA were obtained after removal of the Kan^r gene in a second round of Red recombination which consisted of the following. One-hundred microliters of an overnight culture of the kanamycin-resistant GS1783 cells was inoculated into 2 ml of medium containing 30 μg/ml chloramphenicol. The bacteria were cultured for 3 h at 32 °C, and then equal amounts of LB supplemented with 2% L-arabinose was added to induce I-*Sce*I expression. After 1 h incubation at 32 °C, the culture was transferred into a 42 °C water bath and incubated for 30 min. The culture was then transferred to a 32 °C shaker and cultured with shaking for 1.5 h before being transferred to agar plates containing 30 μg/ml CAM. Kanamycin-sensitive but CAM-resistant clones were selected by plating single clones onto CAM-kanamycin containing plates. Generated BACs were characterized by RFLP and PCR (primers P22-P23) followed by sequence of the amplified product using primers P22 to P24. To produce BAC derived viruses vEHV-3_gErest, vEHV-3_ΔgE and vEHV-3_gErestHA, NBL-6 cells were nucleofected with 2 μg of column purified BAC DNA as described. *In vitro* growth characteristics of reconstituted viruses were evaluated as described for vEHV-3_BAC.

2.7. Immunoblotting

For Western blot analysis, NBL-6 cells were seeded in 6-well plates that were either mock-infected or infected with appropriate viruses. Pellets of cells were resuspended in radioimmunoprecipitation assay buffer (1 mM Tris, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 5 M sodium chloride, 0.5 mM EDTA, 0.1% SDS) and a protease inhibitor cocktail (Roche). Samples were mixed with sample loading buffer (1 M Tris–HCl, pH 6.8, 0.8% SDS, 0.4% glycerol, 0.15% β-mercaptoethanol, 0.004% bromophenol blue), heated at 65 °C for 5 min, and subjected to 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Roth GmbH). The membranes were blocked using 5% nonfat milk and then probed with antibodies diluted in PBS-0.1% Tween-20. Expression of EHV-3 gE was detected with an anti-HA tag monoclonal antibody (Cell Signaling Technology) followed by anti-mouse IgG-peroxidase conjugate (Santa Cruz Biotechnology). Reactive bands were visualized by enhanced chemiluminescence (ECL plus – GE Healthcare)

3. Results

3.1. Generation of EHV-3 BAC and reconstitution of infectious virus

First, we constructed the recombination plasmid pMiniF_ORF19/20 that contained the mini-F with GFP and xgpt and flanked by viral sequences compromising the ORF19, ORF20 and the intergenic region without any nucleotide deletion. Recombination was expected to occur between homologous sequences present in the plasmid and the EHV-3 viral genome. The recombinant plasmid was nucleofected into NBL-6 cells, which was followed by virus infection the day after. A pure EHV-3 recombinant virus stock was obtained after three rounds of purification of fluorescent plaques and its structure was verified by PCR and sequencing. Results obtained by DNA sequencing confirmed that vector DNA sequences were inserted at the expected location on the EHV-3 genome between nucleotides 26,730 and 26,731

of the reference sequence. No alterations around either of the junction sites were observed when compared to the corresponding sequences of parental EHV-3 C175. Circular intermediates of the recombinant viral genome were isolated from infected cells and electroporated into commercially available *E. coli* MegaX DH10B^{TM} T1R cells to increase transformation efficiency. Column-purified pEHV-3 BAC DNA was used to electroporate *E. coli* GS1783 cells to allow BAC manipulation.

3.2. Characterization of the EHV-3 BAC

BAC DNAs were purified from CAM resistant bacterial colonies and characterized by *Hin*dIII digestion and sequencing using the same primers as for rEHV-3. Fig. 2 shows a clone with an expected RFLP pattern predicted from the complete genome sequence of EHV-3 strain AR/2007/C3A (GenBank accession number KM051845). There is no *Hin*dIII recognition site in the 8,8-kb inserted foreign sequence, therefore a 28-kb fragment present in wild-type/parental EHV-3 (Fig. 2A, lane 1 and B) increased to approximately 37-kb in the pEHV-3 clone (Fig. 2A, lane 2 and B). Circularization of the EHV-3 genome created a new fragment with an expected length of approximately 15-kb upon the fusion of the 1392- and a 13,722-bp terminal fragments (Fig. 2A, lane 2). The RFLP pattern obtained using the gel toolbar of Vector NTI version 11.5 (ThermoFisher Scientific) was in perfect agreement with the banding pattern observed on the agarose gel (Fig. 2C) indicating that examined *Hin*dIII restriction sites were completely conserved in the EHV-3 BAC. No mutations were observed in the targeted EHV-3 genomic region between cloned pEHV-3 and the recombinant virus rEHV-3.

