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Functional Profiling of Varicella-Zoster Virus Genome by Use of a Luciferase Bacterial Artificial Chromosome System

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1. Introduction

The varicella-zoster virus (VZV or Human Herpesvirus-3) is a member of the human herpesvirus family. Identified as the causative agent of chickenpox and shingles, VZV is a significant pathogen in the United States, infecting over 90% of the US population (Abendroth & Arvin, 1999). Primary VZV infections generally occur during childhood and result in a relatively benign illness termed varicella (chickenpox). However, like all herpesviruses, VZV will establish latency in the host's sensory neurons. This occurs specifically in the trigeminal ganglia and dorsal root ganglia (Arvin, 1996; Gilden et al., 2000), where the virus can hide from the immune system for years and often a lifetime.

Reactivation of this virus with increasing age or immunosuppression results in herpes zoster (shingles). Herpes zoster is a more painful, neurocutaneous infection associated with acute or chronic neuropathic pain and a significant incidence of post-herpetic neuralgia, a complication causing many patients to continue suffering excruciating pain, lasting anywhere from months to years after infection due to the resulting nerve damage (Cohen et al, 2007). This not only greatly increases the cost of medical care, but also significantly compromises the quality of life in the elderly (Pickering & Leplege, 2010; Opstelten et al., 2010; Burgoon et al, 1957).

1.1 The varicella vaccine

In the early 1970s, Japanese researchers isolated a VZV sample from the blood of a small boy. Through serial passage in cell culture, scientists were able to successfully develop the first live attenuated varicella vaccine (Takahashi et al., 1974; Arvin, 2001; Gershon, 2001). The vaccine strain was termed the Japanese Oka varicella virus (v-Oka). By 1995, this chickenpox vaccine was introduced to the United States and quickly recommended for vaccination in all children. Since then, chickenpox incidence in the US has dramatically declined; the effectiveness of this vaccine is estimated to be between 72% and 98% (Hambleton & Gershon, 2005). Nevertheless, outbreaks of chickenpox are still ever-present. Furthermore, the vaccine may indirectly increase the occurrence of herpes zoster in the elderly population by lessening the number of natural infections, and therefore lowering the exposure to wild-type VZV that would boost natural immunity (Arvin, 2001; Galea et al,

2008; Volpi, 2005). It is also important to note that the current shingles vaccine has been shown to only reduce the risk of shingles by 50% (Oxman et al., 2005). Because of this, VZV continues to be an important public health concern. In order to improve future prevention and treatment of VZV infections, a better understanding of VZV's biology and pathogenesis is critical.

1.2 VZV research methods

VZV contains the smallest genome among the eight human herpesviruses, consisting of a 125-kb double-stranded DNA genome that encodes 70 unique open reading frames (ORFs). The function of most of these ORFs, however, was largely unknown until recent years. This is in part due to the absence of both a genetic tool to efficiently generate mutant clones for loss-of-function studies and a true animal model for large scale screening of *in vivo* virulence factors (Cohen et al., 2007).

Obstacles in mutagenizing VZV include its large genome size, narrow host range, and marked differences in replication cycles when studied *in vitro* versus *in vivo* (Arvin, 1996; Cohen, 2001). A once prevalent technique to create recombinant VZV variants was the four-cosmid system, made by cloning overlapping segments of the VZV genome into four large cosmids (Cohen & Seidel, 1993; Mallory et al, 1997; Niizuma et al., 2003). Co-transfection of these cosmids, one of which containing a mutation in the desired ORF, created a recombinant VZV variant. However, this method alone faced many challenges. For example, research was thwarted because co-transfection of the large cosmids into permissive mammalian cells and multiple homologous recombination events within a single cell were necessary to generate the full-length viral genome (Zhang et al, 2008).

More recent developments have helped to circumvent these problems by cloning the entire VZV genome as a bacterial artificial chromosome (VZV_{BAC}) (Nagaike et al., 2004). This approach provides easy and efficient manipulation of the viral genome and rapid isolation of recombinant viruses, making the systemic deletion of every ORF in the genome feasible. A firefly luciferase cassette is also inserted into the VZV_{BAC} to produce a novel luciferase VZV_{LUC} BAC. This allows us to not only generate VZV ORF deletion mutants, but also monitor its subsequent growth in cultured cells.

2. Generation of a VZV_{BAC}

Viral BACs are created when a BAC vector sequence is inserted into the viral genome. Typically, a BAC vector is roughly 10-kb long and contains an origin of replication, genes essential for BAC replication, and genes to control the rate of replication; ideally the copy number should be limited to one or two BACs per bacterial cell (Warden et al., 2010). An antibiotic resistance marker and selection marker, such as a green fluorescent protein, are also added to select for bacterial colonies harboring the BAC vector and isolate these BAC-containing recombinant viruses. BAC vectors in addition must also be flanked by a 500-1000-bp sequence homologous to the target sequence at the site of insertion. Lastly, loxP sites are commonly included at both ends of the BAC sequence to excise the vector after recombinant viruses are generated, as is required for vaccine production (Zhang et al., 2007, see below).

2.1 The pUSF-6 vector

For our purposes, the VZV_{BAC} was constructed from a pUSF-6 vector. As shown in Fig. 1, this vector contains the prokaryotic replication origin (*ori*), replication and partition function (*repE*, *parA*, *parB*) genes, chloramphenicol resistance (*cam^r*) gene, and a green fluorescent protein (*GFP*) gene. Insertion of a GFP reporter gene in BAC DNA is a popular means to visualize *in vitro* infections in cell culture. Viral GFP is expressed using the SV40 early promoter and polyadenylation signals, which activate the gene during the appropriate stages of viral replication and cause the cell to fluoresce (Marchini et al., 2001). The vector is also flanked by two 500-bp VZV fragments and contains a *loxP* site at each end (Fig. 1).

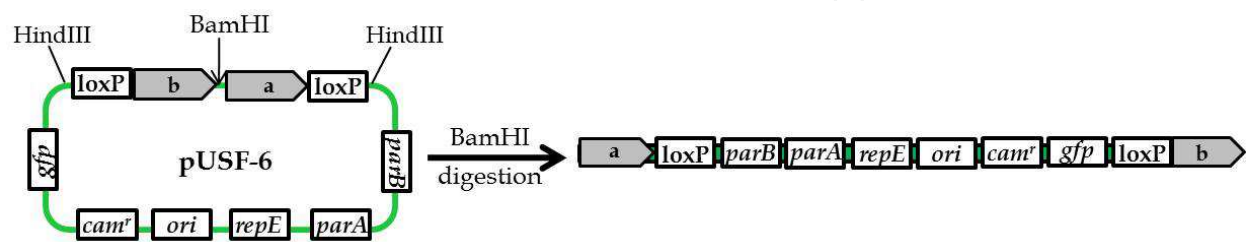


Fig. 1. The BAC vector, pUSF-6. The vector contains the prokaryotic replication origin (*ori*), replication and partition (*repE*, *par*, and *parB*) genes, *cam^r* gene, green fluorescent protein (*GFP*) gene, two *loxP* sites and two VZV homologous sequences, a and b. To insert this BAC vector into a VZV cosmid, pUSF-6 was digested by BamHI, resulting in a linear fragment.

