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Development of a bacterial artificial chromosome (BAC) recombineering procedure using *galK*-untranslated region (UTR) for the mutation of diploid genes

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Abstract

Bacterial artificial chromosome (BAC) recombineering using *galK* selection allows DNA cloned in *E. coli* to be modified without introducing an unwanted selectable marker at the modification site. Genomes of some herpesviruses have a pair of inverted repeat sequences that makes it very difficult to introduce mutations into diploid (duplicate) genes using the *galK* selection method. To mutate diploid genes, we developed a *galK*-UTR BAC recombineering procedure that blocks one copy of the target diploid gene by insertion of a *galK* untranslated region (UTR), which enables the simple mutation of the other copy. The blocked copy can then be replaced with a UTR-specific primer pair. The *IR2* gene of equine herpesvirus 1 (EHV-1) maps within both the internal (IR) and terminal repeat (TR) of the genomic short region and is expressed at low levels because its promoter is TATA-less. Both *IR2* promoters in EHV-1 BAC were replaced with a mutant *IR2* promoter containing three Sp1-binding motifs and a consensus TATA box by *galK*-UTR BAC recombineering. The expression level of the *IR2* protein controlled by the modified promoter increased approximately 4-fold as compared to that of wild-type EHV-1. The *galK*-UTR method will provide a useful tool in studies of herpesviruses.

Keywords

equine herpesvirus 1; diploid *IR2* gene; bacterial artificial chromosome (BAC) recombineering; *galK* selection; *galK* untranslated region (UTR)

1. Introduction

Bacterial artificial chromosomes (BACs) have become indispensable tools for the mapping of genomic sequences, genetic engineering of animals and plants, and studies of the structures and functions of chromosomal elements essential for DNA replication and transcription (Heintz, 2001; Muylers et al., 2001; Poser et al., 2008; Tsyrlunyk and Moriggl, 2008). However, conventional molecular cloning methods using restriction enzymes are unsuitable for modifications of these constructs due to their large sizes (100-300kb). Recombineering (recombination-mediated engineering), which was developed from phage-

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based *Escherichia coli* homologous recombination systems, has enabled a wide variety of modifications of large DNA constructs that were virtually impossible in the past (Zhang et al., 1998; Copeland et al., 2001).

Recombineering is made possible through the use of three λ Red-encoded genes: *exo* (a 5'–3' exonuclease), *bet* (a pairing protein) and *gam* (inhibitor of the *Escherichia coli* RecBCD exonuclease). These genes can be expressed from a stably integrated defective λ prophage, where *exo*, *bet* and *gam* are controlled by the strong phage promoter *pL* under stringent control of the temperature-sensitive repressor, *cI857* (Yu et al., 2000; Lee et al., 2001). At 32°C, the recombination system is inactive because of the active λ repressor. By shifting to 42°C, the λ repressor becomes inactivated, and the recombinases are expressed from the *pL* promoter, thereby allowing homologous recombination to occur.

However, a major limitation to the usefulness of BACs is the ease and efficiency with which one can make subtle and 'seamless' mutations, such as point mutations and clean deletions, or introduce in-frame fusions of cDNAs or epitope tags without leaving a selectable marker or a *loxP/Frt* site at the modification site. A novel and highly efficient *galK* positive/negative selection method for the manipulation of BACs was developed (Warming et al., 2005). The *E. coli* galactose operon consists of four genes: *galE*, *galT*, *galK* and *galM*, all of which are necessary for growth and utilization of galactose as the only carbon source. The *galK* gene product, galactokinase, catalyzes the phosphorylation of galactose to galactose-1-phosphate in the galactose degradation pathway. Galactokinase also efficiently catalyzes the phosphorylation of a galactose analog, 2-deoxy-galactose (DOG). The product of this reaction cannot be further metabolized, leading to a toxic build-up of 2-deoxy-galactose-1-phosphate (Alper and Ames, 1975). Thus, both positive and negative selection can be conferred by *galK*. Because *galK* is used for both selection steps, background following negative selection is reduced, and colony screening is not necessary.

Herpesvirus genomes are classified into groups A to F with regard to their structural features, such as the number and location of repeat and inverted sequences and ability to exist in one, two, or four isomeric arrangements (Roizman and Pellet, 2001). In the genomes of viruses comprising group A, exemplified by channel catfish herpesvirus, a large sequence from one terminus is directly repeated at the other terminus. Herpesviruses with genomes of group D, such as equine herpesvirus 1 (EHV-1), bovine herpesvirus 1 (BHV-1), pseudorabies virus (PRV), and varicellazoster virus (VZV), have a fixed long region (*U_L*) covalently linked to a short (*S*) genomic region comprised of a pair of inverted repeat sequences that bracket a unique short segment (*US*). In group E genomes exemplified by herpes simplex virus (HSV) and human cytomegalovirus (HCMV), sequences from both termini are repeated in an inverted orientation. Recently, Yakushko et al. (2011) showed that a Kaposi's sarcoma herpesvirus (KSHV) genome as a bacterial artificial chromosome (KSHV-BAC36) contains a duplication of a 9 kb fragment of the long unique region in the terminal repeat region. The EHV-1 genome of 150 kb encodes 78 genes and contains a pair of identical internal repeat (*IR*) and terminal repeat (*TR*) sequences (Henry et al., 1981; O'Callaghan and Osterrieder, 2008; Ruyechan et al., 1982; Telford et al., 1992; Whalley et al., 1981). Each inverted repeat harbors six genes (*IR1* to *IR6*). The diploid *IR2* gene is embedded within the sole immediate-early (*IE*; *IR1*) gene (Harty and O'Callaghan, 1991) and encodes the 1165-aa early *IR2* protein (*IR2P*) which inhibits viral gene expression (Kim et al., 2006 and 2011). In comparison to the *IE* protein, *IR2P* is expressed at a low level at the early stage of infection because of its weak promoter (Kim et al., 2006).

