Creating mutant Murine Cytomegalovirus using signature tag mutagenesis

Vu Gia Phong
Hong Bang International University, Vietnam

ABSTRACT
Cytomegalovirus (CMV) belongs to the Beta subclass of Herpes virus family. Human Cytomegalovirus (HCMV) was found to be ubiquitous in human populations. While remaining asymptomatic or only causing mild subclinical consequences in healthy populations, HCMV causes debilitating symptoms in individuals whose immune system is compromised such as blindness, pneumonia in AIDS patients, mental or behavioral dysfunction in neonates, and allograft rejections. To make matters worse, the virus will establish a life-long latent and persistent infection. Currently, there are no effective vaccine or treatment methods for the virus clinically. In addition, HCMV research is challenging because Beta Herpes virus infections are highly species-specific. To overcome this barrier presented by HCMV, researchers make use of Mouse Cytomegalovirus (MCMV) because MCMV shares a high degree of genetic similarity and disease manifestation with HCMV. In this study, Bacterial Artificial Chromosome techniques developed in the Liu Fenyong Lab - UC Berkeley is coupled with signature tag mutagenesis to generate deletion mutants for each of the 170 genes in pSM3fr - our MCMV BAC construct. Using our novel design, we successful generate tagged mutants and demonstrate that we can distinguish each separate tagged virus from one another.

Keywords: *signature mutagenesis, vaccine, virus herpes, virus Cytomegalovirus*

1. INTRODUCTION

Human Cytomegalovirus (HCMV) is a very significant human viral pathogen. It is highly prevalent in underdeveloped populations[1]. Even in developed countries, the infection rate of this virus is quite significant [2]. In the United States alone, HCMV infected about 58.9% of individuals ≥ 6 years old [3]. HCMV infection is opportunistic. It creates mild or subclinical signs in immuno-competent individuals, yet in people with immunodeficiency, HCMV infection can result in high morbidity and mortality [4 - 6]. In newborns, HCMV is the leading cause of birth defects [7]. HCMV is also a prominent cause of allograft transplant complications and rejections [7].For the individuals listed above, the effects of HCMV infection can also be quite fatal.

To make matters worse, once infected with HCMV, the human host retains the virus for the rest of his or her life. In addition, there is no commercialized vaccine with high efficacy. For now, high dosages of acyclovir and valacyclovir are recommended to reduce the risk for CMV infection [8 - 9]. Valacyclovir has also been used to counteract the development of disease when a viral reactivation occurred [9]. Ganciclovir is an effective medication for CMV infection, yet it is rarely used today due to its significant toxicity. Foscarnet or cidovir is used as second-line treatment when previous indicated medications have failed [10 - 12].

Corresponding author: Dr. Vu Gia Phong
Email: phongvg@hiu.vn

Nevertheless, with increasing numbers of resistant incidents reported, it is crucial to develop more therapeutic treatments against this virus. HCMV research is challenging because HCMV infections are highly species-specific. This characteristic creates special challenges for studying HCMV since humans are the only animal models for an HCMV infection. However, Murine Cytomegalovirus (MCMV) is a virus that is very similar to HCMV in genetic composition, viral replication, and overall pathogenesis. Consequently, researchers make use of MCMV to overcome barriers presented by HCMV research [2, 13].

Specifically, MCMV and HCMV have 78 open reading frames (ORFs) that share a high degree of sequence identity [14]. In addition, they also share similar infection spectrum and ranges of disease manifestations. Understanding the role of MCMV and the contribution of genes to the tropisms of the virus will provide us with valuable insights and hints about HCMV pathogenesis.

Our lab has developed a successful protocol to generate viral mutants using bacterial artificial chromosome (BAC.) We have generated unique signature tagged virus mutants for each of 170 ORFs in pSM3fr- a MCMV BAC construct which replicates like wildtype and retain wildtype properties in vitro and in vivo [15].

