Construction of a HSV-1 strain HF Based Replication Defective Vector with LR-Recombination Sites

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Abstract: In this study, we incorporated the lambda phage based specific recombination sites attR into HSV-1 replication-defective vector by using Red recombineering, and constructed the HSV-1 replication-defective vector BAC-HSV1-HF-△ICP27-attR-GK. In addition, a red fluorescence reporter gene DsRed was introduced to the vector to construct the BAC-HSV1-HF-△ICP27-attB-DsRed by LR recombination approach. To conduct research on the expression of exogenous gene integrated to the vector, the plasmid BAC-HSV1-HF-△ICP27-attB-DsRed was transfected to the 2-2 Vero cells, 72hr later, CPE and the red fluorescence protein were observed. These results indicated that we successfully constructed HSV-1 replication-defective vector BAC-HSV1-HF-△ICP27-attR-GK which carrying LR recombination specific sites attR, and the vector can incorporate exogenous gene by a one-step LR recombination in vitro. This method simplified the procedure of site-directed integration of exogenous gene into HSV-1 replication-defective vectors, and greatly facilitated the research of HSV-1 derived vectors for gene therapy.
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Keywords: HSV-1, Red recombineering, LR recombination, replication defective vectors, site-directed integration

Introduction

Herpes simplex virus type 1 (HSV-1) is an enveloped, double-strand (ds) DNA virus, could cause serious infection diseases in humans, including infection of skin, mucous membranes and nervous system, is an important human pathogen[1-2]. Due to its features, such as natural cell tropism, lowly immunogenicity and inability of integration of genome to host chromosomes, HSV-1 derived vectors have been exploited[3-4]. HSV-1 Replication-defective vectors[5-6] are kind of mutant virus with deletions in one or more genes essential for the lytic cycle, such as ICP4 and ICP27, these defective virus could be packaged in the complementary cell lines. With a neurotropic property and the ability of establishing latent infection, replication-defective vectors could steadily and long-termly express the exogenous gene in neurons, have become a good candidate for gene therapy[5-6]. However, because of the huge genome of HSV-1, It's still a question of how to integrate the target gene into replication-defective HSV-1 vectors simply and rapidly.

Typical methods to clone exogenous gene into replication-defective HSV-1 vectors are mainly based on homologous recombination in eukaryocyte, owing to its disadvantages such as multiple manipulation, difficulties in screening recombinant clones, it's not suitable for simple and rapid cloning of target genes. The appearance of recombineering technology[7-8] and development of bacteria artificial chromosome (BAC) technology[9-11]greatly facilitated the research of HSV-1 genome function and HSV-1 derived vectors, and the target gene can be integrated into replicate-defective HSV-1 vectors simply by the recombineering technology in Escherichia. Although this system simplified the procedure of target mutagenesis HSV-1, it still needs to accomplish recombination with the help of recombinase in *Escherichia coli*, which has low efficacy and long screening cycle. How to integrate target gene into HSV-1 derived vectors simply and rapidly in vitro has become a new research area for us.

LR recombination system[12] is a kind of site-specific recombination system based on λ phage, which contains two starting DNAs: an entry clone (attL1-gene-attL2), and a destination vector (attR1-cm-ccdB-attR2). The target DNA fragment flanked by attL in the entry clone could exchange with the DNA fragment between the attR sites in the destination vector with the action of LR recombinase. There hasn't been a report about LR recombination system applied in integrating exogenous DNA fragment into replication-defective HSV-1 vectors so far yet.

We previously[13] constructed the HSV-1 replication-defective vector BAC-HSV1-HF-△ICP27 with Red recombineering technology successfully. In this study, we incorporate the attR recombination sites based on λ phage to the BAC-HSV1-HF-△ICP27 vector to construct a replication-defective HSV-1

vector BAC-HSV1-HF-ΔICP27-attR-GK. In addition, we cloned the red fluorescence reporter gene DsRed into BAC-HSV1-HF-ΔICP27-attR-GK by the one-step LR recombination in vitro. In this paper we provide a new approach for site-directed integration of exogenous gene into replication-defective HSV-1 vectors.

Materials

Cells: African green monkey kindey (Vero) cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The 2-2 cell line was kindly provided by Dr Jia laboratory in The Prostate Centre at Vancouver General Hospital, Vancouver, British Columbia, Canada. All cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplement with 10% fetal calf serum in humidified 37℃, 5% CO_2 incubator.