3.3. Infectious virus was successfully reconstituted from pEHV-3 BAC, showed expected RFLP pattern and had similar in vitro growth properties when compared to parental virus

To reconstitute infectious viruses, the pEHV-3 BAC was nucleofected into NBL-6 cells. Viral plaques appeared after 3 days and full CPE development was observed 6 days after transfection. The DNA profile of the reconstituted virus was assessed by digestion with *Hin*dIII. As shown in Fig. 2A (lane 3), the linear reconstituted virus showed a similar pattern when compared to the parental virus (Fig. 2, lane 1), with the exception of a larger 37-kb (instead of 28-kb band) due to the insertion of the foreign sequences.

To determine whether the insertion of mini-F sequences had an effect on the EHV-3 growth *in vitro*, the plaque sizes and the growth kinetics of the reconstituted vEHV-3_BAC was compared to that of the parent C175 strain. As shown in Fig. 3, no significant difference of plaque sizes was observed between the two viruses ($p > 0.05$, Student's *t*-test). When growth kinetic was assessed, no significant differences were detected with viral titers increasing from 4 to 24 h or 8 to 36 h p.i. in one- and multi-step time course experiments, respectively. The results suggest that insertion of foreign vector sequences in the intergenic region between ORF19 and ORF20 had no detrimental effect on viral growth and cell-to-cell spread of EHV-3.

3.4. The EHV-3 genome can be easily manipulated in bacteria via the Red recombination system

To assess the feasibility of mutagenesis of the cloned EHV-3 genome, we decided to construct an EHV-3 mutant virus with a complete deletion of the gE gene by manipulating the generated EHV-3 BAC. To create such a virus, we first sequenced the predicted ORF74 coding for gE of strain C175 and that of its derivative pEHV-3 BAC (deposited in GenBank under Acc. No KX822029). Based on the nucleotide sequences, expression of a 408 amino acid-truncated version of gE lacking the entire cytoplasmic tail and the transmembrane domain was predicted using the Phobius protein algorithm (http://phobius.sbc.su.se/index.html) when compared to the sequence of EHV-3 strain AR/2007/C3A present in GenBank. Therefore, we first decided to restore the complete gE gene, construct an HA-tagged version and delete completely the gE gene in our infectious clone (Fig. 4). Forward and reverse primers were specifically designed to construct such variants using Red-mediated *en passant mutagenesis* (Table 1). Integrity of the genomic organization before and after recombination events was checked by RFLP (data not shown) and PCR (Fig. 5) followed by sequencing of amplified products (see nucleotide and amino acid sequence alignments in Supplementary Figs. 1 and 2). Reconstituted vEHV-3gErest, vEHV-3ΔgE and vEHV-3_gErestHA viruses were obtained after nucleofection. Inserted modifications confirmed by PCR and sequencing of amplified products were in agreement with those observed for the BAC constructs.

3.5. A gE-null EHV-3 or an EHV-3 lacking the gE cytoplasmic and transmembrane domain cannot efficiently spread in cultured cells

In vitro growth kinetics were performed to assess whether either the truncated version of gE present in vEHV-3_BAC or the gE-null version (vEHV-3ΔgE) had an effect in EHV-3 replication and cell-to-cell spread. The virus vEHV3_gErest containing the full-length version of gE was used as control. The relative plaque sizes produced by either vEHV-3ΔgE or vEHV-3_BAC were similar in size but significantly smaller ($p < 0.001$, Student's *t*-test) than those produced by vEHV3_gErest (Fig. 6). Even though vEHV3ΔgE and vEHV3_BAC produced smaller plaques, they replicated with similar kinetics and produced virtually identical virus yields when compared to the vEHV3_gErest (Fig. 6). The results indicate that in EHV-3 with a deletion compromising either the entire gE coding region or the glycoprotein's cytoplasmic and transmembrane domain are unable to spread efficiently from cell-to-cell *in vitro*.