2.2 Construction of the VZV BAC (VZV_{BAC})

Generally, BAC vectors can be directly inserted into viral genomes via homologous recombination. However, this method cannot be used to construct the VZV_{BAC} because the virus' highly cell-associated nature makes isolation of the VZV genome and purification of recombinant plaques difficult (Nagaike et al., 2004). Instead, VZV_{BAC} clones are constructed using a set of four overlapping cosmids spanning the entire VZV genome (Fig. 2).

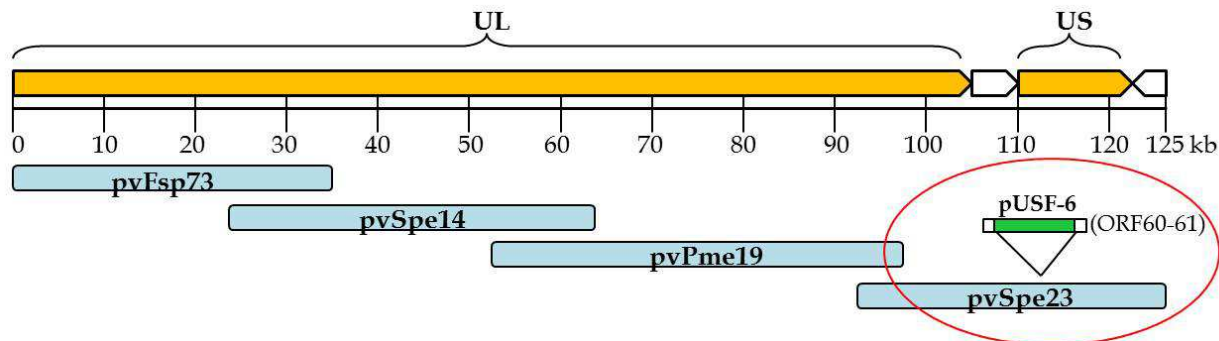


Fig. 2. Schematic diagram the VZV genome and four-cosmid system. The VZV clinical strain, p-Oka, consists of a 125-kb genome with unique long (UL) and unique short (US) segments. Four cosmids with overlapping VZV genomic segments are shown. The BAC vector-containing plasmid, pUSF-6, was inserted between ORF60-61.

First, the pUSF-6 vector was inserted into VZV cosmid pvSpe23, between ORF60 and ORF61 (Fig. 2), via homologous recombination (Yu et al., 2000). The BAC-containing cosmid was

then co-transfected with three complementary cosmids (Niizuma et al., 2003) into human melanoma (MeWo) cells. Because each of the four cosmids contains an overlapping sequence, the cosmids can recombine into one large circular virus genome via homologous recombination to create the recombinant virus. Our use of the GFP selectable marker in the pUSF-6 vector allowed for visualization of the recombinant VZV_{BAC} in plaques that form post-transfection (Fig. 3C). Finally, the VZV_{BAC} DNA from the infected cells was purified and transformed into *E. coli*. Chloramphenicol-resistant colonies were selected for and used to isolate the desired VZV_{BAC} DNA (Zhang et al., 2007).

We used restriction enzyme digestion and DNA sequencing to verify the integrity and stability of the VZV_{BAC} DNA. VZV_{BAC} DNA was digested by HindIII, yielding the predicted digestion pattern with a sum of ~130 kb (Fig. 3G), thus indicating that no large deletions and rearrangements were present. In addition, the ORF62/71 gene was sequenced to check for base-pair changes in the VZV_{BAC} genome after synthesis in *E. coli*. This large duplicated gene, encoding an immediate-early transactivating protein (Perera et al, 1992), was amplified via PCR and cloned into a pGEM-T vector for sequencing. Because the ORF62/71 sequences in the VZV_{BAC} were identical to those of the published p-Oka strain, we can conclude that the BAC DNA in *E. coli* is stable.

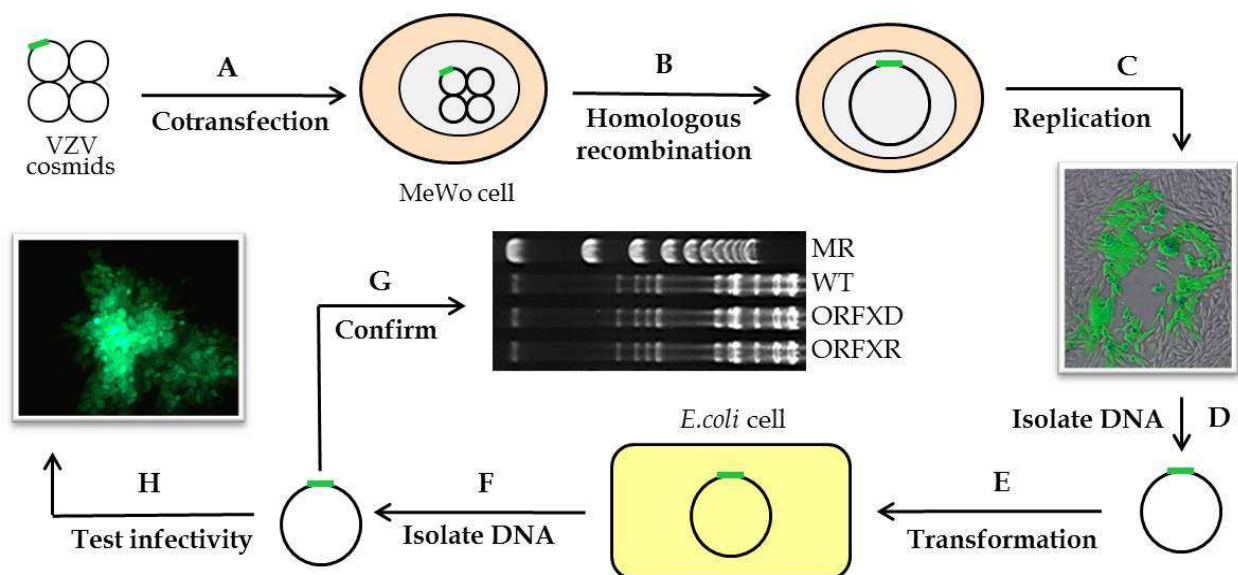


Fig. 3. Construction of VZV BAC. (A) The BAC-containing cosmid was co-transfected with the three complementary cosmids into MeWo cells. (B) Homologous recombination between cosmids formed a circular, full-length VZV_{BAC} genome. (C) The recombinant BAC was replicated, and produced a plaque visualized with the GFP marker. (D) Circular DNA was isolated from infected cells, (E) transformed into *E. coli*, and selected for cm^R colonies. (F) The VZV_{BAC} DNA was isolated from *E. coli* and (G) verified by restriction digestion and partial sequencing. (H) The infectivity and integrity of the VZV_{BAC} were tested by transfecting BAC DNA into MeWo cells to generate the VZV virus.

