RESEARCH ARTICLE

Studying a poxvirus gene capture model through recombination and reactivation

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Keywords
Poxvirus: *Large double-stranded DNA viruses, which infect both vertebrates and invertebrates.*
Vaccinia (VACV): *Poxvirus famously used to vaccinate humans against smallpox. Vaccinia virus is the most widely used model in poxvirus research.*
Recombination: *The breaking and rejoining of genetic material to create new combinations of genetic traits.*
Homology: *Sequence similarity between two (or more) DNA, RNA, or protein molecules.*
Titer: *Concentration of infectious virus expressed in plaque forming units per mL (PFU/mL).*

Abstract

Introduction: Vaccinia poxvirus (VACV) is a double stranded DNA virus that replicates in the cytoplasm of infected cells. Some VACV genes resemble homologs of host genes and appear to have been captured from the cell; however, since poxviruses are confined to the cytoplasm, researchers are unclear as to how these viruses acquire this homology (1). If a cellular mRNA was accidentally reverse transcribed into cDNA, which could occur during retrovirus co-infection, a poxvirus might be able to incorporate this sequence into its own genome through rare non-homologous (homology-independent) recombination.

Methods: We modeled this process using two different recombination systems and substituted a DNA encoding mycophenolic acid (MPA), a selectable marker, for the hypothetical non-homologous host cDNA. We prepared DNA constructs containing this marker along with 20 base pairs homologous to the 5' and 3' flanking regions of the VACV-encoded *Not*I restriction site. A construct without this flanking homology was also prepared. The "passive" recombination system used a helper poxvirus to reactivate VACV DNA; in contrast, VACV infected BSC40 cells were transfected with the construct in the "active" recombination system.

Results: The "passive" recombination system generated 105 PFU/mL of reactivated VACV; however, no recombinants containing the selectable marker were detected. The "active recombination" method generated 106 PFU/mL of total VACV and approximately 10 PFU/mL of recombinant virus for both homology containing and non-homology containing constructs.

Discussions: We were unable to determine the recombination frequency of the "passive system" because recombinant virus was not detected. Based on virus titers determined from plaque assays, we approximated the recombination frequency of the "active system" to be ≤ 10-5. We are currently cloning and sequencing viruses resulting from non-homologous recombination to determine where the MPA marker is located. Preliminary analysis of these types of clones (data not shown in this paper) suggests that the transfected DNAs are being incorporated into a diversity of sites, some located near the boundary of the VACV genome where the right terminal inverted repeat begins. In summary, our findings suggest that the recombination frequencies in both methods are very low and better methods of selection are needed to observe these rare events. Future studies of recombinant clones are needed to gain a better understanding of this non-homologous gene capture process.

Introduction

Poxviruses are large double stranded DNA viruses that replicate solely in the cytoplasm of infected cells. VACV was famously used to vaccinate against smallpox and is very commonly used in poxvirus research. VACV and other members of this family encode all of the enzymes required for viral replication, including DNA replication and transcription. Genetic analysis of VACV and other poxviruses has revealed sequences that appear to have cellular origins (1). Over the course of thousands of years, these viruses presumably captured

copies of cellular genes, which mutated into products that conferred advantages against host immune responses. For example, the VACV CrmE gene encodes a tumor necrosis factor (TNF) receptor, which has significant homology to the mammalian type 2 TNF receptor (1). This mutant receptor allows the virus to evade apoptosis mediated by host produced TNF-α. However, since poxviruses are confined to the cytoplasm, researchers are unclear as to how they are able to acquire these genes. Furthermore, it is also unclear how other large DNA viruses, such as Herpes virus, capture foreign genes. It is unlikely that VACV picked up genes from other sources though, but Fadly *et al.* showed that Fowlpox virus could capture the DNA form of the Reticuloendotheliosis retrovirus genome during replication (5). However, it is possible that this retrovirus DNA was simply mistaken for cellular DNA. In the current study, we investigated a potential mechanism for poxvirus "gene capture" using recombination.

Previous work done by Evans *et al.* showed that recombination occurs frequently during poxvirus replication (2). Gradually, successive recombinations lead to the evolution of new strains as DNA is exchanged between viruses. If a host gene is transcribed, processed, and then reverse transcribed back into a cDNA, a poxvirus could capture this gene through rare illegitimate (non-homologous) recombination. Presumably, the required reverse transcriptase activity would be supplied by endogenous enzymes or by a retrovirus also present within the cell (retroviral co-infection). Viral and host genomes have little sequence similarity; the cDNA fragment would be incorporated into a random location within the viral DNA, but it would likely insert into regions surrounding the telomeres, where non-essential genes are located. We also hypothesized that cDNA containing any flanking homology to a specific region of the viral genome will recombine into that locus even if the homology is only a few nucleotides. The frequency of recombination events will increase when larger sequence homologies are present (3).