Plasmid and bacteria: The plasmid pYD-C255, a GalK-kan (galactokinase-kanamycin) dual-expression cassette and a recombineering E. coli strain SW105 were gifted by Dr YU, Washington University in St.Louis. The DH10B electroporation competent cells were conserved by our laboratory. The plasmid BAC-HSV1-HF-ΔICP27 was constructed by our laboratory as described previously[13], which is able to produce infectious viral particle when transfected into 2-2 complement cell lines. The plasmid pGM-T was purchased from TIANGEN. The plasmid pAd/BLOCK-iT™-DEST (DEST), pENTR™/U6 and a E. coli strain *E. coli* DB3.1 cells were purchased from Invitrogen. The plasmid pDsRed2-C1 was purchased from Clontech.

Enzyme and primers: All the restriction enzyme were purchased from Takara. All the primers were synthesized by Sangon Biotech.

Methods

1 Construction of BAC-HSV1-HF-ΔICP27-attR-GK replication-defective vector by Red recombineering

1.1 Procure of recombinant UL47-UL48 homology arms

To construct pGM-T-UL47-UL48 vector, UL47-UL48 homology arms were amplified by PCR using the follow primers: P1：5'-GACGCGGCCGCGGTAGTCGTCCTCCTCGTA-3' and P2: 5'-CGGGAGCTAAACCACATTCGCGAGCACC-3', and HSV-1 HF virus genome DNA as the template. The 1.5 kb PCR products were cloned to pGM-T vector via TA cloning method.

1.2 Procure of LR recombination specific sites attR

The vector pGM-T-attR1-cm-ccdB-attR2 is a derivative of pGM-T, modified by the insertion of a gene cassette containing attR recombination sites acquired from DEST into the multiple cloning region of the pGM-T. To achieve that, pGM-T was digested with SalI and NotI, and treated with calf intestinal phosphatase (CIP). The plasmid DEST was digested with NheI and SphI to obtain a 1.9kb fragment harboring attR1-cm-ccdB-attR2. The T4 DNA polymerase-blunted fragment was ligated into the linear pGM-T, and transformed into *E. coli* DB3.1 cells.

1.3 Procure of Red recombination selection gene GalK-kan

GalK-kan cassette was amplified by PCR using the P3: 5'-CCTGTTGACAATTAATCATCG-3' and P4: 5'-CTCAGCAAAAGTTCGATTTA-3' as primers, and pYD-C255 plasmid as the template. The PCR products were digested with DpnI and then the 2.3kb PCR products GalK-kan cassette was inserted to the BamHI/PstI sites of pGM-T-attR1-cm-ccdB-attR2 vector by blunt end, with the cm-ccdB DNA sequence deleted.

1.4 Construction of pGM-T-UL47-attR-GK-UL48 vector

The vector pGM-T-UL47-attR-GK-UL48 is a derivative of pGM-T-UL47-UL48, modified by the insertion of a DNA fragment attR1-GalK-kan-attR2. To achieve that, a 2.8kb fragment harboring attR1-GalK-kan-attR2 was acquired by the NcoI/SacI digestion of plasmid pGM-T-attR1-GK-attR2, and then cloned to the BstBI site of pGM-T-UL47-UL48 by blunt end.

1.5 Procure of Red recombination fragment UL47-attR-GK-UL48

The 3.6kb UL47-attR-GK-UL48 DNA fragment was obtained through digesting pGM-T-UL47-attR-GK-UL48 vector with KpnI, SacI and XmnI respectively.

1.6 Transduction of UL47-attR-GK-UL48 DNA fragment into BAC-HSV1-HF-ΔICP27 recombineering bacteria and identification of recombinant colonies

As shown in Figure 1, to induce a homology recombination, electrocompetent SW105 bacteria harboring BAC-HSV1-HF-ΔICP27 was electroporated with UL47-attR-GK-UL48 DNA fragment, after 5 hr resuscitation in the LB, the recombinant bacteria was applied on a MacConkey + cm^+ + $kana^+$ plate over night at 31℃ in a cabinet-type incubator. After incubation, four red colonies were picked and identified by PCR with two pairs of primers, UL47-F/GalK-R: CCTGAATGGTGTGAGTGG/GACATGGTGGCGATAGA and Kan-F/UL48-R: CACGAGCAC ATACATTACAA/GTTGGACGAGTCGGAATC.