The GFP marker in the viral genome was also tested; MeWo cells were infected with VZV_{BAC} and continuously passed four times (1:10 dilution) over two weeks. On examination of the plaques under a fluorescent microscope, all VZV_{BAC}-infected cells fluoresced green,

signifying the stability of the GFP marker in the viral genome. Lastly, the infectivity and integrity of the VZV_{BAC} were confirmed by transfecting BAC DNA into MeWo cells to produce the virus. A summary of the process to construct a VZV BAC and verify its integrity is illustrated in Fig. 3.

3. Application of VZV_{BAC} with a luciferase marker

Visualization markers are often inserted into the viral BAC genome to detect and quantify viral replication. Two methods frequently utilized are fluorescence imaging and bioluminescence imaging. Fluorescence-based imaging, such as through the use of a GFP reporter gene, is a common method to monitor *in vitro* infections and allows researchers to study the interaction of a given virus with its host (Tang, 2008). Visualization of *in vivo* infections on the other hand, can be established by the use of bioluminescence imaging (BLI).

3.1 Background on bioluminescence imaging

Developed over the last decade, bioluminescence imaging is a technology that enables visualization of viral gene expression in live tissues and animals (Tang et al, 2008). With little surprise, BLI has become a powerful technique for studying VZV pathogenesis.

Bioluminescence is the production of light by living organisms, resulting from a chemical reaction in which chemical energy is converted to light energy (Hastings, 1983; Kurfurst et al., 1983). BLI systems generate bioluminescence using two compounds – luciferase and its substrate luciferin. Luciferase is a class of enzymes commonly exploited as a reporter gene for transcriptional regulation studies (Doyle et al., 2004). Most extensively employed is the luciferase of the North American firefly (*Photinus pyralis*), which can be expressed in mammalian cells by inserting the gene under the control of a promoter. Because firefly luciferase generates a bioluminescence wavelength that can efficiently penetrate tissues, it serves as an excellent indicator for *in vivo* studies (Tang et al, 2008).

There are many advantages to employing BLI over other bio-imaging techniques; one of the key factors is its use of luciferin. Not only can luciferin permeate all tissues *in vivo* (including cell membranes and the blood-brain barrier) (Contag et al., 1997; Rehemtulla et al., 2002), the substrate can also be administered numerous times to the same animal and provides great accuracy, due to its low toxicity and high sensitivity, respectively (Contag et al., 1997; Rehemtulla et al., 2000). When exposed to the appropriate luciferin substrate, luciferase will catalyze an oxidation reaction to produce light visible to the human eye. The light's intensity depends on the amount of luciferase present, which can be determined by quantifying the relative amounts bioluminescence emitted *in vivo* via computer-based analysis. For this reason, engineering viral BACs to express luciferase can be especially valuable for monitoring the activities of the promoter that mediates gene regulation, detecting sites of viral infections, and quantifying viral replication in living cultures and animals (Zhang et al., 2007; Zhang et al., 2008; Dulal et al., 2009; Zhang et al., 2010).

3.2 The VZV_{BAC} with a luciferase marker (VZV_{Luc})

To generate a VZV strain expressing luciferase, a firefly luciferase expression cassette was inserted into the intergenic region between ORF65 and ORF66 of the VZV_{BAC} genome. This

clone was transfected into MeWo cells to produce the VZV_{Luc} strain. 24 hours later, cell culture media was replaced with media containing 150 µg/ml D-luciferin. After incubation at 37°C for 10 minutes, bioluminescent signals were observed and quantified using an *In Vivo* Imaging System (IVIS).

Upon analysis (Fig. 4), the growth of VZV_{Luc} closely resembled that of its parental VZV_{BAC} (data not shown). This confirms that the addition of a luciferase reporter to the viral BAC did not change its growth properties. Unlike its parental strain however, only cells infected with VZV_{Luc} expressed high levels of luciferase activity (Fig. 4A) and emitted a strong bioluminescence after the addition of D-luciferin (Fig. 4B).

To explore the possibility of using bioluminescent signals as an indicator of viral growth, bioluminescent assays were compared to the conventional infectious center assay. Plates were inoculated with wild-type (WT) VZV and VZV_{Luc}. Their viral titers were quantified daily via both methods for seven days and the data collected was used to construct viral growth curves (Fig. 4C). As the figure illustrates, the intensity of the bioluminescent signals strongly correlated with the viral titers generated by an infectious center assay. Thus, this data supports BLI as an alternative method for growth curve assays and quantifying viral titers.

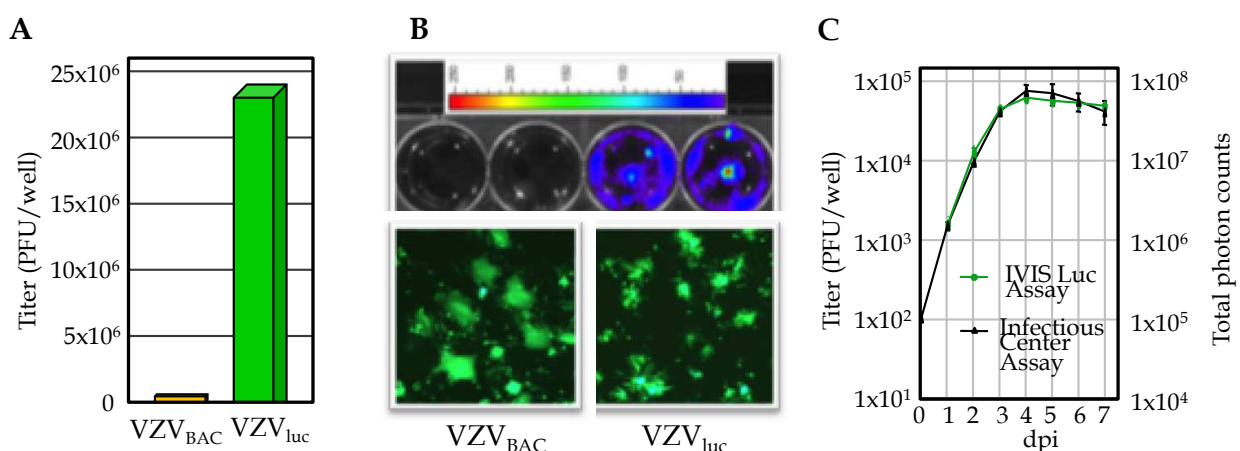


Fig. 4. Analysis of the VZV_{Luc} strain. (A) Luciferase assay. MeWo cells were infected with VZV_{BAC}/VZV_{Luc} for two days; luciferase activity was measured. The cells infected with VZV_{Luc} showed a high level of luciferase activity, while the parental VZV_{BAC} strain showed no activity. (B) Bioluminescence measure. Two wells of MeWo cells were infected with VZV_{BAC} (upper left), and two were infected with VZV_{Luc} (upper right). Two days post-infection, D-luciferin was added to the cultured wells. Bioluminescence was measured and could only be detected in VZV_{Luc}-infected cells. The intensities are indicated by an intensity scale bar at the top; higher intensities are represented by warmer colors, and lower intensities are represented by cooler colors. The infection was verified by the GFP-positive plaques (bottom panel). (C) Correlation of luminescence and plaque numbers. Growth curves generated by an infectious center assay (black curve and left scale) and a bioluminescence assay (green curve and right scale) were compared.