3.6. A 110-kDa gE protein is detected in cell lysates using vEHV-3_gErestHA, the gE-tagged version of restored virus

Lysates from vEHV3_gErestHA-, vEHV3_gErest- and mock-infected cells (Fig. 7) were subjected to immunoblot analysis as described under material and methods. An approximately 110-kDa protein immunoreacted with an antibody against HA in vEHV3_gErestHA (Fig. 7, lane 1) while no reactivity was detected in the others two cell lysates (Fig. 7, lane 2 and 3). The results indicated that the fully processed form of EHV-3 gE is present in lysates of infected cells and that the apparent molecular mass of the mature protein exceeds that predicted by 50 kDa.

4. Discussion

In this study, we successfully cloned and achieved mutagenesis of the EHV-3 genome as an infectious BAC in *E. coli*. The BAC sequence was inserted by homologous recombination between ORF19 and ORF20, the UL41 and UL40 homologues of HSV-1, which encode a virion host shut-off tegument protein and a small subunit of ribonucleotide reductase, respectively. Both genes were shown to be non-essential for viral replication in cell culture for many herpesviruses (Darling et al., 1989; Fenwick and Everett, 1990; Matis and Kúdelová, 2001) and expected to be transcribed in opposite directions relative to each other. Neither disruption of any transcriptional unit nor nucleotide deletions in the ORF19-ORF20 intergenic sequence was, however, expected by applying this strategy. The circular intermediates of the recombinant, mini-F containing EHV-3 genome were isolated and transformed into *E. coli*. An isolated BAC clone exhibited the expected complete DNA