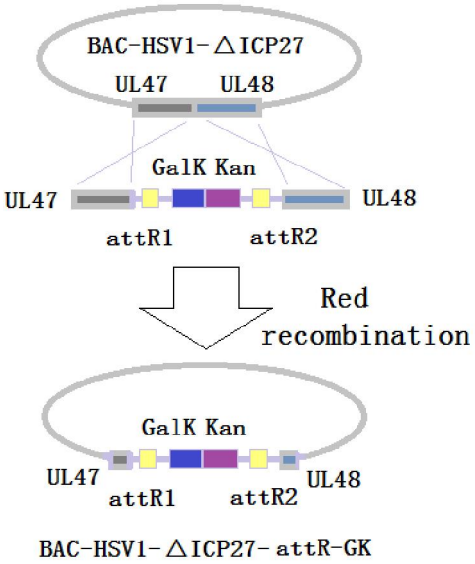

Fig.1. Outline of Red recombination

2 Functional analysis of BAC-HSV1-HF-ΔICP27-attR-GK replication-defective vector

2.1 Construction of BAC-HSV1-HF-ΔICP27-attB-DsRed by LR recombination technique

1) Procure of reporter gene DsRed

To introduce the reporter gene to the entry vector pENTR™/U6 with attL sites, a 1.5kb reporter gene DsRed cassette was obtained by AseI/MluI digestion of plasmid pDsRed2-C1 and then cloned to the SmaI site of pENTR™/U6 by blunt end to produce a new entry vector pENTR-attL1-DsRed-attL2 with DsRed reporter gene.

2) LR recombination integrate reporter gene to BAC-HSV1-HF-ΔICP27-attR-GK and identification of recombinant clones

As shown in Figure 2, plasmids pENTR-attL1-DsRed-attL2 and BAC-HSV1-HF-ΔICP27-attR-GK were incubated at 25℃ with the help of LR recombinase for 5 hr, followed by being digested with protease K for 10min, the LR recombination system was electroporated into DH10B competent cells, and the recombinant bacteria was applied on Cm^+ LB plate over night at 37℃ in a cabinet-type incubator. After incubation, some recombinant colonies were picked and streaked onto a $Kana^+$ LB plate and a Cm^+ LB plate respectively for another 24hr, four colonies which could grow on Cm^+ but not $Kana^+$ LB palte were picked and identified by PCR with primers DsRed-F/DsRed-R: CGGCTGCTTCATCTACAA/ACCACCTGTTCCTGAGA
T.

2.2 Functional analysis of testing infective BAC-HSV1-HF-ΔICP27-attB-DsRed vector virus generation and reporter gene expression in Vero and 2-2 cells

Vero cells and 2-2 cells (5.0×10^5cells/well) grown on the 6-well plate were respectively transfected with 2 μg of BAC-HSV1-HF-ΔICP27-attB-DsRed and BAC-HSV1-HF-ΔICP27-attR-GK plasmid DNA using lipofectamin 2000 following the manufacturer's protocol. 72 hr later, observing the CPE and the expression of red fluorescence protein under the fluorescence microscope.

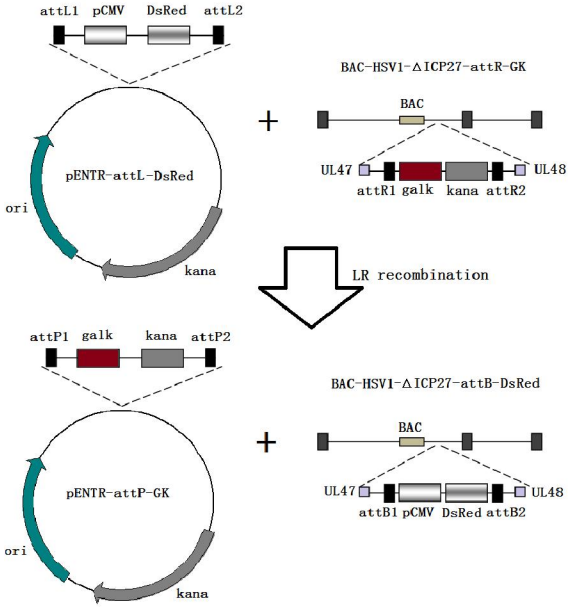

Fig.2. Outline of LR recombination

Results

1. Construction of BAC-HSV1-HF-ΔICP27-attR-GK replication-defective vector by Red recombineering

Through positive selection of the Red recombination in SW105 bacteria strain, we obtained the recombinant bacteria colonies, which were grown on MacConkey + Cm^+ + Kan^+ indicator plate. In Figure 3, recombinant bacteria colonies were shown in red color.