3.3 Bioluminescence imaging for studying VZV in SCID-hu mice

Another useful application of bioluminescence imaging is the live-image analysis of VZV replication in severe combined immunodeficient mice with human tissue xenografts (SCID-hu

mice). Because VZV only infects human cells, *in vivo* studies of VZV pathogenesis have been limited to the use of immunodeficient mice with human tissue implants. However, although SCID-hu mice are established as appropriate models for studying VZV pathogenesis (Besser et al, 2003; Ku et al., 2005; Zerboni et al., 2005), collecting quantitative data has been a major challenge. Since measuring viral growth required the mice to first be euthanized, it was impossible to monitor the progression of the viral infection in the same mouse. In addition, viral titers tend to vary from animal to animal because of the differently sized implants (Moffat & Arvin, 1999), thus hindering not only the frequency of data collection, but also the accuracy as well. These factors greatly impeded efforts to study large numbers of VZV variants and made it difficult to discern minor phenotypic differences leading to pathogenesis.

The development of BLI has been extremely helpful to circumvent these obstacles. Luciferase provides a visible marker for detecting VZV in human tissues within living animals. By using VZV_{Luc}, the SCID thymus-liver mouse model, and *In Vivo* Imaging System (IVIS, Xenogen), the spread of the VZV infection can be frequently monitored in the same mouse over an extended period of time; thereby, allowing the generation of credible growth curves to gain accurate insights into VZV's growth kinetics *in vivo*.

We applied this method to explore VZV replication and measure its spread *in vivo*. Human fetal thymus and liver tissue were implanted under the left kidney capsule of the SCID mouse. Over the course of the next few months, the implanted tissue developed into a thymus-like organ consisting mainly of T cells. VZV-infected cells were then inoculated into the SCID-hu mice with thymus-liver implants. VZV replication was measured *in vivo* after the injection of the luciferin substrate, using an IVIS. Each mouse was imaged daily, starting four hours after inoculation (i.e day zero), for eight days (Fig 5A).

Our data depicts the daily increase in bioluminescence emitted from the infected implants (Fig. 5B). The quantified signals were plotted to generate an *in vivo* growth curve (Fig. 5C). As shown, VZV grew rapidly in human T cells, doubling approximately every 12 hours and peaking at seven days postinfection. The exponential growth curve is then followed by a steady state where the viral infection reaches the saturation limit of the implant.

We also tested the VZV_{Luc} viruses for their spread and detection in human fetal skin xenografts *in vivo*. Similar to the process outlined above, human skin tissues were introduced into SCID mice. Four weeks after implantation, VZV_{Luc} virus was inoculated into the skin tissues and viral growth was monitored every two to three days for 15 days using an IVIS. High luciferase activity was detected in the implants (data not shown), verifying VZV_{Luc}'s ability to grow in skin tissue *in vivo*.

In short, by engineering VZV to express luciferase enzymes, bioluminescence imaging can be used to monitor the progression of viral growth and quantify viral replication in organ cultures and SCID-hu mice. Compared to the traditional infectious center assay, BLI not only saves time and labor, but also significantly increases the reproducibility of results (Doyle et al., 2004). Moreover, the presence of luciferase activity indicates viral replication in cells and not free-viral particles (Zhang et al., 2010), making BLI the most suitable method for studying this particular cell-associated virus. Consequently, the development of BLI has greatly facilitated our ability to investigate aspects of VZV infection in the SCID-hu mouse model and has significantly advanced our understanding of VZV pathogenesis and virus-cell interactions (Zerboni et al., 2010; Arvin et al, 2010; Zhang et al; 2010; Moffat & Arvin; 1999; Arvin, 2006).

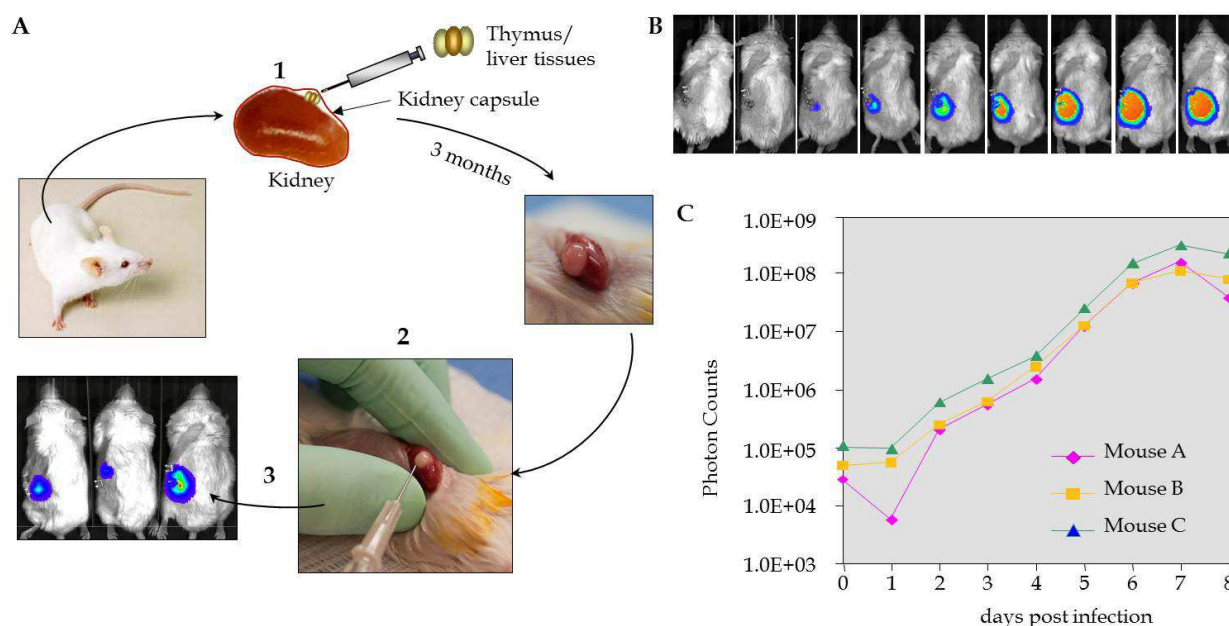


Fig. 5. Monitoring VZV_{Luc} virus replication in SCID-hu mice. (A) SCID-hu model. 1. Human fetal thymus/liver tissues were implanted under SCID mouse kidney capsule. 2. Two to three months later, the implant was inoculated with VZV_{Luc}. 3. Viral replication in human T cells was detected by IVIS. (B) Replication and progression of VZV_{Luc} in human thymus/liver implants in SCID mice. Three SCID-hu mice with thymus/liver implants were inoculated with VZV_{Luc}. Using IVIS, each mouse was scanned daily (from day 0 to day 8). Measurements were taken 10 minutes after i.p. injection with luciferin substrate. Only images from one mouse are shown. Warmer colors indicate higher viral load; colder colors indicate lower viral load. (C) VZV growth curves *in vivo*. Bioluminescence from three SCID-hu mice in the above experiment (B) was measured and VZV growth curves in human thymus/liver implants were generated.