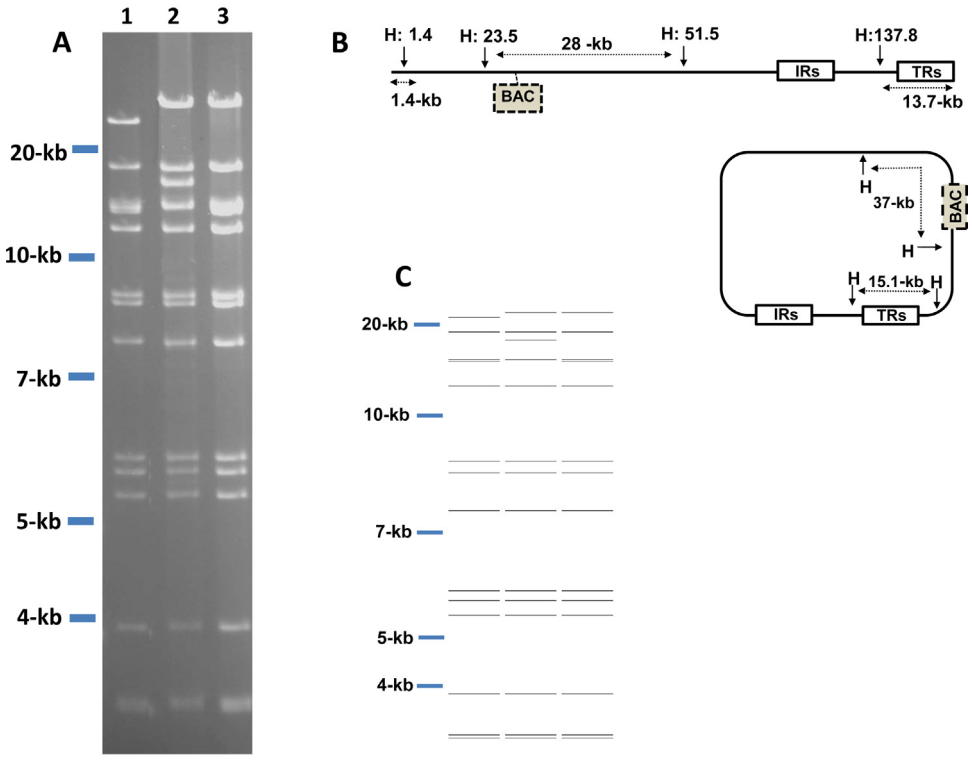

Fig. 2. (A) Structural analysis of parental EHV-3 strain C175 (lane 1), pEHV-3 BAC (lane 2) and the reconstituted virus vEHV-3_BAC (lane 3). Genomic and BAC DNAs digested with *Hin*dIII, separated by 0.8% agarose gel electrophoresis and stained with ethidium bromide. Marker bands are indicated on the left. (B) Predicted structure of the EHV-3 BAC plasmid. The positions of relevant *Hin*dIII sites (H) and the sizes of expected fragments are shown. (C) Predicted RFLP pattern based on the gel toolbar of Vector NTI Advance® 11.5 software (ThermoFisher Scientific) using the whole genome sequence of strain AR/2007/C3A deposited in GenBank under Acc. No KM051845. Superposition of two bands is shown as high intensity. Marker bands are indicated on the left.

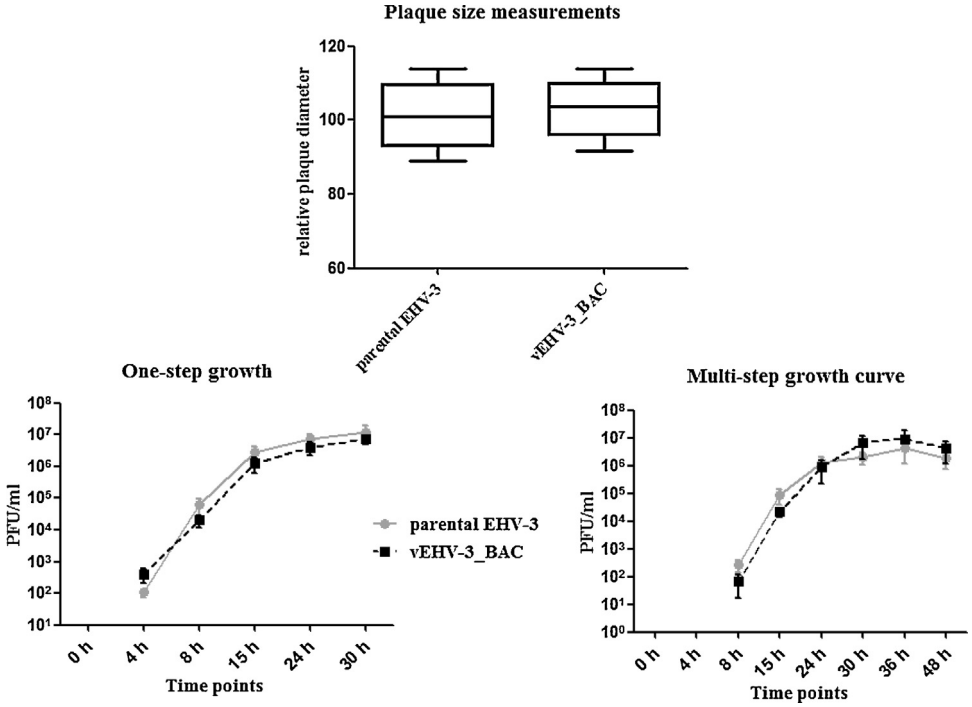

Fig. 3. *In vitro* growth properties of parental EHV-3 strain C175 and the BAC-derived reconstituted vEHV3_BAC virus. For plaque size measurements, box-and-whisker plots of plaque diameters of 50 plaques relative to parental virus are shown. One- and multi-step growth curves are displayed at the bottom of the figure.