Previously, signature mutagenesis (STM) makes use of transposon insertion mutagenesis and allows screening of multiple mutants by negative selection[16]. A short and unique DNA sequence is tagged with each mutant so that each mutant can be subsequently identified within a pool of mutants. By comparing the presence of mutants in the output (in vivo or stressed conditions) with the input (in vitro or non-stress conditions,) one can easily identify which mutants failed to persist. In this study, we took advantage of the quantitative PCR (qPCR) techniques to push for further boundaries of signature tagged mutagenesis. qPCR will allows us not only to detect the absence/ presence of viruses but also allow us to compare the degree of effectiveness of each unique mutant to persist in each organ, or assess the tropism of each mutants in a pool setting. In addition, we generated full-length deletion of each ORF and coupled each with a unique DNA barcode tag instead of generating a signature-tagged transposon mutant.

2. MATERIALS AND METHODS

2.1. Cells, Viruses

NIH3T3 cells were propagated in growth medium containing 10% Nu-Serum, essential and nonessential amino acids, penicillin-streptomycin, and Sodium Bicarbonate (Invitrogen) and cultured according to the guidelines set by ATCC. pSM3fr MCMV BAC virus was a gift from the Zhu Lab.

pSM3fr MCMV BAC virus and zeocin-tagged BAC mutants were propagated in NIH-3T3 cells. To generate stock virus, 2 T175 flasks of NIH3T3 cells at 90% confluence were infected with a low multiplicity of infection (M.O.I) of 0.1-0.5. When the cytopathogenic effect (CPE) is at 100%, the cells were separated from the flask with a cell scrapper. The infected cells and media in the flask were spun down at 300 x g for 10 minutes. Afterward, each pellet was re-suspended in a solution containing 10% non-fat dry milk that has been autoclaved. Each stock virus was sonicated three times on ice, and 1mL of the stock virus was aliquoted in cryovials. The stocks were stored at -80oC for long-term storage. Viral titers were obtained using standard plaque assays. Viral titers of stock ranged from 2x105 to 5x107plaque forming unit (PFU) per mL.

2.2. Generation of viral progeny from BAC DNA constructs

NIH3T3 cells of passage 20 or below were grown to 90-100% confluence. Cells were

trypsinized, pelleted and resuspended in DMEM. 3-5 million cells in 260µL DMEM were mixed with 4.5 µg of MCMV BAC DNA in a 4mm electroporation cuvette, and electroporated with a BTX ECM630 electroporator set at 250 volts, 960 µF, and 75 ohms. The expected pulse time was between 30 to 40 milliseconds. Immediately following electroporation, 1mL of DMEM media was added to the cell/DNA mixture in the cuvette. The contents of the transfection were then transferred to a T-25 flask. On the following day post transfection, the media in the T-25 flask was removed and fresh DMEM was added. The cells were then monitored for CPE. Cells transfected with viral BAC construct with wildtype-like replication kinetics would often exhibit plaque formation 3 to 5 days post transfection, indicating the presence and spread of infectious viral progeny from the BAC DNA. To remove the BAC vector from the viral genome, the virus was passaged five times in NIH3T3 cells.

2.3. Construction of the viral ORF-deletion mutants

To generate a deletion mutant for each open reading frame (ORF), two oligonucleotide primers (30 in Zeo 5' and 30 in Zeo 3') were constructed and contained the following genetic segments (from 3' to 5'): 64 homologous nucleotides (nt) to zeocin LoxP cassette, 20 nt unique barcode tag, and a common 19 nt primer. The 30 in Zeo 5' and 30 in Zeo 3' primers were used to amplify the Zeocin LoxP cassette, which contains the zeocin resistance gene flanked by LoxP sites. After the first round of PCR, the amplicon that contains the zeocin resistance cassette with LoxP site flanked by the barcode tag end and the common 19, was then subject to a second round of PCR with primers 50 up 5' and 50 dn 3'. Each primer contained 20 homologous nucleotides to the 1st PCR product and 50 bases of homology to the region upstream and downstream of the target ORF, respectively. The subsequent product was a zeocin LoxP cassette flanked by 50-nt homologous sequence targeting the ORF to be deleted in pSM3fr BAC. This PCR-amplified zeocin LoxP barcode tag cassette was then transformed into the DY380- a strain of bacteria carrying pSM3fr BAC. The DY380 was engineered from DH10B. DY380 strain expressed phage-derived recombination genes under the control of a temperature-sensitive repressor [17]. Trans-formation of pSM3fr-bearing DY380 strain with the PCR product resulted in the replacement of ORF based on selection for zeocin resistance. The 2 unique 20-mer barcode sequences were covalently linked to the sequence that targeted the MCMV genome, creating a permanent association and genetic linkage between a particular deletion strain and the tag sequences.