4. Generation of recombinant VZV using a highly efficient homologous recombinant system

To test the novel VZV_{Luc} system for studying VZV pathogenesis, five single ORF deletion mutants were first generated, starting from ORF0 to ORF5, via the homologous recombination system harbored in DY380 *E. coli*. Afterwards, the VZV_{Luc} was used for genome-wide mutagenesis to systematically delete each individual VZV ORF for functional characterization of the VZV genome.

4.1 The DY380 *E. coli* strain

The DY380 *E. coli* strain offers a highly efficient homologous recombination system for chromosome engineering by enabling efficient recombination of homologous sequences as short as 40-bp (Yu et al., 2000). A defective lambda prophage supplies the function that protects and recombines linear DNA. In addition, the system is strictly regulated by a temperature-sensitive lambda repressor. This allows homologous recombination between two sequences to be transiently induced by activating the prophage through incubation at 42°C for 15 minutes.

4.2 Generation of a VZV deletion clone

The entire process to engineer a VZV ORF deletion mutant (ORFXD) is illustrated in Fig. 6. VZV_{Luc} BAC DNA was first introduced into DY380 by electroporation. Homologous recombination functions were transiently induced by increasing the culturing temperature to 42°C for 15 minutes during electroporation-competent cell preparation. A kan^R expression cassette was amplified from pGEM-oriV/kan^R by PCR using two primers containing 40-bp homologous sequences flanking the target ORF (ORFX). The PCR product was then transformed into the DY380 harboring the VZV_{Luc} BAC via electroporation. As expected, homologous recombination occurred between the ORF flanking sequences of the cassette and targeted ORF, replacing the ORFX with the kan^R gene and generating an ORFXD VZV clone (Zhang et al., 2008).

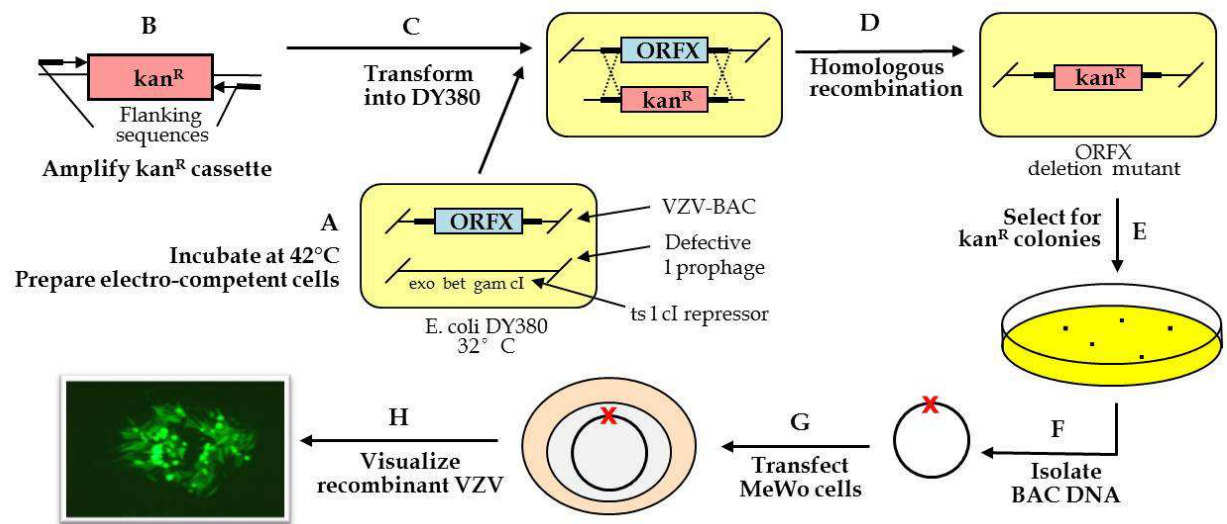


Fig. 6. Generation of a VZV deletion clone. (A) The DY380 strain permits transient induction of recombination system by incubation at 42°C for 15 min during electro-competent cell preparation. VZV_{Luc} BAC DNA was introduced into DY380 by electroporation. (B) Amplification of the kan^R expression cassette by PCR using a primer pair to add 40-bp homologous sequences flanking ORFX. (C) 200ng of the above PCR product was transformed into DY380 carrying the VZV_{Luc} BAC by electroporation. (D) Homologous recombination between upstream and downstream homologies of ORFX replaced ORFX with the kan^R cassette, creating the ORFX deletion VZV clone. (E) Recombinants were selected on LB agar plates. (F) The deletion of ORFX DNA was isolated and confirmed by testing antibiotic sensitivity and PCR analysis. The integrity of the viral genome after homologous recombination was examined by restriction enzyme digestion. (G) Purified BAC DNA was transfected into MeWo cells. (H) 3-5 days after transfection, the infected cells were visualized by fluorescence microscopy.

Successful ORF deletion clones were confirmed by three sequential procedures: 1. antibiotic sensitivity selection, 2. mini-preparation of BAC DNA with PCR verification, and 3. maxi-preparation of BAC DNA with HindIII digestion profiling. Firstly, recombinants were selected on LB plates with chloramphenicol or kanamycin for resistant colonies. It was also important to verify that the deletion clones were sensitive to ampicillin since ampicillin-resistant circular pGEM-oriV/kan^R was used as the PCR template. Multiple colonies were then selected for

mini-preparation of BAC DNA to confirm the ORF deletion and kan^R replacement by PCR. Lastly the PCR-verified clones were chosen for maxi-preparation of BAC DNA and digested with HindIII to ensure that only the targeted sequence was deleted. When the digestion pattern of the deletion clone was compared to that of the parental WT VZV_{Luc} clone, no additional deletions from the genome were detectable (as shown in Fig. 3G).

Finally, to generate VZV deletion mutant viruses, these verified clones were transfected into MeWo cells, along with WT VZV_{Luc} DNA. The size and growth kinetics of the virus as measured by resultant plaques, or absence of plaques, are indicative of the essentiality of a particular VZV ORF for viral replication, discussed later.

4.3 Generation of a VZV rescue clone

VZV ORF deletion rescue clones were also generated (Fig. 7) in order to show that growth defects observed in analyses of the deletion mutants are a direct result of the deleted genes, as opposed to potential mutations in other regions of the genome. Ideally, the wild-type phenotypes should be fully restored in these rescue viruses.