It was considered almost impossible to mutate duplicate genes in the BAC DNA by use of BAC recombineering using *galK* selection. When mutating the second copy of a diploid gene, it is very difficult to distinguish the mutation site or even confirm the mutation itself.

To mutate the diploid *IR2* gene, we developed a *galK*-UTR BAC recombineering method and successfully generated the IR2 mutant virus EHV-1 ST2/ST1 that contains three Sp1-binding motifs and a consensus TATA box in the IR2 promoter.

2. Materials and Methods

2.1. Recombineering and *galK* selection

Recombineering was performed as previously described (Warming et al., 2005). SW106 *E. coli* (Warming et al., 2005) harboring the RacL11 BAC (Rudolph et al., 2002) was used for recombineering. 500 µl of an overnight culture was diluted in 25 ml LB medium with or without chloramphenicol selection (12.5 µg/ml) in a 50 ml flask and allowed to grow at 32°C until the bacteria were in log-phase growth ($A_{600} \approx 0.6$). Then, 10 ml was transferred to another baffled 50 ml conical flask and heat-shocked at 42°C for 15 min. The remaining culture was left at 32°C as the uninduced control. After 15 min, the induced and uninduced samples were briefly cooled in an ice/waterbath slurry and pelleted using $5,000 \times g$ at 0°C for 5 min. The pellet was resuspended in 1 ml ice-cold ddH₂O. After the second washing with 9 ml ice-cold ddH₂O, the pellet (~50 µl each) was kept on ice until electroporated with PCR product. An aliquot of 25 µl was used for each electroporation in a 0.1 cm gap cuvette (BioRad, Hercules, CA) at 25 µF, 1.75 kV and 200 Ω. After electroporation the bacteria were recovered in 1 ml LB for 1 h at 32°C. For the counter selection step, the bacteria were recovered in 10 ml LB and incubated for 4.5 h at 32°C. After the recovery period, the bacteria were washed twice in 1× M9 salts (6g Na₂HPO₄, 3g KH₂PO₄, 1g NH₄Cl, 0.5g NaCl per liter), and the pellet was resuspended in 1 ml of 1× M9 salts before plating serial dilutions on minimal medium plates (Warming et al., 2005).

2.2. PCR amplification of the *galK* targeting cassette and *galK* untranslated region (UTR)

Primers used in the experiments are shown in Table 1. PCR was performed using AccuPrimer Pfx polymerase (Invitrogen, San Diego, CA). The UTR and *galK* were amplified using 1 ng pGalK (Warming et al., 2005) as templates in the following conditions: 94°C for 15 s, 60°C for 30 s and 68°C for 1.5 min, for 35 cycles. The PCR product was purified by PCR purification kit (Qiagen, Valencia, CA) and eluted in 40 µl ddH₂O. An aliquot of 3 µl was used for each experiment.

2.3. Viruses and cell culture

The pathogenic RacL11 EHV-1 was used as the parental virus (Ahn et al., 2010). Mouse fibroblast L-M, rabbit kidney RK-13, and equine dermis NBL6 cells were maintained at 37°C in complete Eagle's Minimum Essential Medium (EMEM) supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, nonessential amino acids, and 5% fetal bovine serum.

2.4. Plasmids

Plasmids were constructed and maintained in *Escherichia coli* JM109. Plasmids pSVIR2 and pIR2-Luc have been described previously (Kim et al., 2006). To generate plasmid pIR2-T-Luc, IR2 promoter sequences -384 to +43 containing consensus TATATTA were synthesized by Integrated DNA Technologies (Coralville, IA), digested with *KpnI* and *HindIII*, and cloned into the *KpnI* and *HindIII* sites of pGL3-basic-Luc (Promega). To generate plasmid pIR2-ST-Luc, IR2 promoter sequences -384 to +43 containing three Sp1 binding motifs and a consensus TATATTA were synthesized by Integrated DNA Technologies, digested with *KpnI* and *HindIII*, and cloned into the *KpnI* and *HindIII* sites of pGL3-basic-Luc.

2.5. Luciferase reporter assays

The luciferase reporter assay was performed as previously described (Kim et al., 2006). Briefly, L-M cells were seeded at 50% confluency in 24 well plates, and co-transfected with 0.12 pmol of reporter vector and 0.08 pmol of effectors. At 43 h posttransfection, luciferase activity was measured by using a luciferase assay kit (Promega, Madison, WI) and a Polarstar Optima plate reader (BMG LABTECH Inc., Cary, NC).