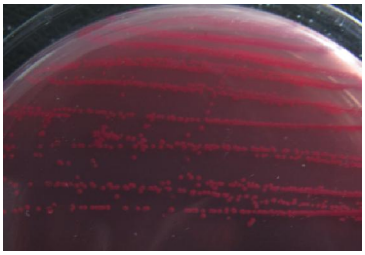

Fig.3. Recombinant bacteria colonies showing in red color on MacConKey + Cm^++ $Kana^+$ indicator plate

PCR analysis of Galk-kan gene from the selected recombinant colonies showed the Galk-kan gene was integrated to BAC-HSV-HF-ΔICP27 genome at the right sites. As shown in Figure 4, agarose gel electrophoresis showed the correct PCR products of Galk-kan gene with the primers of Kan-F/UL48-R in A1 and UL47-F/Galk-R in A2.

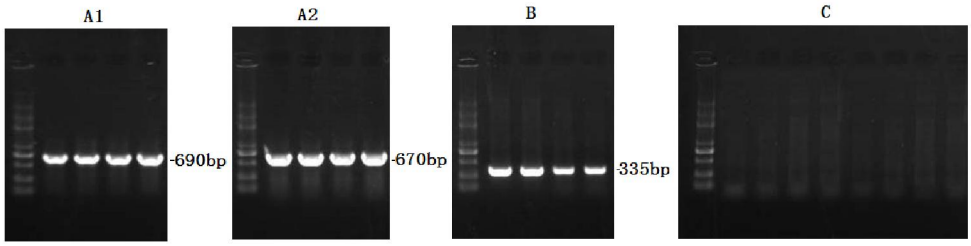

Fig.4. Agarose gel electrophoresis of PCR products
A1 primers：Kan-F/UL48-R；A2 primers：UL47-F/GalK-R；
B primers：DsRed-F/DsRed-R；C primers：Kan-F/UL48-R and UL47-F/GalK-R

2. Construction of BAC-HSV1-HF-ΔICP27-attB-DsRed replication-defective vector by LR recombination

After LR recombination between pENTR-attL1-DsRed-attL2 and BAC-HSV1-HF-ΔICP27-attR-GK, the recombination reaction was electroporated into DH10B competent cells, and the recombinant bacteria was applied on Cm^+ LB plate over night at 37℃ in a cabinet-type incubator. We obtained recombinant bacteria colonies, which could grow on Cm^+ but not $Kana^+$ LB plate were picked. PCR analysis of DsRed gene and the Galk-kan gene from the selected recombinant colonies showed the DsRed gene was inserted and Galk-kan gene was replaced from BAC-HSV1-HF-ΔICP27-attR-GK genome. In Figure 4, agarose gel electrophoresis showed the correct PCR products of DsRed gene with the primers of DsRed-F/DsRed-R in B, Kan-F/UL48-R and UL47-F/Galk-R in C.

3. **Functional identification of BAC-HSV1-HF-ΔICP27-attB-DsRed replication-defective vector**

As shown in Figure 6, after transfection of the Vero cells and 2-2 cells with BAC-HSV1-HF-ΔICP27-attR-GK and BAC-HSV1-HF-ΔICP27-attB-DsRed plasmid DNA, CPE was observed in 2-2 cells, but not in Vero cells; red fluorescence was observed in both Vero and 2-2 cells transfected with BAC-HSV1-HF-ΔICP27-attB-DsRed plasmid DNA, but not the cells transfected with BAC-HSV1-HF-ΔICP27-attR-GK plasmid DNA.