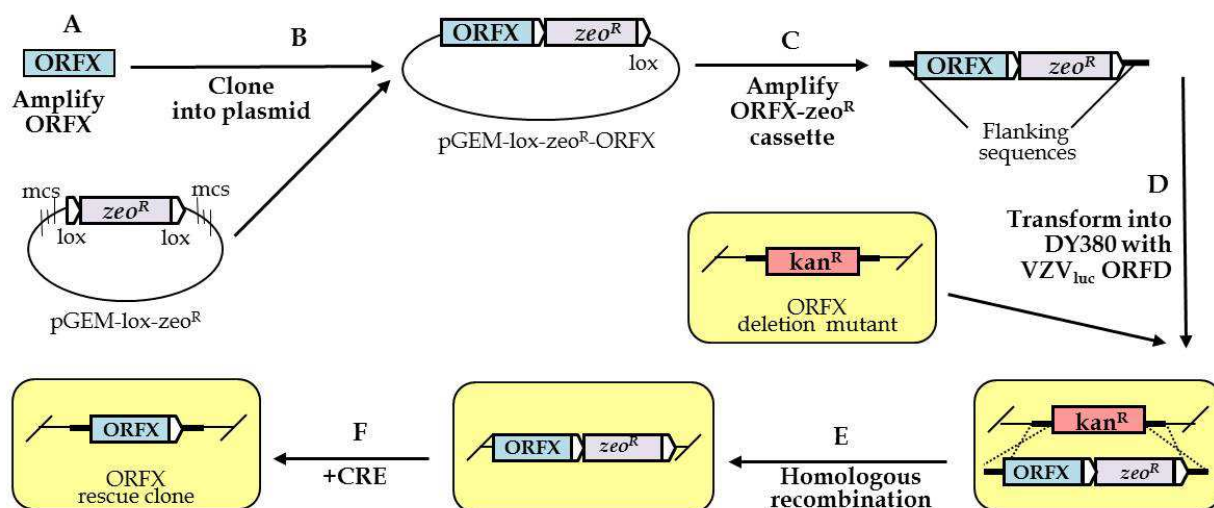


Fig. 7. Generation of a VZV rescue clone. (A) ORFX was amplified by PCR from the WT VZV BAC DNA and (B) directionally cloned into plasmid pGEM-lox-zeo to form pGEM-lox-zeo-ORFX. (C) Amplification of the ORFX-zeo^R rescue cassette by PCR using a primer pair adding 40-bp homologies flanking ORFX. (D) The PCR product was transformed into DY380 carrying the VZV_{Luc} ORFX deletion via electroporation. (E) Homologous recombination between upstream and downstream homologies of ORFX replaced kan^R with the ORFX-zeo^R rescue cassette. (F) Zeo^R and BAC vector sequences were removed post-verification by co-transfecting a Cre recombinase-expressing plasmid, creating the ORFX rescue clone.

To generate ORF deletion rescue clones (ORFXR), the targeted ORF deletion was amplified from wild-type VZV_{Luc} BAC DNA by PCR. Next, the ORFX was directionally cloned into plasmid pGEM-lox-zeo to produce pGEM-zeo-ORFX. This was then used as the template to generate the ORFX-zeo^R cassette via PCR using a primer pair to add 40-bp sequences, homologous to the kan^R cassette flanking ORFX. In a process similar to the homologous recombination system described earlier, the PCR product was transformed into DY380 carrying the ORFX deletion genome (Fig. 7C). The kan^R cassette was replaced with the ORFX-zeo^R rescue cassette by homologous recombination, thus allowing for positive

selection of zeocin^R colonies. Because the zeo^R gene within the rescue cassette is flanked by two loxP sites, it can be removed along with the BAC vector, through Cre-mediated recombination, generating the desired ORFX rescue virus. Full recovery from the marked growth defects is expected after using this rescue method to restore the wild-type ORF.

5. Functional profiling of VZV genome

Even though VZV has the smallest genome among all human herpesviruses, less than 20% of the VZV genome had been functionally characterized (Cohen et al, 2007). In order to investigate VZV ORF function, we created an entire library of single VZV ORF deletion mutants using the DY380 *E. coli* recombination system. The individual functions of each ORF were determined by transfecting MeWo cells with mutant DNA, and observing the subsequent growth of viral plaques. If a VZV ORF is nonessential for viral replication, plaques corresponding to the deleted ORF should be detectable 3-5 days after transfection and resemble the growth of the wild-type virus. Plaques that grow significantly smaller and later suggest that the ORF strongly influences optimal growth. The absence of a plaque entirely implies that the ORF is essential for viral replication.

5.1 Essential VZV ORFs

The results indicate that among VZV’s 70 unique ORFs, 44 ORFs are essential for viral replication in cultured MeWo cells, while 26 ORFs are nonessential (Zhang et al., 2010). Fig. 8 provides a visual representation of the entire VZV genome and categorizes the essentiality of each ORF based on the growth properties of its corresponding deletion mutant virus.

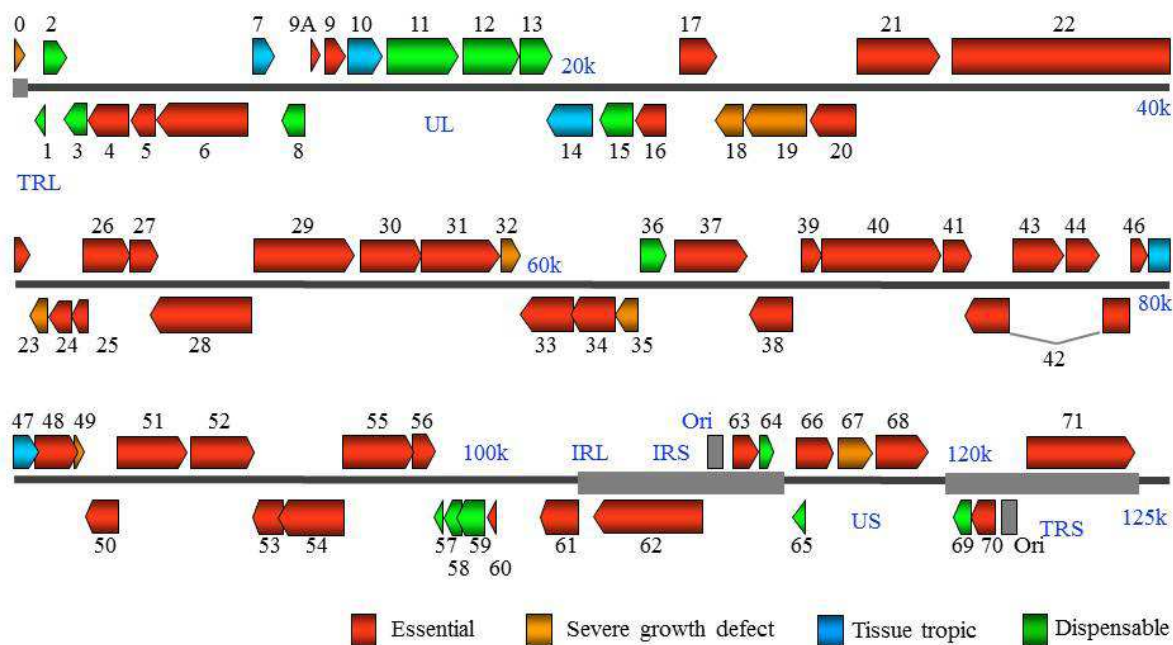


Fig. 8. VZV genome-wide functional profiling based on analysis of single viral ORF deletion mutants. Each VZV ORF is color-coded according to the growth properties of its corresponding virus gene-deletion mutant in cultured MeWo cells and human fetal skin organ cultures. The grey lines for ORF42 represent a splicing junction. For all growth curves, wild-type infections served as positive controls and mock infections served as negative controls.