2.4. Bacteria transformation procedures

Recombination and electro-competent cells for transformation were generated by culturing DY380 bacteria at 30oC in low salt Luria Broth (LSLB) media and shaking at 250 rpm for approximately 2 to 3 hours until the culture reached the OD600 of 0.4 and 0.6. Then the bacteria were incubated at 42oC for 15 minutes in shaking water bath rotating at 250 rpm. At 42oC the recombination protein is allowed to express to permit the degradation of temperature sensitive repressor. This process makes the bacteria recombination competent. After the 15 minutes induction period, bacteria cultures were then immediately placed in an ice water slurry for 10 minutes and gently swirled to ensure uniform cooling of the bacteria. Bacteria cells were then recovered by centrifugation in a pre-cooled (4oC) centrifuge and washed with 100 mL of ice-cold sterile distilled water. Bacteria cells were then pelleted again and washed with 10% ice cold glycerol and subsequently re-pelleted. Afterward, the bacterial pellet was re-suspended in 10% ice-cold glycerol, aliquoted at a volume of 40 µL, and stored at -80oC for

long-term storage.

Bacteria competent cultures were electroporated with the PCR DNA product that contained the zeocin loxP cassette flanked by the regions targeting the specified ORF for deletion. Approximately 1 to 5µg of linear PCR product was mixed with 40 µL of the competent bacterial cultures in a 1 mm elec-troporation cuvette. A BTX ECM 630 electroporator was used with settings at 1.6 kV, 25 µF, and 150 ohms. The transformed bacteria were incubated for 1 to 2 hours at 30oC and plated on LSLB agar plates (12.5 µg/mL chloramphenicol/ 50 µg/mL zeocin) at 30oC. BAC DNAs were isolated from the surviving colonies and were screened with PCR assay, restriction profiling, and Southern analysis.

3. RESULTS

3.1. Generation of an STM MCMV Mutant Library

In order to assess the contribution of each ORF in viral replication, we took advantage of a rapid bacterial homologous recombination system and generated a collection of mutants in Escherichia coli by using a PCR-based mutagenesis approach to delete each of the predicted ORFs from pSM3fr MCMV BAC. Each gene was precisely deleted from the start to stop codons and replaced with a zeocin loxP resistance cassette, which was PCR-amplified using primers containing 50nt bordering the regions of the targeted ORF. Each deletion was verified using PCR screening, restriction digest profiling, and Southern analysis. All of the predicted 170 ORFs were deleted to create mutants with unique bar-code tag.

The mutant BAC DNA was isolated from the bacteria and transfected into cultured NIH3T3 cells. Of the 170 constructed mutants, 110 produced viral progeny, indicating that these mutated genes are not essential for MCMV replication in NIH3T3 cells. However, 60 mutants did not yield infectious progeny following repeated transfection and one month of incubation. In the experiments, two additional independent DNA preparation and transfections were conducted to confirm the no-growth phenotype of the construct.