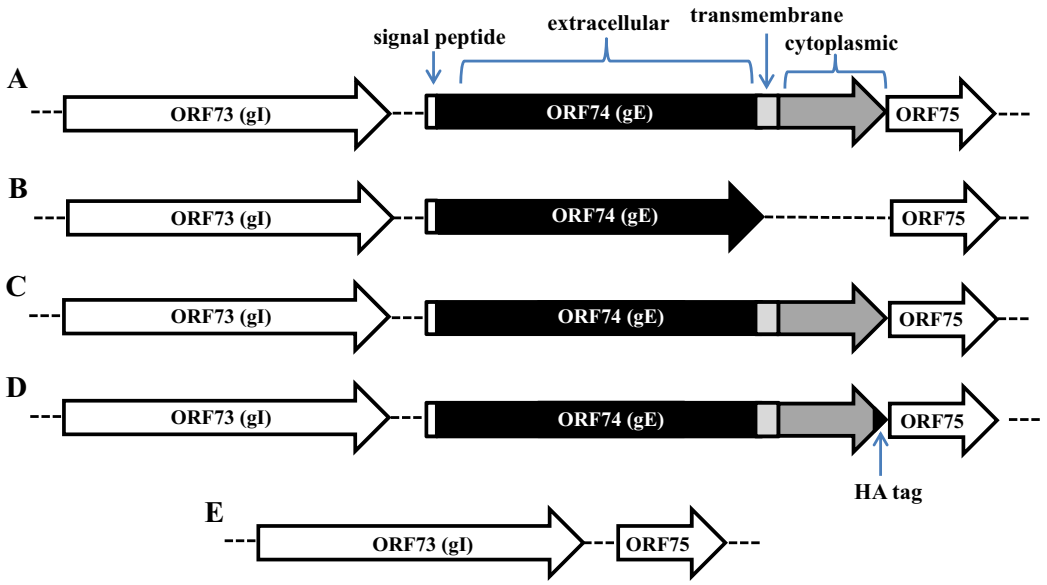

Fig. 4. Predicted gE and its domains from (A) EHV-3 AR/2007/C3A strain (GenBank, Acc. No KM051845) and (B) EHV-3 strain C175 used in the study. Generated EHV-3 infectious clones using the red recombination system are shown in C (pEHV-3_gErest), D (pEHV-3_gErestHA) and E (pEHV-3_ΔgE).

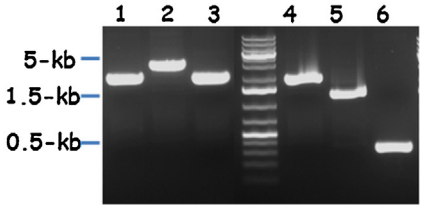

Fig. 5. Verification of the recombination events for the construction of pEHV-3_gErest (lanes 1–3) and pEHV-3_ΔgE (lanes 4–6). PCR amplification of pEHV-3 using P22 and P23 ORF74 franking primers gave a DNA band of 2057-bp (lane 1 and 4). Intermediates of 3043- (lane 2) and 1378-bp (lane 5) resulted after 988-bp Kanr/I-*Sce*I cassette integration. A second recombination event removed the Kanr/I-*Sce*I cassette and either restored (2055-bp band, lane 3) or delete (390-bp band, lane 3) ORF 74. A 1-kb plus DNA Ladder (ThermoFisher Scientific) is included in the middle lane. Size of bright reference bands are indicated on the left. BAC pEHV-3_gErestHA had a similar PCR pattern as pEHV-3_gErest (data not shown).

sequence as predicted by its RFLP pattern and when compared to the parental virus. Infectious virus was successfully reconstituted by transfecting NBL-6 cells with the generated full-length EHV-3 BAC. The insertion of foreign genes did not affect viral growth in cell culture; hence, we concluded that the intergenic region of the ORF19 and ORF20 of EHV-3 is a suitable site for the insertion of foreign sequences and that EHV-3 is capable of harboring approximately 9-kb of foreign sequence without any deleterious effect on virus growth.

To assess the feasibility of mutagenesis of the cloned EHV-3 genome, we decided to construct an EHV-3 mutant virus with a complete deletion of the gE gene by manipulating the generated EHV-3 BAC. The reason to construct such a virus mutant was based on: (1) the consequences of the deletion can be tested *in vitro* since gE mutants of other alphaherpesviruses have distinct plaque-size phenotypes (Damiani et al., 2000; Matsumura et al., 1998; Tirabassi et al., 1997; Trapp et al., 2003; Wisner et al., 2000) and (2) a potential vaccine candidate for protection against disease induced by EHV-3 can be provided, given that gE-negative viruses are attenuated but highly immunogenic in related viruses and used as commercial vaccines (Jacobs and Kimman, 1994; Kaashoek et al., 1995; Tsujimura et al., 2009; van Engelenburg et al., 1994). Homologous glycoproteins of HSV-1, pseudorabies (PRV) and Varicella Zoster Virus (VZV) associate with gI forming a noncovalent complex in infected cells, functioning as an immunoglobulin G (IgG) Fc binding protein and involved in cell-to-cell spread (reviewed in Johnson and Huber, 2002).