This study provides preliminary data and method optimization for our future recombination experiments. Although VACV is a widely used model system in virology, little is known about its evolution or the origins of poxvirus genes. This work represents the first step in understanding this process and testing our proposed gene capture model, showing that poxviruses can integrate foreign DNA found in the cytoplasm into their genomes via non-homologous recombination.

Methods

Plasmids, Primers and PCR Conditions:

Two PCR products were constructed from the pDGloxP(del) plasmid, kindly provided by Don Gammon. One of these constructs contained 20 base pairs of flanking homology to the NotI restriction digest site in the VACV genome and the other did not have any flanking homology to the NotI site (Fig. 1). Notably, there is only one NotI digestion site in the VACV genome. Both of these constructs also contained a yellow fluorescent protein-guanine phosphoribosyltransferase mycophenolic acid selectable marker (YFP/gpt) controlled by an early poxvirus promoter. A control construct was also created, which did not contain the selectable marker, but instead contained approximately 600 base pairs of homology flanking either ends of the NotI restriction site (Fig. 1).

PCR Product A (gpt/*Not*I) was constructed using the following primers:

5'TAGACAACACACCGACGATGAGGGCGAATTGAGTGAAGGCCG3' (forward) and 5'TAGTCATATTCGTGTCTGTGGAGTCAGTGAGCGAGGAAGCGG3' (reverse) (Fig. 1).

PCR Product B (gpt) was constructed using the following primers:

5'AGGGCGAATTGAGTGAAG3' (forward) and 5'GAGTCAGTGAGCGAGGAA3' (reverse) (Fig. 1).

PCR product C was constructed from VACV DNA using the following primers, kindly provided by Li Qin:

5'ACACCCAAAAACAACCGA3' (forward) and 5'ACATACCATCGACATCCA3' (reverse) (Fig. 1).

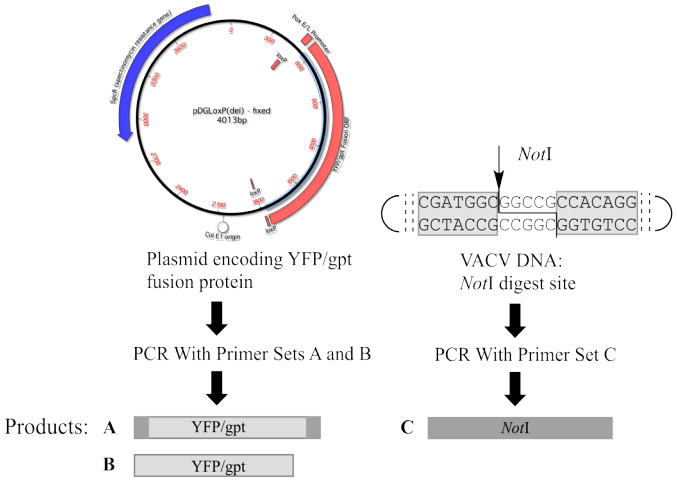

Fig. 1
Constructed PCR products containing a YFP-gpt fusion protein (MPA selectable marker). Product A is flanked by 20 base pairs of homology to the VACV NotI digestion site. Product B does not have homology to the NotI digestion site. Product C is a control for recombination experiments and encodes 600 base pair homology to the NotI digestion site.

All PCR products were verified by gel electrophoresis and purified using spin columns (Fermentas).

Plaque Assay Using Crystal Violet Staining

All viruses were titered by infecting mycoplasma-free African green monkey kidney epithelial (BSC40) cells in six well culture plates with 1mL of ten-fold dilutions (10-1 to 10-7) of virus. Titer plates were cultured for 1 day at 37°C to allow for plaque formation and then stained using crystal violet. To stain, cell medium was removed and then crystal violet was added at room temperature for 30 minutes. The stain was removed by rinsing plates with distilled water. Virus titer in plaque forming units (PFU) was calculated by selecting the well that contains approximately 10-20 plaques and then accounting for the dilution. For example, if the 10-4 dilution well had 10-20 plaques, then virus titer would be 105 PFU/ mL considering that cells were infected with 1mL of virus.