Various studies, cumulatively, have found that the essential VZV ORFs encode genes for viral structural proteins, transcriptional regulatory proteins, and enzymes involved in DNA replication. The majority of these crucial ORFs encode proteins with imperative functions in maintaining the viral life cycle. For example, some ORFs are a part of the viral tegument and encode immediate-early proteins with transcriptional regulatory activity (Perera et al., 1992; Defechereux et al., 1993; Moriuchi et al., 1994). Other ORFs encode phosphoproteins primarily contained in the nuclei of infected cells (Moriuchi et al., 1993). It has also been reported that most of the VZV ORFs encoding glycoproteins also belong in this group of genes indispensable for viral replication (Mallory et al., 1998; Yamagishi et al., 2008).

Upon further analysis, we found that essential VZV genes have significantly different enrichment for functional categories than nonessential genes. As depicted by the distribution of functional annotations (Fig. 9A), essential VZV genes are significantly enriched for DNA replication and DNA packaging. These include genes encoding the subunits of VZV DNA polymerases, DNA binding proteins, DNA packaging proteins, and nucleocapsid proteins.

5.2 Nonessential VZV ORFs

As previously mentioned, 26 of the 70 unique VZV ORFs were deemed nonessential for VZV replication in MeWo cells (Zhang et al., 2010). Of these, 8 ORFs appeared to significantly affect viral growth. In viral growth assays, the peak signals from their corresponding plaques were at least 5-fold less than the peak signals from the WT parental strain. Furthermore, atypical morphology of virally infected cells, such as reduced plaque sizes and altered syncytia formation, were also frequently observed. Studies have shown that some of these ORFs affecting optimal growth encode the small and large subunit of ribonucleotide reductase (Heineman & Cohen, 1994) and specific phosphoproteins that are post-translationally modified by protein kinases (Reddy et al., 1998).

The plaques corresponding to the remaining 18 nonessential VZV ORF deletions exhibited wild-type growth in cultured MeWo cells (Zhang et al., 2010). *In vitro* growth curve analysis for viral replication showed that these ORF deletion mutants have the same growth kinetics as their wild-type parental strain, VZV_{Luc}. Nonessential genes, in general, are significantly enriched for other and unknown functional categories (Fig. 9B).

5.3 Other findings

Despite major differences between herpesvirus genomes, all the herpesviruses studied thus far have a similar number of essential genes, but varying number of nonessential genes. For example, our study suggests that the VZV genome encodes 44 essential genes and 26 nonessential genes. The herpes simplex virus 1 (HSV-1) genome encodes 37 essential genes and 48 nonessential genes (Roizman et al., 2007). Similarly, the human cytomegalovirus (HCMV), one of the largest human DNA viruses, has a genome that encodes 45 essential genes and 118 nonessential genes (Dunn et al., 2003). Furthermore, 26 of the 44 essential VZV genes have essential homologues in HSV and 18 of the 44 have essential gene homologues in HCMV, alluding that some essential genes may perform core functions for all of these herpesviruses.

Another observation worth noting is the size of the essential ORFs as compared to the size of the nonessential ORFs. Essential ORFs are significantly larger in size, averaging 1250-bp, while nonessential ORFs have an average size of 970-bp. Moreover, the ten largest VZV ORFs are all essential, while of the 11 smallest VZV ORFs, eight are nonessential.

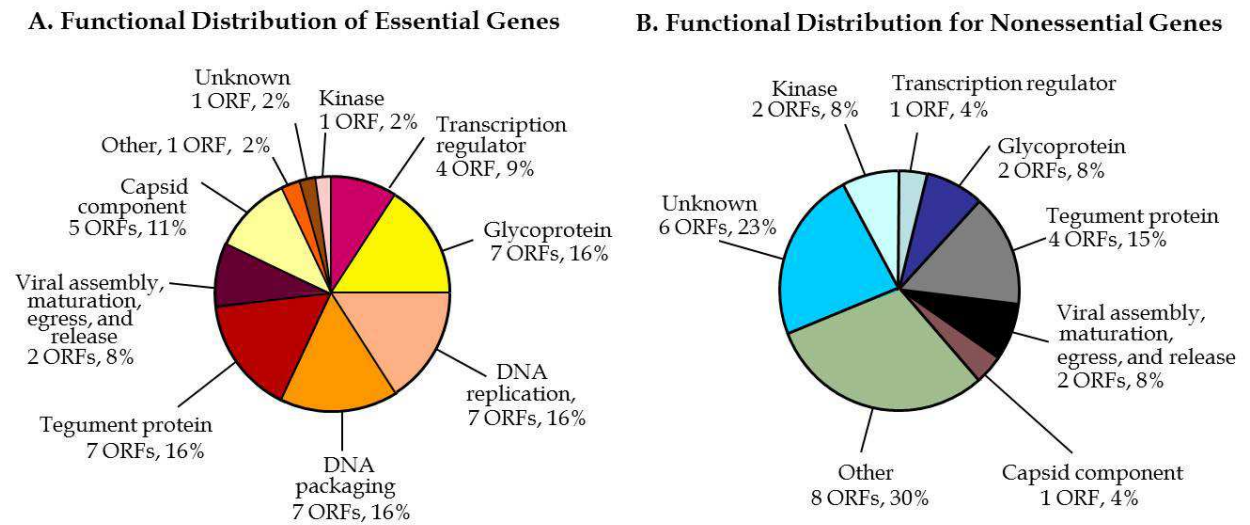


Fig. 9. Distribution of functional annotations for essential and nonessential genes. (A) Distribution of functional annotations for essential genes. Essential genes are significantly enriched for DNA replication (Bonferroni corrected p -value $<10^{-4}$) and DNA packing (corrected p -value $<10^{-4}$) functional categories. (B) Distribution of functional annotations for nonessential genes. Nonessential genes are significantly enriched for other (corrected p -value $<10^{-3}$) and unknown (corrected p -value <0.01) functional categories. Statistical significance was determined by a hypergeometric test.

6. Identification of VZV tissue tropic genes

Although 26 VZV ORFs are shown to be dispensable for viral replication in cultured MeWo cells, it is possible that some of these viral genes may encode proteins critical for optimal viral infection in skin tissue. To test this hypothesis, all of the nonessential ORF deletion mutants were further tested in human fetal skin organ culture (SOC). SOC is a reliable alternative to the SCID-hu mouse model (Taylor & Moffat, 2005), and is especially convenient for initial genome-wide screening of skin-tropism determinants.

We found that all VZV deletions which demonstrated severe growth defects in cultured MeWo cells also exhibited the same growth defects in SOC samples. Interestingly however, among the 18 VZV ORFs believed to be completely dispensable for viral replication in cultured MeWo cells, four ORFs displayed significant growth defects in SOC (Fig. 8) (Zhang et al., 2010). Because these ORFs are trivial for viral replication in MeWo cells but prove crucial for optimal viral replication in skin tissue, they evoked further investigation as potential skin tropism factors.

Rescue viruses were generated for two of these four ORF deletions to ensure that growth defects in skin culture are in fact due to the functions of the deleted genes. As expected, the growth curve analyses showed that in MeWo cells, rescue viruses grew indistinguishably from wild-type VZV, and in SOC, they were able to fully recover the growth defects of their

corresponding deletion mutants (Zhang et al., 2010). Three of these four ORFs (ORF10, ORF14, ORF47) have previously been identified as tissue-tropic factors (Cohen & Seidel, 1994; Heineman & Cohen, 1995; Moffat et al., 1998). Our findings verified these previous studies and additionally identified ORF7 as a novel skin-specific virulence factor. To confirm our original finding here, we also produced a premature stop-codon mutant (ORF7S) by mutating the 5th codon from TGT to the TGA stop codon. Like ORF7D, ORF7S displayed wild-type growth in MeWo cells, but had a growth defect in SOC, indicating that ORF7 may function as a VZV skin-tropic factor.