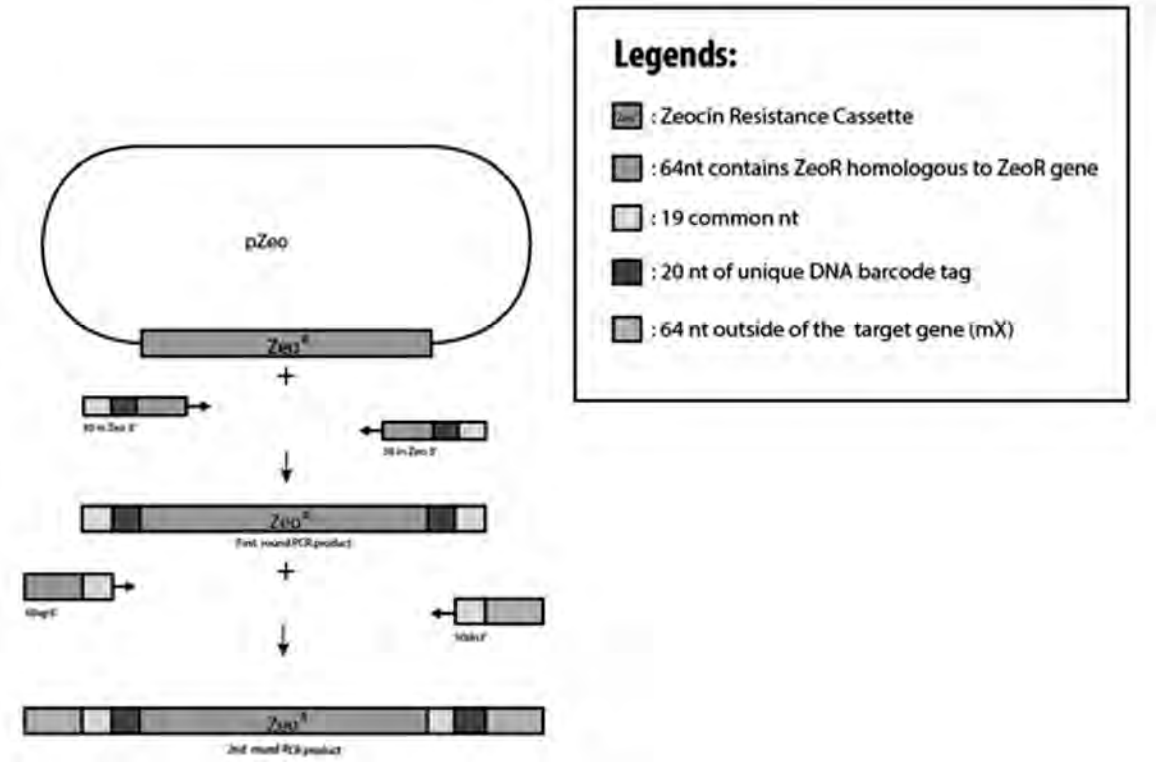

Figure 1. Generation of Zeocin Resistance Cassette that have a unique DNA barcode tag

To generate the first round PCR product, 2 primers, 30 in Zeo 5' and 30 in Zeo 3' were constructed containing from 3' to 5': 64 nucleotides homologous to Zeocin LoxP cassette which contains the zeocin resistance gene flanked by LoxP sites, 20 nt unique barcode tag, and a common 19 nt primer. After the first round of PCR, the amplicon resulted would contain the zeocin resistance cassette with LoxP site flanked by the barcode tag end and the common 19.

To generate the second round PCR product, we used 2 primers -50 up 5' and 50 dn 3'- contain 20 homologous nucleotides to the 1st PCR product and 50 bases of homology to the region upstream and downstream of the target ORF, respectively. The final product was a zeocin LoxP cassette flanked by 50-nt homologous sequence targeting the ORF to be deleted in pSM3fr BAC.