Active Recombination: Live Virus Recombination

Mycoplasma-free BSC40 cells were cultured in Minimal Essential Medium (MEM, Life Technologies), which was supplemented with 5% fetal bovine serum, 1% nonessential amino acids, 1% l-glutamine, and 1% antibiotic at 37°C. These cells were infected with VACV (Western Reserve strain) at a multiplicity of infection of one for 1 hour in phosphobuffered saline (PBS) at 37°C, which is enough time for the majority of cells to become infected. As a negative control, BSC40 cells were also mock infected with PBS for 1 hour at 37°C. VACV infected cells were then transfected with either of the PCR constructs using the following protocol. DNA and Lipofectamine 2000 transfection reagent (Invitrogen) were incubated separately in minimal serum medium (Opti-MEM, Life Technologies) for 5 minutes at room temperature and then gently added together and incubated for an additional 20 minutes at room temperature. PBS was removed from the cells and then the transfection mixture was added, along with MEM, immediately 1 hour after the VACV infection period. Cells were transfected for 1 hour at 37°C. These time frames were chosen based on work carried out by G. McFadden *et al.*, which showed that replication and recombination occur concurrently during the first phase of the VACV replication (replication persists up to 6-8 hours post-infection) (2). The transfection mixture was then removed and fresh medium was added. Cells were cultured at 37°C for 1 day to allow for viral replication and then recombinants were selected for by adding 1 X mycophenolic acid (MPA) (Fig. 2).

Recombinant virus was cloned and purified by re-plating in the presence of 1 X MPA three additional times, which ensures elimination of wild type VACV. To approximate recombination frequency, plaque assays were performed on virus samples taken from the initial round of MPA selection. Total virus (recombinant + non-recombinant) was determined by titering samples from transfected cells that were plated without drug. Approximate recombination frequency was calculated using

$$\frac{\text{Recombinant virus } (\frac{\text{PFU}}{\text{mL}})}{\text{Total virus } (\frac{\text{PFU}}{\text{mL}})}$$

Passive Recombination: VACV Reactivation Using a Helper Poxvirus

Poxvirus DNA alone is not infectious because it does not have access to either viral or cellular DNA replication machinery. Passive recombination is a reactivation strategy where new VACV is produced from viral genomic DNA. Yao *et al.* showed that VACV DNA could be reactivated into live virus if the cell is infected with another helper poxvirus (4). The helper poxvirus provides all necessary replication machinery. Furthermore, since VACV can be reactivated from a series of overlapping DNA fragments, recombination should also occur between a foreign PCR product with homology to the VACV genome and the other corresponding VACV fragments during reactivation (Fig. 3). The PCR product simply resembles another fragment of viral DNA if significant homology is present. We tested whether recombinants could be produced if the PCR product did not have any homology. This allowed us to investigate whether gene capture is a "passive" process and whether it could have occurred while the virus was inactivated (e.g. by UV light) within a host cell. We also investigated whether a double stranded break promotes recombination, by digesting VACV DNA with *Not*I before transfection (Fig. 3).

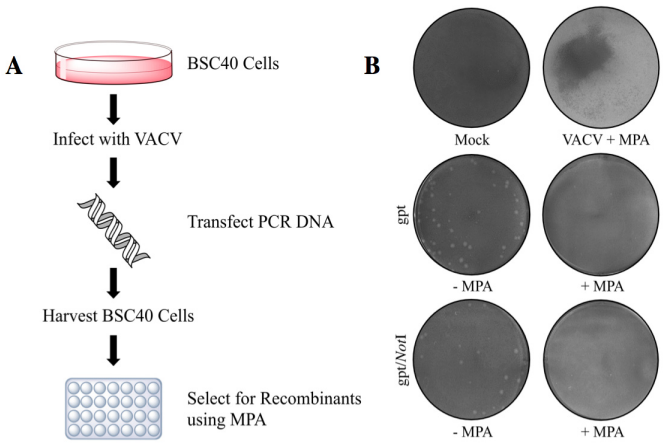

Fig. 2
(A) Active recombination system method using BSC40 cells. Only recombinant VACV containing the YFP/gpt fusion gene can survive in the presence of MPA.
(B) From top left to right: mock infected BSC40 cells; VACV (strain WR) infected BSC40 cells cultured for 3 days with MPA; VACV infected BSC40 cells transfected with PCR product B (gpt, no NotI site homology) with and without MPA selection; VACV infected BSC40 cells transfected with PCR product A (gpt/NotI, 20 base pairs homology) with and without MPA selection.