2.6. Western blot analysis

Cytoplasmic and nuclear extracts of transfected cells were prepared as previously described (Kim et al., 2001). Samples were boiled in 2X Laemmli sample buffer and separated by 7 or 10% SDS-PAGE. Proteins were transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH). Blots were incubated for 1 h with an IE peptide-specific antibody OC33 (Harty and O'Callaghan, 1991). The blots were washed three times for 10 min each in TBST and incubated with secondary antibody (anti-rabbit IgG [Fc]-alkaline phosphatase [AP] conjugate [Promega]) for 1 h. Proteins were visualized by incubating the membranes containing blotted protein in AP conjugate substrate (AP conjugate substrate kit, Bio-Rad) according to manufacturer's directions.

3. Results

3.1. Development of *galK*-UTR BAC recombineering to mutate diploid genes

BAC recombineering using *galK* selection (Warming et al., 2005) is a two-step system: *galK* cassette insertion (positive selection) and mutant replacement (counter selection) by homologous recombination in bacteria. In order to introduce a mutation into the EHV-1 BAC, the *galK* expression cassette is amplified by PCR using primers with 50 bp of homology to either side of the mutation site. *GalK* recombinant bacteria are able to grow on minimal medium with galactose as the only carbon source. Next, the *galK* cassette is replaced with the PCR product containing the mutation by homologous recombination and selected against *galK* by 2-deoxy-galactose (DOG; turns into a toxic intermediate by *galK* phosphorylation). The *galK* selection method allows DNA to be modified without introducing an unwanted selectable marker at the modification site. However, it is very difficult to mutate diploid (duplicate) genes in the BAC DNA using *galK* selection. In the case of equine herpesvirus 1, the diploid *IR2* gene lies in both the internal repeat (IR) and terminal repeat (TR) of the genome (Fig. 1A). One of the *IR2* genes can be easily mutated by *galK* cassette insertion and the ST (three Sp1-binding motifs and a consensus TATA box) mutant replacement (Fig. 1B, steps i and ii). However, it is very difficult to discern the mutation site or even confirm the mutation itself as the PCR bands of both wt and mutant sequences will appear identical. When the other copy of the target gene is replaced, the *galK* cassette can go to either the IR or the TR (Fig. 1B, step iii). The location of the *galK* cassette also cannot be discerned. If the correct clone is randomly picked, after the 2nd mutant replacement, the same problem still exists.

To overcome these obstacles, we developed a *galK*-UTR BAC recombineering method, a modified version of the *galK* selection method, using two templates (ST1 and ST2) and two primer pairs (OUT-PCR and IN-PCR primers) for PCR (Fig. 1C). As shown in Fig. 1C, OUT-PCR primer pair anneals outside the IN-PCR primer pair. The two templates ST1 and ST2 contain the same three Sp1-binding motifs and a consensus TATA box. ST1 has an additional mutation in the restriction enzyme site *StuI* (aggcct to aggact), which is needed to discern the location of the mutation site, as well as to confirm the mutation site. The key idea is to block one of the target diploid genes by the insertion of *galK* untranslated region (UTR) (Fig. 1C, steps i and ii). Then, the other copy of the diploid genes can be easily

mutated (Fig. 1C, steps iii and iv). The blocked copy can also be mutated with UTR-specific primers (Fig. 1C, steps v and vi). These mutation steps will be explained in detail below.

3.2. The selection of the IR2 ST mutant promoter

First, we introduced point mutations in the promoter region of the IR2 gene which was difficult because the *IR2* gene is embedded within the *IE* gene. The *IR2* is expressed at a low level at the early stage of infection because its promoter is TATA-less (Kim et al., 2006). The TATA-like sequences catttta (nt -32 to -26) of the IR2 promoter were mutated to a consensus TATA box tatattta which was linked to a luciferase gene to generate pIR2-T-Luc (Fig. 2A). The IR2 promoter DNA containing three Sp1-binding motifs ggagcgg and a consensus TATA box was also cloned upstream of luciferase gene to generate pIR2-ST-Luc (Fig. 2A). In luciferase reporter assays, the addition of the consensus TATA box (T) increased luciferase activity 1.5-fold as compared to that of the wild-type (wt) IR2 promoter (Fig. 2A). The addition of three Sp1 sites and a consensus TATA box (ST) increased the promoter activity 3-fold. The IR2 ST mutant was used for generating an IR2 mutant virus EHV-1 ST2/ST1.