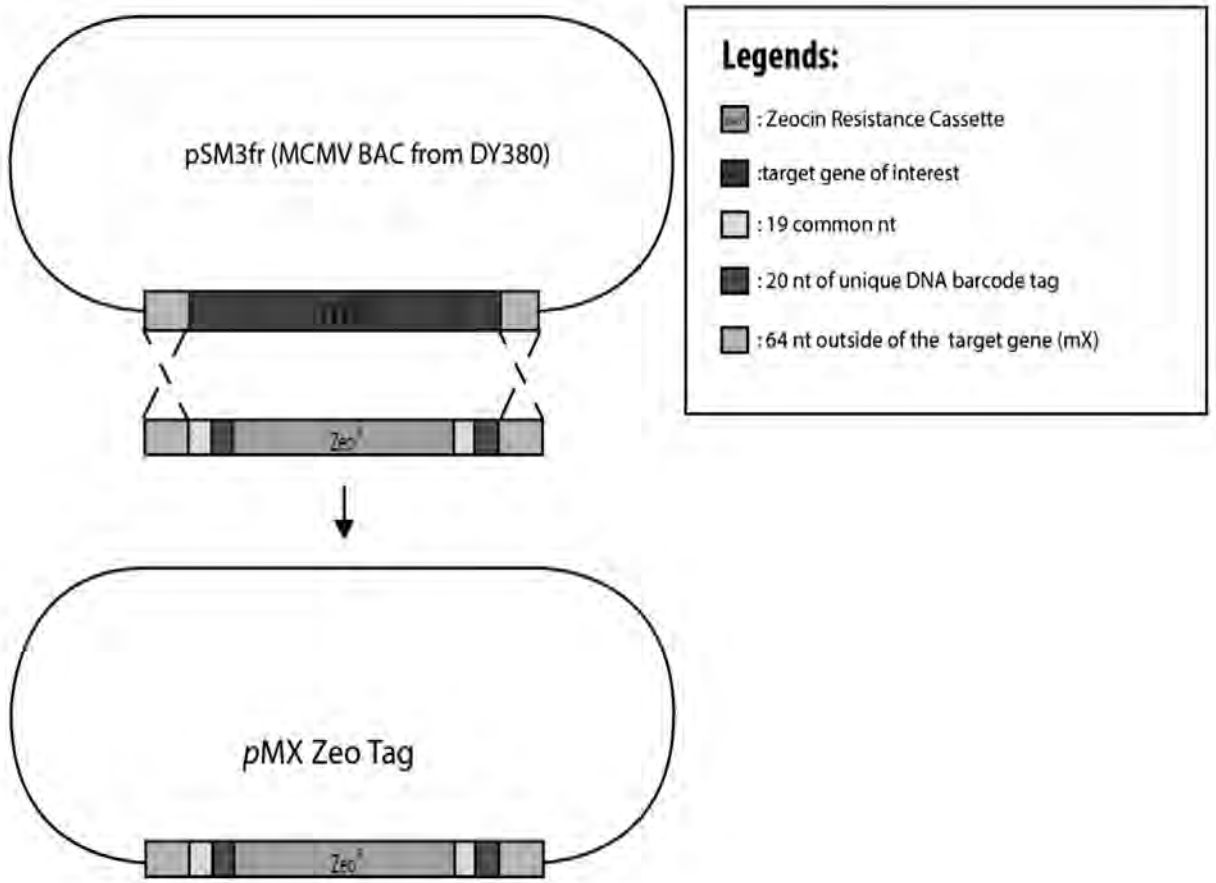

Figure 2. Generation of Signature Tag Bacterial Artificial Chromosome using unique barcod tag resistance cassette

This PCR-amplified zeocin LoxP barcode tag cassette was then transformed into the DY380- a strain of bacteria carrying pSM3fr BAC. The DY380 was engineered from DH10B. DY380 strain expressed phage-derived recombination genes under the control of a temperature-sensitive repressor. Transformation of pSM3fr-bearing DY380 strain with the PCR product resulted in the replacement of the targeted ORF based on selection for zeocin resistance. The resulting bacterial strain would have an altered BAC plasmid which contained a mutant MCMV genome with targeting gene deleted and permanently linked to 2 unique 20-mer barcode sequences.