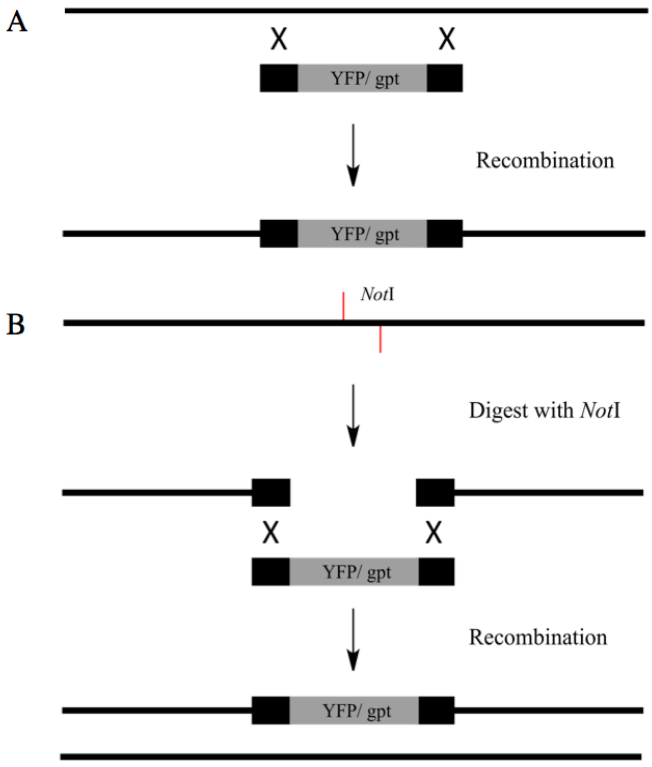

Fig. 3
(A) Homologous recombination of selectable marker and VACV DNA without digestion (double stranded break independent).
(B) Homologous recombination of selectable marker and VACV with *Not*I digestion (double stranded break dependent).

For the reactivation, mycoplasma-free Buffalo green monkey kidney (BGMK) cells were cultured in Minimal Essential Medium (MEM, Life Technologies), which was supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% l-glutamine, and 1% antibiotic at 37°C. BGMK cells were infected with Shope Fibroma poxvirus (SFV) at a multiplicity of infection of 0.1 for 1 hour in PBS at 37°C. As a negative control, BGMK cells were also mock infected with PBS for 1 hour at 37°C. BGMK cells support the growth of both VACV and SFV (Fig. 4). SFV infected BGMK cells were then transfected with either NotI digested or undigested VACV DNA and either of the PCR constructs according to the aforementioned protocol. Cells were cultured at 37°C for 1 day to allow for VACV reactivation and replication. Reactivated virus was selected by plating on BSC40 cells, which only support VACV growth; virus was plated with 1 X MPA to select for recombinants and without 1 X MPA to determine total reactivated VACV. After culturing for 3 days at 37°C, resulting VACV was titered using plaque assay with crystal violet staining. Virus was given 3 days to replicate because very little reactivated virus is initially present.

Results

Active Recombination: Live Virus Recombination

We investigated whether replicating VACV could capture DNA containing a selectable marker. BSC40 cells were infected with VACV and then recombinants were isolated by culturing with MPA. Notably, recombinant viruses were cultured under a total of four rounds of drug selection to eliminate wild type VACV even though no plaques were visible above after the first round (Fig. 2). For cells transfected with PCR Product B (gpt, no *Not*I site homology), the viruses present in - MPA conditions are most likely wild type VACV (titer of 10^6 PFU/mL) because no recombinants were detected (Fig. 2). However, we were able to propagate recombinant virus from these + MPA samples that have very low titers (approximately 10 PFU/mL). + MPA titers increased with continued culture as the small amount of recombinant virus present replicated to detectable levels. Plaque assays of recombinant virus after four rounds of MPA selection showed high virus titers of approximately 10^7 PFU/mL (data not shown here); non-homologous recombination between the DNA and VACV was successful. VACV infected BSC40 cells transfected with PCR Product A (gpt/*Not*I, 20 base pairs homology) yielded similar results as the gpt transfected virus. The 20 base pair homology does not appear to have had a significant effect on recombination rates because comparable amounts of recombinant and total virus were recovered (10 PFU/mL and 10^6 PFU/mL). However, based on previous work done by the Evans's lab, we expect larger homologies, such as 100 base pairs, to have a more pronounced affect on recombination (3). We expect higher initial titers of recombinants with this amount of homology (> 10 PFU/ml). The overall recombination frequency for the active system was calculated to be 10^{-5} (0.00001).