3.3. Blocking one copy of the diploid IR2 promoter by the UTR

First, to generate the *IR2* gene mutant virus *EHV-1 ST2/ST1* using *galK*-UTR BAC recombineering, one of the IR2 promoters was blocked by *galK* UTR flanked by OUT-PCR primers (Fig. 1B, steps i and ii). In order to introduce the UTR into wt EHV-1 BAC, 1,351-bp OUT-*galK* PCR product, a *galK* cassette flanked by 60-bp OUT PCR primers, was amplified by OUT-PCR primer pair OUT *galK*-F1 and OUT *galK*-R1 (Table 1) and was inserted into IR2 promoter region by homologous recombination in SW106 harboring wt EHV-1 BAC (Fig. 1B, step i; positive selection). *GalK* recombinant bacteria were able to grow on minimal medium with galactose as the only carbon source. The recombinant EHV-1 OUT-*galK* BAC was screened by PCR using four PCR primer pairs P1, P2, P3, and P4 (Fig. 2B). As expected, PCR products 1,351 bp, 633 bp, and 598 bp were amplified with *galK*-specific primers P1, P2, and P3 (Fig. 2B, lanes 2, 3, and 4, respectively). In contrast, no PCR product was amplified with the P1, P2, and P3 primer pairs from the control wt EHV-1 BAC (Fig. 2B, lane 6, 7 and 8, respectively). With P4, PCR products of 1,570 bp from the *galK*-inserted IR2 promoter and 768 bp from the other wt IR2 promoter were obtained (Fig. 2B, lane 5). As expected, only the 768-bp PCR product was amplified with P4 primers from the wt EHV-1 BAC (Fig. 2B, lane 9).

To replace the OUT-*galK* cassette with the *galK* untranslated region (UTR) which is located 30-bp downstream of the *galK* open reading frame, 830-bp UTR sequences were amplified by OUT-PCR primer pair OUT utr-F2 and OUT utr-R2 (Table 1). Then the OUT-*galK* cassette was replaced with the UTR by homologous recombination (Fig. 1B, step ii; counter selection). *GalK* negative recombinant bacteria were selected against *galK* by including the DOG. The recombinant EHV-1 UTR BAC (II) was screened by PCR (Fig. 2C). The correct PCR products 830 bp, 393 bp, and 605 bp were amplified with UTR-specific primer pairs P1, P2, and P3 (Fig. 2C, lanes 2, 3, and 4, respectively). In contrast, no PCR product was amplified with the P1, P2, and P3 primer pairs from the control wt EHV-1 BAC (data not shown). With P4, PCR products of 1,048 bp from UTR-inserted IR2 promoter and 768 bp from the other copy of diploid IR2 promoter were obtained (Fig. 2C, lane 5).

3.4. Mutation of the other copy of the IR2 promoter by IN-PCR primers

Blocking one copy of the IR2 promoter by the UTR with OUT-PCR primers enabled us to easily replace the other copy of the IR2 promoter using IN-PCR primers. The 1,351-bp IN-*galK* PCR product was amplified by the IN-PCR primer pair IN *galK*-F3 and IN *galK*-R3 (Table 1) and then was inserted into the IR2 promoter region (Fig. 1B, step iii; positive

selection). The recombinant EHV-1 UTR/IN-galK BAC was screened by PCR (Fig. 2D). As expected, PCR products 1,351 bp, 703 bp, and 696 bp were amplified with galK-specific primers P1, P2, and P3 (Fig. 2D, lanes 2, 3, and 4, respectively). In contrast, no PCR product was amplified with the P1, P2, and P3 primer pairs from the control wt EHV-1 BAC (data not shown). With P4, PCR products: 1,739 bp from *galK*-inserted *IR2* promoter and 1,048 bp from the UTR-inserted *IR2* promoter were obtained (Fig. 2D, lane 5).

For the *IR2* ST1 replacement, the 380-bp mutant *IR2* promoter DNA was amplified from the ST1 template containing three Sp1-binding motifs, a consensus TATA box, and a mutated *StuI* site (aggcct to aggcgt) with IN-PCR primer pair IN-F4 and IN-R4 (Table 1). Following homologous recombination (Fig. 1B, step iv), the recombinant EHV-1 UTR/ST1 BAC (III) was screened by PCR (Fig. 2E). The expected PCR products 380 bp and 435 bp were amplified with ST1-specific primer pairs P1 and P2 (Fig. 2E, lanes 2 and 3, respectively). With P3, PCR products of 671 bp from the ST1-inserted *IR2* promoter and 951 bp from the UTR-inserted *IR2* promoter were obtained (Fig. 2E, lanes 4 and 6). In contrast, only 671-bp PCR product was amplified from the control wt EHV-1 BAC (Fig. 2E, lane 8). ST1 mutation was confirmed by restriction enzyme digestion and DNA sequencing. When the two P3 PCR products from UTR/ST1 (671 bp and 951 bp) were digested with restriction enzyme *StuI*, none of the PCR products was cut due to a mutation of the *StuI* site (Fig. 2E, lane 7). The 671-bp wt PCR product was cut into two bands 389 bp and 282 bp (Fig. 2E, lane 9). All bands in lanes 6 to 9 were eluted and confirmed by DNA sequencing (data not shown; (DNA sequencing facility, Iowa State University).

3.5. Mutation of the blocked copy by UTR-specific PCR primers

The blocked copy of the *IR2* promoter was replaced with the ST2 mutant by UTR-specific primers. First, the 1,351-bp UTR-galK PCR product was amplified by the UTR-specific primer pair UTR galK-F5 and UTR galK-R5 (Table 1) and was inserted into the UTR of the EHV-1 UTR/ST1 BAC (Fig. 1B, step v). In the screening of the recombinant EHV-1 UTR-galK/ST1 BAC, the correct PCR products 1,351 bp, 691 bp, and 658 bp were amplified with galK-specific primer pairs P1, P2, and P3 (Fig. 3A, lanes 2, 3, and 4, respectively). In contrast, no PCR product was amplified with the P1, P2, and P3 from the control wt EHV-1 BAC (Fig. 3A, lane 6, 7 and 8, respectively). With P4, PCR products of 1,690 bp from *galK*-inserted *IR2* promoter and 768 bp from the ST1-inserted *IR2* promoter were obtained (Fig. 3A, lane 5). Only the 768-bp PCR product was amplified with the P4 from the wt EHV-1 BAC (Fig. 3A, lane 9).