Interestingly, sequencing of the gE gene revealed that pEHV-3 and its parental virus specify a truncated version of the gene and lack the cytoplasmic tail and transmembrane domain. Deletions in the US region of alphaherpesviruses including the coding region of gE through serial passages in cell culture and/or animals models is not an uncommon event (Bartha, 1961; Kaashoek et al., 1994; Stokes et al., 1989; Yang et al., 2015). The number of passages in equine cells of our EHV-3 C175 strain could not be traced since its first isolation, but the absence of a full gE coding region may indicate a substantial number of passages that may have gone to the same extent as described for other members of the subfamily and be at the root of the genomic modification. Therefore, first the entire sequence coding for gE was reconstituted in the original EHV-3 BAC. Next, a HA tag sequence was inserted at the C-terminus of the gE coding sequence of the newly generated BAC to allow protein detection. Finally, a gE-null EHV-3 virus was created. All generated BACs yielded infectious virus when transfected into NBL-6 cells demonstrating that viruses either devoid of gE or containing a gE-truncated version can be efficiently propagated *in vitro* as those containing a complete gE. However, when cell-to cell spread was assessed, the viruses expressing a truncated gE exhibited a small-plaque phenotype, which was indistinguishable from the gE-null EHV-3 mutant. Plaques produced by either virus were significantly smaller when compared to those produced by the EHV-3 virus expressing the restored full-length version of gE. The small-plaque phenotype of the virus having the truncated version of gE shows that the transmembrane and/or the cytoplasmic domains of gE are important for its function in efficient cell to cell spread of EHV-3 *in vitro*. It has been shown that the cytoplasmic domains of gE are similarly important for cell-to-cell spread of BHV-1, PRV and HSV-1 (Brack et al., 2000; Tyborowska et al., 2000; Wisner et al., 2000). However, it should be noted that the effect of such genomic alterations might have had on the transcription of downstream genes has not been evaluated. An only positional ORF75 homolog, predicted to encode a 141 amino acids-long type II putative membrane protein US8A (Sijmons et al., 2014), initiates immediately downstream of the gE gene. No sequence similarity to ORF75 of other alphaherpesviruses is found by using the BLAST algorithm.