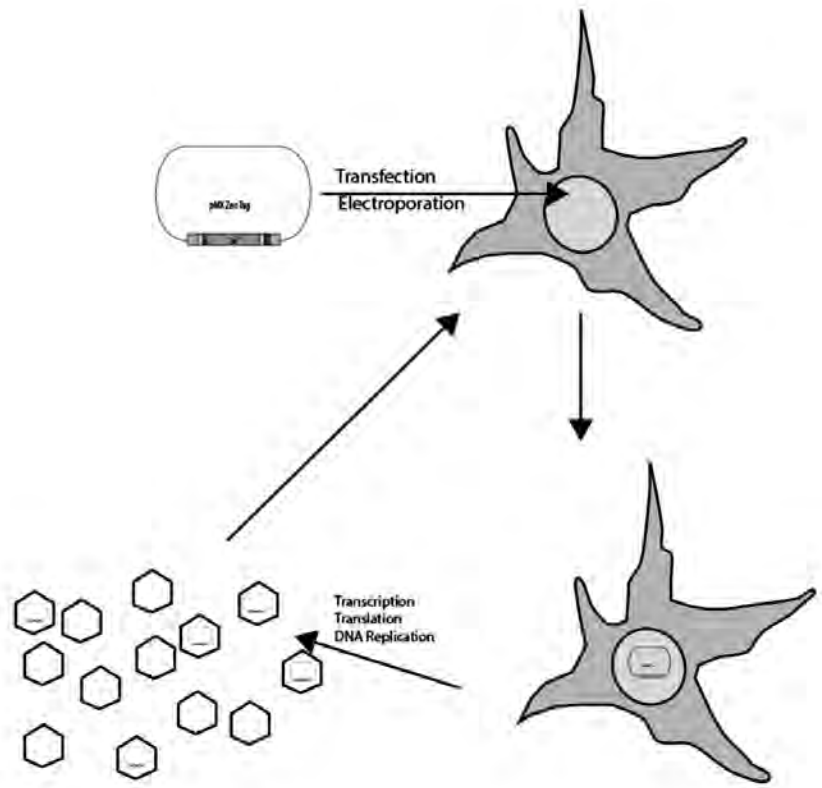

Figure 3. Generation of STM mutant Virus

The mutant BAC DNAs were isolated from bacteria and transfected into cultured NIH3T3 cells. NIH3T3 cells belong to the cell line that is most permissive for MCMV growth. In bacteria, the pSM3fr BAC act like a plasmid. However, in the NIH3T3 intra-cellular environment, the BAC DNA is replicated. MCMV genes will be able to undergo transcription, translation and DNA replication allowing for the production of Signature Tag Mutant viruses to take place.

3.2. In-vitro confirmation of unique barcode tag Mutants and their unique primer pairs

Following the generation of viruses that do not have the unique signature bar code tag inserted in the essential genes for replication and production in NIH3T3, we performed quantitative Real-time quantitative PCR assays followed by gel electrophoresis assays to determine whether it is possible to detect each unique barcode tag mutant using its respective set of primer pair.

We confirmed that when a mutant or a mutant pool contains the virus which uniquely associated with the detection primer pair (abbreviated as dPSM3fr +mX – X is the numerical nomenclature of the MCMV ORF), we were able to detect a band with the size of 650bp (lane 2-9 Figure 2.) On the other hand, if the mutant of interest was not present (even in the presence of other STM Mutants MCMV), no band would be visualized at the location associated with 650bp (lane 11-18). Band#1 is the pool reaction PCR that used the primers associated with m107. The bands' presence implied that MCMV DNA is in the pool since it matches with predicted size for m107- 695 base pairs.

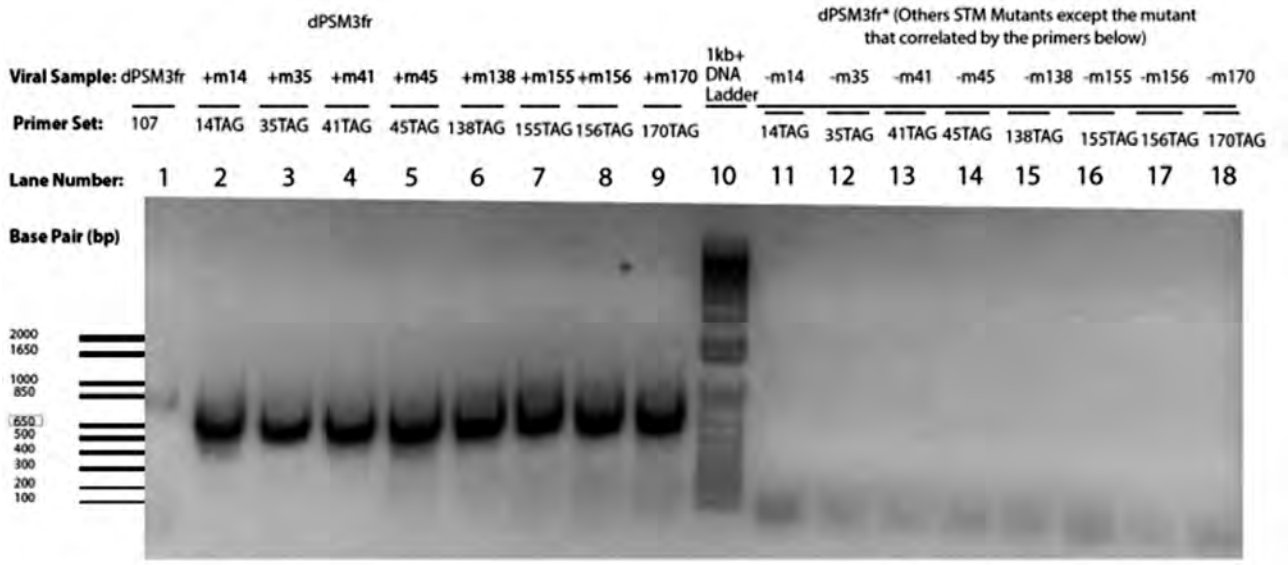

Figure 4. Confirmation of STM Mutant with primer pair associated with the respective barcode tag

Using PCR amplification, the insertion of the zeocin unique tag barcode was verified.