The *galK* cassette of EHV-1 UTR-galK/ST1 BAC was replaced with 550-bp mutant *IR2* promoter *IR2* ST2 amplified from the ST2 template containing three Sp1-binding motifs, a consensus TATA box, and a *StuI* site with OUT-PCR primer pair OUT-F6 and OUT-R6 (Table 1) (Fig. 1B, step vi). The final recombinant EHV-1 ST2/ST1 BAC (IV) was screened by PCR (Fig. 3B). As expected, the same PCR products 550 bp, 435 bp, and 671 bp were amplified from the ST2/ST1 mutant and wt EHV-1 BAC with primer pairs P1, P2, and P3 (Fig. 3B, lanes 2 and 5, lanes 3 and 5, lanes 4 and 7, respectively). ST2/ST1 mutation was confirmed by restriction enzyme digestion and DNA sequencing. When the 671-bp P3 PCR product from ST2/ST1 was digested with *StuI*, half of the PCR products was resistant to cut due to a mutation of the *StuI* site in the ST1, resulting in three bands of 671 bp, 389 bp, and 282 bp (Fig. 3B, lane 10). In wt, the 671-bp PCR product was completely cut into two bands of 389 bp and 282 bp (Fig. 3B, lane 12). All bands in lanes 10 and 12 were eluted and examined by DNA sequencing (Fig. 3C). Three Sp1-binding motifs, a consensus TATA box, and a *StuI* site were observed in the ST2 mutation site (Fig. 3C). As expected, the mutated *StuI* site was observed in the ST1.

3.6. The expression of IR2P was increased in equine NBL6 cells infected with EHV-1 ST2/ST1

WT EHV-1 BAC (I) and three recombinant EHV-1 UTR BAC (II), EHV-1 UTR/ST1 BAC (III), and EHV-1 ST2/ST1 BAC (IV) were analyzed by restriction enzyme digestion (Fig. 4A). *Bam*HI cut the outside IR2 promoter mutation area. Only a difference of 280 bp by the insertion of the UTR sequences in the IR2 promoter region occurred in the EHV-1 UTR BAC (II) and EHV-1 UTR/ST1 BAC (III). One band of higher molecular weight can be seen around 4.6 kb in the EHV-1 UTR BAC (II) and EHV-1 UTR/ST1 BAC (III) (Fig. 4A, lanes 4 and 5, respectively). However, *Stu*I cuts inside the mutation site. A diagram was prepared to analyze the *Stu*I-digested DNA patterns (Fig. 4B). The insertion of the UTR in the IR2 promoter regions resulted in the loss of one *Stu*I site in the EHV-1 UTR BAC (II). As seen in Fig. 4A and 4B lane 10, the 8.5-kb DNA fragment a and one of the 12-kb double DNA fragment b were merged and shifted up to the 20.5-kb DNA fragment c as compared to those of wt EHV-1 BAC (I). The replacement of the other copy of the IR2 promoter with the ST1 mutant containing a mutated *Stu*I site resulted in additional loss of the *Stu*I site in the EHV-1 UTR/ST1 BAC (III). The 11.5-kb DNA fragment d and 18.5-kb DNA fragment e were merged and shifted up to the 30-kb DNA fragment f (Fig. 4A and 4B, lane 11). The replacement of the blocked copy of the IR2 promoter with the ST2 mutant containing *Stu*I site resulted in a regain of the *Stu*I site in the EHV-1 ST2/ST1 BAC (IV). The 20.5-kb DNA fragment g was cut back into the 12-kb DNA fragment h and 8.5-kb DNA fragment i (Fig. 4A and 4B, lane 12). These results indicated that three recombinant BACs, EHV-1 UTR BAC (II), EHV-1 UTR/ST1 BAC (III), and EHV-1 ST2/ST1 BAC (IV) were correctly generated by the *galK*-UTR BAC recombineering method.