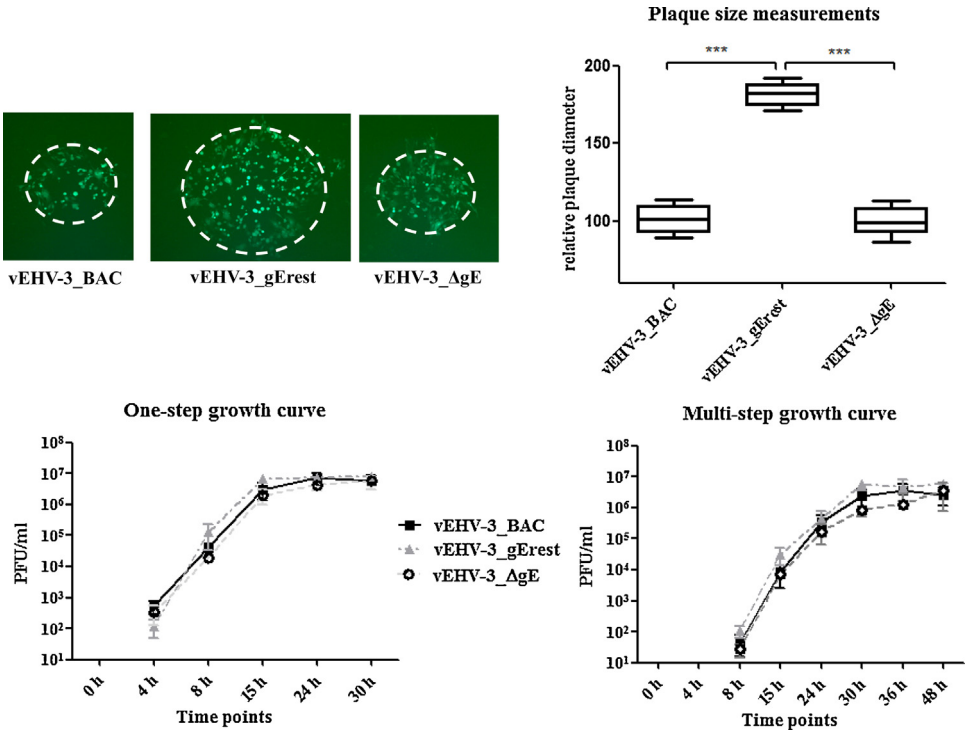

Fig. 6. *In vitro* growth properties of vEHV-3_BAC, vEHV-3_gErest and vEHV-3_ΔgE. Green fluorescence plaques, with shapes indicated by dashes, were visualized under the fluorescence microscope after 2 days in culture. For plaque size measurements, box-and-whisker plots of plaque diameters of 50 plaques relative to vEHV-3_BAC virus are shown. One- and multi-step growth curves are displayed at the bottom of the figure.

Transcripts initiating upstream of ORF74 and using poly(A) signals located at the end of both ORF75 and ORF76, which are present in the EHV-3 genome (Sijmons et al., 2014), have been described in other members of the subfamily (Damiani et al., 1999; Rixon and McGeoch, 1985; Willemse et al., 1995). Hence, a similar organization could be expected with transcription of ORF75 and ORF76 unaltered by the genetic modifications introduced in the generated viruses. It is worth to mention that given the impaired capacity of strain C175 to spread, the EHV-3 gE mutant derived by serial passage in cell culture may be a good candidate for a marker vaccine to control EHV-3 infection. Live and killed gE-deleted marker vaccines are now used worldwide, in combination with gE-based diagnostic tests to control bovine herpesvirus type 1 (BHV-1 – van Drunen Littel-van den Hurk, 2006), EHV-1 (Andoh et al., 2013; Tsujimura et al., 2009) and PRV (Müller et al., 2011) in cattle, horses and swine.

Using a gE-tagged virus version of EHV-3, gE was detectable as an approximately 110-kDa protein in infected cells. The observed molecular mass of gE is considerably larger than that predicted from the primary sequence (62-kDa). As predicted from it deduced amino acid sequence using NetOglyc 4.0 and NetNglyc 1.0 servers (http://www.cbs.dtu.dk/services/), EHV-3 gE contains 4 and 12 N- and O-linked sites for glycosylation, respectively. It is evident from the discrepancy in molecular mass as determined by SDS-PAGE, glycosylation seems quite extensive. Further work, however, is necessary to understand the migration behavior of the glycoprotein after SDS-PAGE, but mature forms of gE of 110-, 98-, and 92-kDa have been detected for PRV (Tirabassi and Enquist, 1999), VZV (Olson et al., 1997) and BHV-1 (Whitbeck et al., 1996), respectively.

In summary, we successfully constructed a full-length EHV-3 BAC and reconstituted infectious virus after transfection from cloned DNA. We have shown that the constructed BAC could be efficiently manipulated in *E. coli*. Derived virus mutants created using this strategy would help us understand the functions of genetic elements present in the EHV-3 virus genome and study their roles in viral pathogenesis, with the potential of generate a preventative vaccine against EHV-3-induced disease in horses.

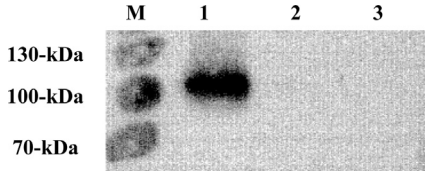

Fig. 7. Detection of a 110-kDa gE protein using lysates of vEHV-3_gErestHA (lane 1) infected cells. Mock- and vEHV-3_gErest-virus infected cells are shown respectively in lanes 3 and 2.

Competing interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2016.11.012.

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