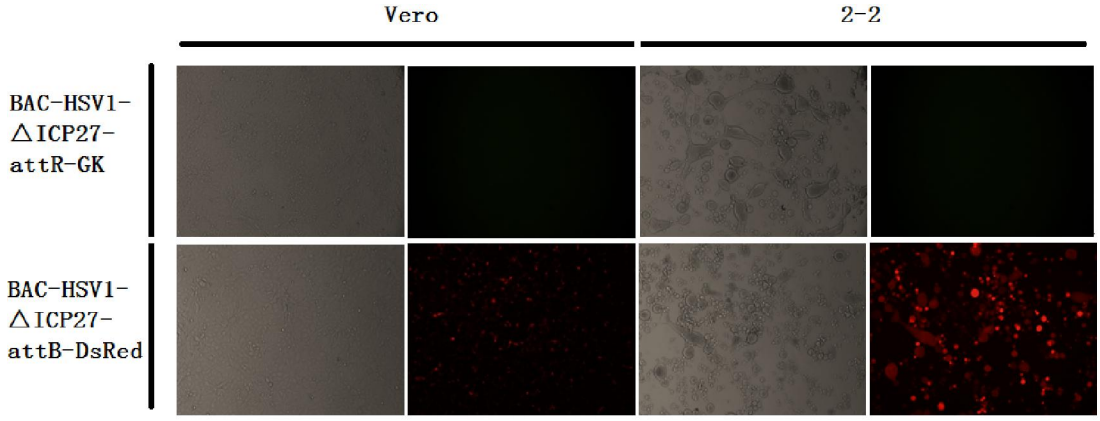

Fig.5. CPE and red fluorescence observation in Vero and 2-2 cells transfected with plasmids 72hr later(X 100)

Discussions

Insertion of exogenous DNA sequences into HSV-1 genome can be achieved in a number of ways. PHILIP J[14] and coworkers utilized the cre-loxp site-specific recombination system based on P1 phage, and lacZ (Escherichia coli β-galactosidase) as reporter gene, successfully inserted exogenous DNA sequences into HSV genome in vitro. DM Krisky[15] and coworkers used homologous recombination technology and lacZ cassette flanked by PacI restriction recognition sites as the reporter gene, made any modification of HSV-1 genome to be possible. However, the two methods mentioned above still need to screen recombinant clones in eukaryotic cells, the procedure remained complex. The emergence of recombineering technology and development of BAC technology further simplified the process of targeting mutation of HSV-1 genome. Our previous study[6]

successfully knockout the HSV-1 replication essential gene ICP27 from BAC-HSV1-HF plasmid and constructed the HSV-1 replication-defective vector BAC-HSV1-HF-ΔICP27. However, the integration of exogenous genes still need to be carried out in *E. coli* with a two-step recombination and selection, the process remained complex.

In this study, we constructed HSV-1 replication-defective vector BAC-HSV1-HF-△ICP27-attR-GK by recombinating the specific sites attR with selection gene galactokinase and kanamycin into the HSV-1 replication-defective vector BAC-HSV1-HF-△ICP27 through Red recombineering. As the integration site was between HSV-1 late gene UL47 and UL48, without deleting any gene, it will not affect the gene function. And then, to identify the function of HSV-1 replication-defective vector BAC-HSV1-HF-△ICP27-attR-GK, we integrated the reporter gene DsRed into BAC-HSV1-HF-△ICP27-attR-GK by a one-step LR recombination in vitro. After transfection 2-2 cells with plasmid DNA BAC-HSV1-HF- △ICP27-attB-DsRed, infectious virus particle was generated, and red fluorescence protein was expressed. These results indicated that the method of using LR recombination to integrate exogenous gene into HSV-1 replication-defective vector was feasible.

The advantages of BAC-HSV1-HF-△ICP27-attR-GK are: (i) The target genes can be site-directed integrated into HSV-1 replication-defective vector with only one-step recombination in vitro; (ii) Any DNA fragment flanked by specific sites attL can be integrated into HSV-1 replication-defective vector rapidly and efficiently, and it is promising in gene therapy research; (iii) This method can be applied to the studies of other viral related vectors and gene function in theory.

The establishment of this method further simplifies the procedure of integration of target gene into HSV-1 related vectors; it is a good candidate for research of target gene therapy and viral gene function. In addition, this method will play a great role in the development of commercial viral vectors in view of its features such as highly efficiency, site-directeded integration and recombination in vitro. Although this study provides a new idea for construction of HSV-1 and other viral vectors, it still has some shortages, such as integration of the target gene can only be inserted to the sites between attR1 and attR2. We believe that with the development of recombineering technology, target modification of the virus genome will become increasingly simple, and further facilitate the development of HSV-1 and other virus related vectors in gene therapy.

Conclusion

We constructed a HSV-1 HF derived replication-defective vector carrying the lambda phage based specific recombination sites attR. Through using this vector, site-directed integration of exogenous gene could be achieved by a simple one-step LR recombination in vitro. It simplified the procedure to integrate exogenous gene into HSV-1 replication-defective vector.

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