To reconstitute EHV-1 ST2/ST1 BAC (IV) as a standard virus, the BAC sequences were replaced with viral DNA sequences for the EUs4 gene by co-transfecting RK-13 cells with both the BAC and a plasmid pGEX-gp2 (Rudolph et al., 2002) containing the EUs4 sequences. Plaques lacking green fluorescent protein (GFP) expression were screened by three rounds of plaque purification, confirmed by DNA sequencing (data not shown), and then propagated to high titer on equine NBL6 cells. Equine NBL6 cells were infected with wt EHV-1 and EHV-1 ST2/ST1 and harvested at 0, 3, 5, and 8 h postinfection (hpi). Western blot analyses were performed with the anti-IEP antibody OC33 which detected both the IEP and IR2P. As a positive control, IR2P was expressed from the IR2 expression vector pSVIR2 (Fig. 4C, lane 10). Several species of antigenically cross-reactive IEP characterized in previous studies (Caughman et al., 1985) were detected in nuclear extracts of EHV-1-infected cells (Fig. 4C, lanes 3 to 8). In wt EHV-1-infected cells, a protein identical in size to the 150-kDa IR2P expressed as the positive control appeared at 5 hpi (Fig. 4C, lane 4) and was clearly seen at 8 hpi (Fig. 4C, lane 5). In EHV-1 ST2/ST1-infected cells, the expression level of IR2P was increased approximately 4-fold (Fig. 4C, lane 9) as compared to that of wt EHV-1 (Fig. 4C, lane 5). When using the IEP *trans*-activation domain (TAD)-specific antibody OC89, which detects only IEP (Kim et al., 2011), IR2P was not detected in the cells infected with wt EHV-1 and EHV-1 ST2/ST1 (Fig. 4C, lanes 12 to 19), indicating that the 150-kDa band is IR2P. We also generated an IR2 knowndown virus (*EHV-1 IR2ΔC*) (data not shown). These results demonstrated that a diploid IR2 mutant virus EHV-1 ST2/ST1 was generated successfully by use of *galK*-UTR BAC recombineering.

4. Discussion

In this study, we developed a *galK*-UTR BAC recombineering method, a modified version of BAC recombineering using *galK* selection, to mutate the diploid *IR2* gene of equine herpesvirus 1. BAC recombineering using *galK* selection allows DNA cloned in *E. coli* to be modified via lambda Red-mediated homologous recombination without introducing an unwanted selectable marker at the modification site. Introduction of mutations into diploid

genes using the *galK* selection method was considered almost impossible due to the difficulty of discerning the mutation site or even confirming the mutation itself. To overcome these problems, four modifications were made to the *galK* selection method. First, one of the target diploid genes was blocked by *galK* untranslated region (UTR), which is not homologous to *galK* open reading frame, or to any EHV-1 gene. The blocking enabled the simple mutation of the other copy. Second, the length of UTR (830 bp) inserted in the BAC was designed as half sizes of the 1,351-bp *galK* cassette which enabled the differentiation of amplification products as the mutation was confirmed. Third, when the blocked copy of the diploid IR2 promoter was replaced with the ST2 mutant, the UTR-specific primer pair was used for the insertion of *galK* cassette into the UTR of the BAC. Fourth, two templates and two primer pairs were used for PCR. One of the two templates has an additional restriction enzyme site to discern the location of the mutation site as well as to confirm the mutation itself.

Diploid genes can be mutated using BAC technology and RED recombination in which two antibiotic resistant genes will additionally be inserted into the BAC genome for mutant selection. Since the antibiotic resistant genes may influence gene expression in the genomic region, the selectable genes need to be removed from the mutant BAC DNA by Cre (loxP/Cre; Zhang et al., 1998)- and FLP-recombinase (FRT/FLP; Nakano et al., 2001) in which loxP and FRT sequences are inserted as flanking sequences of the two antibiotic genes, respectively. However, 34-bp sequences of loxP and FRT will still remain in the BAC genome after removing antibiotic genes. The unwanted sequences might also complicate the interpretation of experimental data. To solve this problem, two-step positive/negative selection schemes were developed, allowing precise modifications of BAC constructs. A method using neomycin and *SacB* as positive and negative selection markers, respectively, was developed (Muyrers et al., 2000). A major drawback of this selection system is that spontaneous point mutations in the *sacB* portion of the *sacB-neo* fusion gene can occur without influencing the bacteria's ability to grow on kanamycin. These *sacB* mutants significantly increase the background after negative selection. Thereafter, the Copeland laboratory developed a *galK* positive/negative selection method which allows DNA to be modified without leaving selectable markers at the modification site (Warming et al., 2005). A procedure that combines Red recombination and cleavage with the homing endonuclease I-SceI was developed to allow highly efficient, PCR-based DNA engineering without retention of unwanted foreign sequence (Tischer et al., 2006). Another two-step recombineering procedure using kanamycin/streptomycin, which does require the use of minimal medium and DOC (Wang et al., 2009), could be an alternative to the *galK* selection scheme.

Herpesviruses, such as Epstein-Barr virus (EBV), with genomes of group C have four internal repeats (R1 to R4) (Roizman and Pellet, 2001). The *galK*-UTR BAC recombineering can also be applied for mutation of triplicate or quadruplicate genes. In the case of triplicate gene, one more additional blocking step is required (Fig. 5). Three templates and three primer pairs are also needed for mutant amplification.

In conclusion, the *galK*-UTR BAC recombineering can be used to mutate diploid genes of herpesviruses without leaving behind any unwanted sequences. This method should be very useful for gene manipulation of diploid genes of herpesviruses.

Acknowledgments

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Research Highlights

We developed a *galK*-UTR BAC recombineering method to mutate the diploid *IR2* gene of EHV-1. > The key idea is to block one of the target diploid genes by *galK* untranslated region (UTR) > The IR2 mutant virus was successfully generated. > The *galK*-UTR method will provide a useful tool in studies of herpesviruses.