Passive Recombination: VACV Reactivation Using a Helper Poxvirus

We investigated whether a segment of DNA could be incorporated into VACV DNA during reactivation by a helper poxvirus. BGMK cells were infected with SFV and then transfected with *Not*I digested or undigested VACV DNA and a PCR construct. The resulting virus was then cultured in BSC40 cells with and without drug selection.

Approximately 10^2 PFU/mL of total reactivated VACV was recovered from BGMK cells transfected with undigested VACV DNA and PCR Product B (gpt, no *Not*I site homology) (Fig. 4). However, recombinants were not detected, and we were unable to propagate the virus further as with virus from the active recombination system (Fig. 4). Interestingly, 10^5 PFU/mL of reactivated virus was recovered from cells transfected with undigested VACV DNA and PCR Product C, which has 600 base pair homology (data not shown). Less than 10^2 PFU/mL of total reactivated VACV was recovered from BGMK cells transfected with NotI digested VACV DNA and PCR Product A (gpt/*Not*I, 20 base pair

homology) (Fig. 4). Recombinants were not detected, and we were unable to propagate neither total virus nor recombinant virus further. However, the reactivation system still functions with digested DNA because approximately 10^2 PFU/mL of reactivated virus was recovered from BGMK cells transfected with digested VACV DNA and PCR Product C (data not shown).

Discussions

The active recombination system produced 10^6 PFU/mL VACV without MPA selection (total VACV) and 10 PFU/mL with MPA selection (recombinant VACV). These titers were very similar for both homology containing PCR Product A and non-homology PCR Product B recombinant viruses. The passive recombination system generated 10^2 PFU/mL of reactivated VACV for BGMK cells transfected with PCR Product B and undigested VACV DNA, but no recombinants were detected. 10^5 PFU/mL of reactivated virus was recovered from BGMK cells transfected with undigested VACV DNA and PCR Product C. These results were similar for cells transfected with PCR Product A and undigested VACV DNA. For BGMK cells transfected with NotI digested VACV DNA and PCR Product A, less than 10^2 PFU/mL reactivated VACV was recovered and no recombinants were detected. We could not recover reactivated virus for BGMK cells transfected with digested VACV DNA and PCR Product B; however, 10^2 PFU/mL reactivated VACV was recovered from BGMK cells transfected with digested VACV and PCR Product C.

Although we did not detect recombinant VACV from the reactivation system and detected few recombinants from the active recombination system, it is possible that these rare recombination events occurred more frequently than suggested. For the active system, we were able to propagate the recombinant virus even though we were not able to titer it; we did not see plaques in the 10^{-1} dilution. At low titers, it is possible to take samples of the stock that do not contain the virus. A titer using this sample would then produce a misrepresentation of the actual number of recombinants. Taking this into account, we approximated the viral titer to be 10 PFU/mL. To avoid this sampling error in future experiments, the entire stock of virus could be plated out on larger dishes, giving a more accurate reflection of the virus titer.

Yao *et al.* demonstrated that VACV reactivation is limited by the number of fragments that need to recombine to reconstruct the genome (4). With an increasing degree of fragmentation, VACV reactivation would be severely reduced or would not occur at all. To avoid this, we grew VACV and purified new DNA to ensure minimal shearing; however, reactivation frequencies did not improve. The reactivation system is also limited by transfection efficiency. Helper poxvirus infected cells must contain all appropriate overlapping DNA fragments in order to reactivate VACV. Recombination between VACV DNAs may still occur in infected cells; however, they may fail to produce live VACV if some fragments are missing. Thus, the reactivation system depends on how much DNA is taken up by helper poxvirus infected cells. With a more efficient transfection, we are more likely to find correct fragments inside a single cell. We will continue to optimize transfection efficiency by varying the concentration of the transfection reagent used and by changing the amount of DNA transfected. However, we expect that increasing the transfection efficiency will not change the recombination frequency because modern transfection protocols introduce large molar amounts of DNA into cells; there will be enough DNA present for recombination to occur optimally. The reactivation system primarily depends on having the right DNA fragments present within the cell, the likelihood of which increases if more DNA is introduced into the cell.