Lane 1 corresponds to 695bp, which corresponds to the length of ORF m107, demonstrates the presence of virus in the pool.

Lane 2-9 corresponds to 650bp. When the primers used matched with the corresponding virus, the product of the PCR would be 650bp in length.

Lane 10 corresponds to 1kb+ DNA Ladder purchased from Invitrogen.

Lane 11-18 have no band presented at the location of 650bp when the STM virus is absent even in the presence of other tagged viruses. These observations demonstrated the specificity of each unique DNA barcode tags.

Table 1. Primers for generated these 12 unique 20-mer DNA barcode tag mutant MCMV

Primer Name	DNA Sequence
m07 30 in Zeo 5'	GATGTCCACGAGGTCTCTAACCGGAGAAACTACGCCGTATAACTTCGTATAATGTATGCT ATACGAAGTTATCAAGTTTCGAGGTCGAGTGTCAGTCCTGCT
m07 30 in Zeo 3'	CGGTGTCGGTCTCGTAGGGAGTCTCTGATCTGCTGTCATAACTTCGTATAATGTATGCTA TACGAAGTTATGGAACGGACCGTGTTGACAATTAATCATCG
m07 50 up 5'	gacactcttgtctgagtcttttggcgtcgtcgggtctatcgagtgagagaGATGTCCACGAGGTCTCTAA
m07 50 dn 3'	gtgtccaggtgtatctgatacgtaccgtcgcacctgcggcggtcggccctCGGTGTCGGTCTCGTAGGGA
m09 30 in Zeo 5'	GATGTCCACGAGGTCTCTTAGTTATACTGTACGTGGCCATAACTTCGTATAATGTATGCT ATACGAAGTTATCAAGTTTCGAGGTCGAGTGTCAGTCCTGCT
m09 30 in Zeo 3'	CGGTGTCGGTCTCGTAGGCGTTAGACTACGTTGACTGATAACTTCGTATAATGTATGCTA TACGAAGTTATGGAACGGACCGTGTTGACAATTAATCATCG
m09 50 up 5'	cggcgcacctgttgcgccggtatccatttgttcacattaaatgatacataGATGTCCACGAGGTCTCTTA
m09 50 dn 3'	actcggacaggtgtgggcggttccaggtgtacgtagaaaatgtgggcaaaCGGTGTCGGTCTCGTAGGCG

2 primers, 30 in Zeo 5' and 30 in Zeo 3' were constructed containing from 3' to 5': 64 nucleotides homologous to Zeocin LoxP cassette which contained the zeocin resistance gene flanked by LoxP sites, 20 nt unique barcode tag, and a common 19 nt primer. The 2 primers were used to synthesize the first round of PCR product. The amplicon resulted would contain the zeocin resistance cassette with LoxP site flanked by the 20-mer barcode tag end and the common 19.

2 primers -50 up 5' and 50 dn 3'- contained 20 homologous nucleotides to the 1st PCR product and 50 bases of homology to the region upstream and downstream of the target ORF, respectively. The final product was a zeocin LoxP cassette flanked by 50-nt homologous sequence targeting the ORF to be deleted in pSM3fr BAC.

Table 2. Primers to detect the presence of corresponding STM MCMV mutant

Primer Name	DNA Sequence
m07 TAG-up	GATGTCCACGAGGTCTCTAACCGGAGAAACTACGCCGT
m07 TAG-dn	CGGTGTCGGTCTCGTAGGGAGTCTCTGATCTGCTGTC
m09 TAG-up	GATGTCCACGAGGTCTCTTAGTTATACTGTACGTGGCC
m09 TAG-dn	CGGTGTCGGTCTCGTAGGCGTTAGACTACGTTGACTG

TAG-up and TAG-dn were constructed from 19 nucleotides of common tag and 20 nucleotides of unique DNA barcode tag in order to detect the corresponding mutant. When the DNA from STM mutant MCMV was present, the PCR reaction could take place to produce 650bp amplicon.