Fig. 1. Overview of the *galK*-UTR BAC recombineering scheme

(A) The EHV-1 genome consists of a unique long region (U_L) and a short region, which are covalently linked to each other. The short region is composed of unique sequences (U_S) flanked by two identical internal repeat (IR) and terminal repeat (TR). Six genes (IR1 to IR6) and the origin (ORI) of replication are in both the IR and TR. (B) Mutation of diploid (duplicate) gene by use of BAC recombineering using *galK* selection. Top diagrams show PCR products of *galK* cassette and the ST mutant which are flanked by the same homology arms for recombination. In the EHV-1 BAC, six genes are in both the internal repeat (IR) and terminal repeat (TR). The *galK* selection scheme is a two-step selection: *galK* cassette insertion (positive selection) and mutant replacement (counter selection) by homologous recombination in bacteria. The mutation of the second copy of the diploid *IR2* gene cannot be proceeded due to the inability to discern the location of the *galK*-inserted site. (C) Mutation of diploid gene by the *galK*-UTR scheme which uses two templates and two primer pairs for PCR. ST1 template contains three Sp1-binding motifs, a consensus TATA box, and a mutated *StuI* (aggcct to aggact). ST2 template contains three Sp1-binding motifs, a consensus TATA box, and a *StuI* site. The *galK*-UTR scheme has one more step for blocking one of the target diploid genes by *galK* untranslated region (UTR).

Fig. 2. Confirmation of the recombinant EHV-1 BACs

(A) Activity of the mutant IR2 promoter containing three Sp1 sites and a consensus TATA box (ST mutant) is increased by 3-fold. Top diagram shows the IR2 promoter region which is embedded with the *IE* gene. WT and mutant IR2 promoters were linked to a luciferase (Luc) gene. TATA-less, lack of consensus TATA box; CCAAT, a transcription factor NF-Y-binding site. L-M cells were co-transfected with 0.12 pmol of the reporter plasmids (control empty reporter pGL3-basic-Luc, pIR2-Luc, pIR2-T-Luc, and pIR2-ST-Luc) and 0.08 pmol of effector plasmid pSVEICP0. Data are averages and are representative of three or more independent experiments. The firefly luciferase signals were normalized to the internal secreted alkaline phosphatase (SEAP) transfection control (Kim et al., 2011). (B) The recombinant EHV-1 OUT-galK BAC was screened by PCR in which four PCR primer pairs P1, P2, P3, and P4 were used. The galK-specific primers P1 (OUT galK-F1 and OUT galK-R1), P2 (IR2-F and galK-R), and P3 (galK-F and IR2-R) and IR2 promoter-specific primer P4 (IR2-F and IR2-R) (Table 1) were used for PCR amplification, and the PCR products were analyzed by agarose gel analysis. OUT, EHV-1 OUT-galK BAC; wt, wt EHV-1 BAC. (C) The recombinant EHV-1 UTR BAC (II). The UTR-specific primers P1 (OUT utr-F2 and OUT utr-R2), P2 (IR2-F and utr-R), and P3 (utr-F and IR2-R) and IR2 promoter-specific primer P4 (IR2-F and IR2-R) (Table 1) were used for PCR amplification. UTR, EHV-1 UTR BAC. (D) The recombinant EHV-1 UTR/IN-galK BAC. The galK-specific primer pairs P1 (IN galK-F3 and IN galK-R3), P2 (IR2-F and galK-R), and P3 (galK-F and IR2-R) and IR2 promoter-specific primer P4 (IR2-F and IR2-R) (Table 1) were

used for PCR amplification. UTR/IN-galK, EHV-1 UTR/IN-galK BAC. (E) The recombinant EHV-1 UTR/ST1 BAC (III). The ST1-specific primer pairs P1 (IN-F4 and IN-R4) and P2 (IR2-F and IR2-R2) and IR2 promoter-specific primer P3 (IR2-F and OUT-R6) (Table 1) were used for PCR amplification. ST1 mutation was confirmed by restriction enzyme *StuI* digestion (lanes 6 to 9) and DNA sequencing (data not shown). UTR/ST1, EHV-1 UTR/ST1 BAC; U, untreated with *StuI*. Numbers to the left represent 1 kb plus DNA ladder (Invitrogen).

Fig. 3. Mutation of the blocked copy of the diploid IR2 promoter with the ST2 mutant

(A) Confirmation of the recombinant EHV-1 UTR-galK/ST1 BAC by PCR. The gal-specific primers P1 (UTR galK-F5 and UTR galK-R5), P2 (IR2-F and galK-R), and P3 (galK-F and IR2-R) and IR2 promoter-specific primer P4 (IR2-F and IR2-R) (Table 1) were used for PCR amplification. UTR-galK/ST1, EHV-1 UTR-galK/ST1 BAC. (B) Confirmation of EHV-1 ST2/ST1 BAC (IV) by PCR and restriction enzyme digestion. The ST1-specific primers P1 (OUT-F6 and OUT-R6) and P2 (IR2-F and IR2-R) and IR2 promoter-specific primer P3 (IR2-F and OUT-R6) (Table 1) were used for PCR amplification, and the PCR products were analyzed by agarose gel analysis. ST1 and ST2 mutations were confirmed by *Stul* digestion (lanes 9 to 12). ST2/ST1, EHV-1 ST2/ST1 BAC; wt, wt EHV-1 BAC; U, untreated with *Stul*. Numbers to the left represent 1 kb plus DNA ladder (Invitrogen). (C) Sequence analysis of the IR2 promoter regions in the wt EHV-1 BAC and EHV-1 ST2/ST1 BAC. WT nucleotide sequences are underlined. Sp1 motif, mutated *Stul* site, and consensus TATA box are marked by boxes.