Interestingly, reactivated VACV was produced in samples transfected with the non-homology containing PCR product (PCR Product B). Recombination is favored when there is homology between the overlapping DNA sequences; correspondingly, the likelihood of recombination decreases significantly between non-homologous sequences (4). Yao *et al.* showed that recombination frequencies increase when the overlap in sequence between recombining DNAs is greater (3). Since recombinants were not detected in the presence of MPA, it is possible that the plaques found on drug free plates were formed by wild type VACV. This could be confirmed by DNA sequencing. Notably, SFV + VACV DNA was not under selective pressure until cell extracts were plated on BSC40 cells. Since MPA selection was not present, VACV

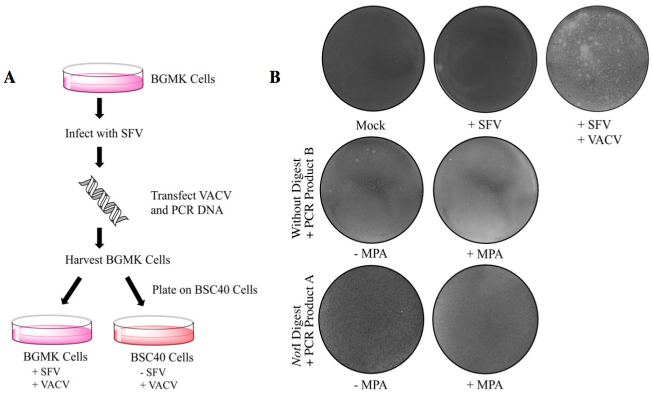

Fig. 4
(A) Passive recombination system method using BGMK and BSC40 cells. Only recombinant reactivated VACV containing the YFP/gpt fusion gene can survive in the presence of MPA.
(B) From top left to right: Mock infected BSC40 cells; SFV infected BSC40 cells; SFV and reactivated VACV infected BGMK cells; reactivated virus from BGMK cells transfected with undigested VACV DNA and PCR product B (gpt, no *Not*I site homology), with and without MPA selection; reactivated virus from BGMK cells transfected with NotI digested VACV DNA and PCR product A (gpt/*Not*I, 20 base pair homology), with and without MPA selection.

did not have to pick up the YFP/gpt marker during reactivation. If transfected BGMK cells were plated with MPA, then only virus in cells expressing the gpt marker would grow. Presumably, this is one potential method of increasing the likelihood of producing recombinant reactivated VACV.

We hypothesized that passive recombination would be favoured by the presence of a double stranded DNA break. To test this, VACV DNA was digested at a unique genome locus with the *Not*I restriction enzyme before transfection. We detected very little reactivated VACV in samples transfected with the selectable marker constructs and digested VACV DNA. Approximately 10^5 PFY/mL of total VACV was recovered for BGMK cells transfected with undigested VACV DNA and PCR product C versus 10^2 PFU/mL for cells transfected with digested VACV DNA. The digested DNA was perhaps too fragmented for reactivation to occur efficiently. Although *Not*I only cleaves the DNA at one locus, the DNA may have sheared during the purification process following digestion. Our protocol could be modified to minimize shearing, for example, by shaking gently during DNA extraction. Overall, the reactivation system must be further optimized and repeated in order to investigate whether poxviruses acquire new genes through reactivation and whether double stranded DNA breaks impact recombination and reactivation frequencies. Taking all of the above factors and titer results into account, we approximate the recombination frequency of the passive system to be less than or equal to 10^{-9}. We could not mathematically approximated the recombination frequency because no recombinants were detected.

Conclusions

Our preliminary study showed that actively replicating poxviruses are able to capture cDNAs present in the cytoplasm under selective pressure. This work suggests that poxviruses are more likely to capture genes while they are functionally replicating within infected cells. With such a low recombination frequency, it is unlikely that poxviruses acquire new genes through reactivation.

Future experiments will focus on the recombinants generated through the active method. We will continue to search for new methods that may improve efficiency and selectivity of both recombination systems. Furthermore, after purifying DNA from recombinant VACV clones and using PCR to confirm the presence of YFP/gpt fusion gene, we can subsequently sequence the recombinants to determine the regions where recombination occurred. Quantitative PCR could also be used to study these rare recombination events. Sequencing results will be confirmed via Southern blot: digestion of recombinant VACV DNA with various restriction enzymes compared with a digest of wild type VACV. Further experiments to see whether VACV can capture a stably expressed selectable marker from cells could be conducted after optimization of these recombination systems and further analysis of recombinant clones is done.

While VACV is a commonly used model in poxvirus biology, little is known about its evolution. Further research in the field of viral genetics will yield a better understanding of how poxviruses acquire mechanisms of immune evasion and about other viral genes that appear to have cellular homology.

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