4. DISCUSSION

This effort of using Signature Tag Mutagenesis to create global deletion of MCMV genome provides a list of many important viral genes for pathogenesis *in vitro* (NIH3T3) and *in vivo* (SCID mice.) In NIH3T3, arguably the most permission cell line for MCMV growth, the mutant viruses revealed very diverse growth phenotypes ranging from absolutely essential to unnecessary for viral production.

The advance of our primers' designed approach using two sets of primers with both unique and common primers allow for switching of the unique bar code tag if necessary. For example, if the bar code tag for m02 is effective, it is possible to reuse this barcode tag for m102. Nevertheless, if two mutants share the same bar code tag, it would not be possible to tell the two mutants apart using the detection primers.

We have confirmed that each unique DNA bar code tag primer pairs only detects the unique bar-code tag that it associated with resulting in a band at 650bp.

Transfection of the BAC library will allow us to determine the essential genes required for successful production of virus which would allows us to use other engineer tool to treat viral infections. In addition, further experiment in animals will help us to understand which mutants that have attenuated production which can subsequently allow us to engineer live-attenuated viral vaccines.

ACKNOWLEDGEMENTS:

This research was funded by the National Institute of Health of America, the University of California at Berkeley and was primarily done in Liu Fenyong Lab's School of Public Health, University of California, Berkeley.

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Tạo dòng Murine Cytomegalovirus (MCMV) đột biến bằng kỹ thuật 'đột biến tạo mã nhận biết chuyên biệt' (DNA signature tag mutagenesis)

Vũ Gia Phong

TÓM TẮT

Cytomegalovirus (CMV) thuộc phân lớp Beta của họ virus Herpes. Cytomegalovirus ở người (HCMV) được phát hiện các quần thể người. Trong khi vẫn không có triệu chứng hoặc chỉ gây ra hậu quả cận lâm sàng nhẹ ở những người khỏe mạnh, HCMV gây ra các triệu chứng suy nhược ở những người có hệ thống miễn dịch bị tổn hại như mù lòa, viêm phổi ở bệnh nhân AIDS, rối loạn chức năng tâm thần hoặc hành vi ở trẻ sơ sinh và thúc đẩy từ chối tiếp nhận các mô cấy ghép. Còn tồi tệ hơn thế nữa, virus sẽ hình thành một bệnh nhiễm trùng tiềm ẩn và dai dẳng suốt đời. Hiện tại, không có vaccine hoặc phương pháp điều trị hiệu quả cho virus HCMV trên lâm sàng. Ngoài ra, nghiên cứu HCMV là một thách thức cực lớn vì virus Beta Herpes có tính đặc trưng cao.HCMV không gây nhiễm ở các động vật hữu nhũ khác ngoài người. Để vượt qua rào cản này do HCMV đưa ra, các nhà nghiên cứu sử dụng Cytomegalovirus gây bệnh trên chuột nhắt (MCMV) vì MCMV có chung mức độ tương đồng di truyền và biểu hiện bệnh cao với HCMV. Trong nghiên cứu này, Bacterial Artificial Chromosome (nhiễm sắc thể vi khuẩn nhân tạo) được phát triển trong Phòng thí nghiệm Liu Fenyong - UC Berkeley được kết hợp với kỹ thuật đột biến tạo mã chuyên biệt (signature mutagenesis) để tạo ra đột biến xóa cho từng gene một trong số 170 gene trong pSM3fr - cấu trúc MCMV BAC.Với thiết kế mới lạ của mình, chúng tôi đã tạo thành công các đột biến được gắn thẻ và chứng minh rằng chúng tôi có thể phân biệt từng virus được gắn mã nhận biết DNA (DNA signature) riêng biệt với nhau. Nghiên cứu này tạo tiền đề kỹ thuật để phát triển các virus suy giảm độc tính tạo tiền đề cho việc phát triển vaccine và xác định độ quan trọng của từng gene của virus để tạo.

Từ khóa: *signature mutagenesis, vaccine, virus herpes, virus Cytomegalovirus*

Received: 15/09/2022
Revised: 20/10/2022
Accepted for publication: 20/11/2022