Fig. 4. Confirmation of the three recombinant BACs and a diploid IR2 mutant virus EHV-1 ST2/ST1

(A) WT EHV-1 BAC (I) and three recombinant BAC genomes EHV-1 UTR BAC (II), EHV-1 UTR/ST1 BAC (III), and EHV-1 ST2/ST1 BAC (IV) were digested by restriction enzymes *Bam*HI and *Stu*I. I, wt EHV-1 BAC; II, EHV-1 UTR BAC; III, EHV-1 UTR/ST1 BAC; IV, EHV-1 ST2/ST1 BAC. Numbers to the left represent 1 kb plus DNA ladder M1 and 1 kb DNA extension ladder M2 (Invitrogen). (B) The *Stu*I-digested DNA pattern was analyzed by a schematic diagram. The two DNA fragments b and h are double DNA bands. Numbers to the left represent 1 kb DNA extension ladder M2 (Invitrogen). (C) The expression of IR2P was determined by western blot analysis. Equine NBL6 cells were infected with wt EHV-1 and EHV-1 ST2/ST1, harvested at 0, 3, 5, and 8 h postinfection (hpi), and analyzed by western blot with the anti-IEP antibody OC33, which detects both the IEP and IR2P, and the anti-IEP *trans*-activation domain (TAD) antibody OC89, which detects only IEP. As a positive control, IR2P was expressed from the IR2 expression vector pSVIR2 (lanes 10 and 20). As a loading control, actin was detected in cell lysates by using anti-actin polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Numbers to the left represent molecular weight standards (kDa) (Bio-Rad). The position of IR2P is indicated with arrows and arrowheads. ST2/ST1, EHV-1 ST2/ST1.

Fig. 5.

Mutation of triplicate gene by the *galK*-UTR scheme which uses three mutant templates and three primer pairs for PCR. The three mutant templates have different restriction enzyme sites. The two blocking steps will be performed by *galK* untranslated regions 1 and 2 (UTR1 and UTR2).

Table 1

List of primers used for generating mutant virus

Primer name ^a	Sequence (5'-3') ^b
OUT galk-F1	<i>ggtgccggggcgagtagctcctcttcgtcgtcctccgacgacagcgacagcgacgaaggc</i> <u>cctgttgacaattaatcatcggcatag</u>
OUT galk-R1	<i>gggatgagttcatgtccagcaagtcccacacggccgtctgcggggcctcctcgccgggtg</i> <u>tcagcactgtcctgctccttggatgg</u>
OUT utr-F2	<i>ggtgccggggcgagtagctcctcttcgtcgtcctccgacgacagcgacagcgacgaaggc</i> <u>cgcgggtggagctccagctttgttccctt</u>
OUT utr-R2	<i>gggatgagttcatgtccagcaagtcccacacggccgtctgcggggcctcctcgccgggtg</i> <u>cttggagcgaacgacctacaccgaactgag</u>
IN galk-F3	<i>agaccctcgcccgcggcactcgcagaacgccggaagaccccgctcgccgccggctctc</i> <u>cctgttgacaattaatcatcggcatag</u>
IN galk-R3	<i>cttcgcttcgacgtcccgcagagccgatttcggacgtggtccttggggagccgggtg tca</i> <u>gcactgtcctgctccttggatgg</u>
IN-F4	<i>agaccctcgcccgcggcactcgcagaacgccggaagaccccgctcgccgccggctctc</i>
IN-R4	<i>cttcgcttcgacgtcccgcagagccgatttcggacgtggtccttggggagccgggtg</i>
UTR galk-F5	<i>cgcgggtggagctccagctttgttcccttagtgagggttaatttcgagcttggcgtaat</i> <u>cctgttgacaattaatcatcggcatag</u>
UTR galk-R5	<i>cttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgc</i> <u>tcagcactgtcctgctccttggatgg</u>
OUT-F6	<i>ggtgccggggcgagtagctcctcttcgtcgtcctccgacgacagcgacagcgacgaaggc</i>
OUT-R6	<i>gggatgagttcatgtccagcaagtcccacacggccgtctgcggggcctcctcgccgggtg</i>
IR2-F	<i>cagcccgagttccatcctcg gcctccccg</i>
IR2-R	<i>ggacggggtgagaagcggtcgcgctggtgta</i>
IR2-R2	<i>gacgccctccagtgactctaaatgcgagc</i>
galk-F	<i>tgcggcacattgccgctgatcacatgtcc</i>
galk-R	<i>ttccagcagccagccctgcgtgatgtcacc</i>
UTR-F	<i>agaggcggtttgcgtattggcgctcttcc</i>
UTR-R	<i>actggaagcgggcagtgagcgcaacgcaa</i>

^aF, forward primer; R, reverse primer; OUT, OUT PCR primer; IN, IN PCR primer^bHomology to BAC sequence (homology arms; OUT, IN, and UTR) is in italics and the underlined sequences are for annealing to the *galK* or UTR template.