7. Ongoing research

As mentioned previously, after a primary VZV infection, the virus will remain dormant in the sensory ganglia of its host. When reactivated, VZV will erupt from the sensory neurons and infect surrounding skin tissue, causing characteristic rashes and severe pain due to nerve damage. Therefore, identifying the VZV factors responsible for not only skin-tropism, but also neurotropism is of great importance.

7.1 Screening for VZV neurotropic factors

We proposed that the VZV genome also encodes factors required for efficient invasion and egress from specific tissues during natural infection, such as neurotropic factors. Using our newly created VZV deletion mutants, we screened each of the 18 dispensable VZV ORFs to determine which are implicated with VZV neuronal infection. First, the mutant DNAs were confirmed to be replication competent in a primary permissive cell line, the human retinal epithelial ARPE19 cells (Schmidt-Chanasit et al., 2008). Then, the deletion mutants were screened in human neuroblastoma, SH-SY5Y, following a similar transfection approach as described earlier for MeWo cells, to establish their essentiality for replication in human neurons.

Of the mutants tested, only the ORF7 deletion mutant was unable to form viral plaques (data not yet published). To confirm this, we also infected differentiated neuroblastoma and human embryonic stem cell-derived neurons with wild type and 7D cell-free particles. As expected, the WT infection exhibited robust proliferation, while the 7D infection yielded no visible plaques. Our finding here establishes ORF7 as the only known VZV factor required for viral spread in human neurons.

7.2 Future applications

While the current VZV vaccine is sufficient to prevent chickenpox, many issues still surround this live attenuated vaccine. Firstly, bulk vaccine production using the vOka strain is difficult and costly due to vOka virus's relatively low yield (Schmid & Jumaan, 2010; Gomi et al., 2002). The vaccine is also not entirely effective at eliminating chickenpox outbreaks. Despite seroconversion after vaccination, varicella infections still occur in some children and adults exposed to wild-type VZV (Schmid & Jumaan, 2010; Bernstein et al., 1993; White et al., 1991). Most significantly however, the currently marketed vaccine strain, v-Oka, while highly attenuated in the skin, still retains its neurovirulence (Hambleton et al., 2008). This means that the virus will continue to establish latency in the sensory nerve ganglia of the immunized host and can potentially reactivate later to trigger herpes zoster and post-herpetic neuralgia. Furthermore, while drug treatments available to date can alleviate some symptoms of VZV-elicited diseases and shorten the disease duration, they

cannot clear the virus or prevent establishment of latency (Miwa et al., 2005; Hatchette et al., 2008). For these reasons, developing a new neuro-attenuated vaccine is imperative to prevent future herpes zoster in both elderly people and vaccinated children.

The use of viral BACs has great potential for novel vaccine development and future treatment of viral diseases. Our studies using the VZV_{Luc} BAC uncovered the first, and plausibly the only, VZV neurotropic factor, ORF7. Because the ORF7D strain is incapable of infecting both human skin and nervous tissue *in vivo*, the deletion virus may serve as an ideal vaccine candidate for the next generation of chickenpox and shingles vaccines. Aside from using the ORF7 deletion virus to produce a safer neuro-attenuated vaccine for the prevention of herpes zoster, the same deletion virus may also be utilized as a potential viral vector for the production of vaccines against other pathogens as well.

Additional research into this BAC-based candidate is needed to lead us closer to designing neuro-attenuated vaccines that do not establish latency in sensory neurons, and thereby eliminate the risk of recurrent herpes zoster and its complications. In time, the development of safe and effective neuro-attenuated vaccines will decrease the likelihood of herpes zoster in the contemporary susceptible population, reduce herpes zoster-associated costs, and potentially eradicate VZV and VZV-related diseases (Lydick et al., 1995; Drolet et al., 2010). Essentially, these new vaccines will change the future of VZV altogether.

8. Conclusions

The use of a bacterial artificial chromosome system has proven to be an invaluable tool in human herpesvirus studies, without which, our genome-wide VZV mutagenesis could not have been possible. Not only can BACs clone the large viral genomic DNA, their slow replication rate and relative ease and accuracy of producing and reproducing stable viral mutants make BACs the ideal method for functional analysis of the VZV genome. Furthermore, the addition of a luciferase marker has greatly improved the efficiency, accuracy and reproducibility of our results. Combined, this luciferase BAC approach has truly facilitated genetic studies of VZV and provided vital insights into the replication and pathogenesis of the virus.

In this study, a global functional analysis of the entire VZV genome was carried out, focusing on the identification of ORFs essential for viral replication in cultured MeWo cells and human fetal skin organs. In all, our study has distinguished novel functional annotations for 36 VZV genes and shed light on the essentiality of each of the 70 unique VZV ORFs. More importantly, our findings have identified ORF7 as both a skin-tropic and neurotropic factor, implicating the ORF7 deletion virus as an ideal vaccine candidate to prevent both VZV-elicited diseases, chickenpox and shingles.

As our research progresses, future VZV_{Luc} BAC studies will continue to provide more exciting discoveries and help identify new antiviral targets. Soon, effective vaccines and improved therapy for the prevention and treatment of a wide array of infections will be tangible.

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10. References

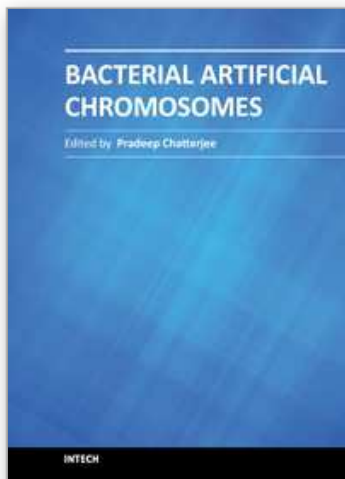
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Bacterial Artificial Chromosomes

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This book focuses on the numerous applications of Bacterial Artificial Chromosomes (BACs) in a variety of studies. The topics reviewed range from using BAC libraries as resources for marsupial and monotreme gene mapping and comparative genomic studies, to using BACs as vehicles for maintaining the large infectious DNA genomes of viruses. The large size of the insert DNA in BACs and the ease of engineering mutations in that DNA within the bacterial host, allowed manipulating the BAC-viral DNA of Varicella-Zoster Virus. Other reviews include the maintenance and suitable expression of foreign genes from a Baculovirus genome, including protein complexes, from the BAC-viral DNA and generating vaccines from BAC-viral DNA genomes of Marek's disease virus. Production of multi-purpose BAC clones in the novel *Bacillus subtilis* host is described, along with chapters that illustrate the use of BAC transgenic animals to address important issues of gene regulation in vertebrates, such as functionally identifying novel cis-acting distal gene regulatory sequences.

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