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Simian Varicella Virus Gene Expression During Acute and Latent Infection of Rhesus Macaques

Christine Meyer^{1,2}, Amelia Kerns¹, Alex Barron¹, Craig Kreklywich^{2,4}, Daniel N. Streblow^{1,2}, and Ilhem Messaoudi^{1,2,3,*}

¹Vaccine and Gene Therapy Institute, Oregon Health & Science University

²Department of Molecular Microbiology and Immunology, Oregon Health & Science University

³Division of Pathobiology and Immunology, Oregon National Primate Research Center

⁴Department of Surgery, Oregon Health & Science University

Abstract

Varicella zoster virus (VZV) is a neurotropic α -herpesvirus that causes chickenpox during primary infection and establishes latency in sensory ganglia. Reactivation of VZV results in herpes zoster and other neurological complications. Our understanding of the VZV transcriptome during acute and latent infection in immune competent individuals remains incomplete. Infection of rhesus macaques with the homologous simian varicella virus (SVV) recapitulates the hallmarks of VZV infection. We therefore characterized the SVV transcriptome by quantitative real-time reverse transcriptase PCR (RT-qPCR) during acute infection in bronchial alveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMC), and during latency in sensory ganglia obtained from the same rhesus macaques. During acute infection, all known SVV open reading frames (ORFs) were detected and the most abundantly expressed ORFs are involved in virus replication and assembly such as the transcriptional activator ORF 63 and the structural proteins ORF 41 and ORF 49. In contrast, latent SVV gene expression is highly restricted. ORF 61, a viral transactivator and latency-associated transcript, is the most prevalent transcript detected in sensory ganglia. We also detected ORFs A, B, 4, 10, 63, 64, 65, 66 and 68 though significantly less frequently than ORF 61. This comprehensive analysis has revealed genes that potentially play a role in the establishment and/or maintenance of SVV latency.

Keywords

Herpesvirus; simian varicella virus; gene expression; latency; rhesus macaque

Introduction

Primary infection with varicella zoster virus (VZV), a neurotropic α -herpesvirus, results in chickenpox (varicella). VZV then establishes a life-long latent infection in sensory ganglia such as the trigeminal and dorsal root ganglia. Reactivation of VZV leads to herpes zoster (HZ, shingles), which causes significant morbidity and occasionally mortality in aged and immunocompromised individuals (Cohen *et al*, 2007). The currently approved vaccine Zostavax® reduces the incidence of shingles by 51% (Oxman *et al*, 2005) leaving a significant portion of the vaccine recipients still susceptible to VZV reactivation. To

*Corresponding author: Ilhem Messaoudi Vaccine and Gene Therapy Institute Oregon National Primate Research Center 505 NW 185th Avenue Beaverton, OR 97006 Phone: 503-418-2734 messaoud@ohsu.edu.

improve vaccine efficacy and develop novel therapeutics, we need to increase our understanding of the viral factors that control VZV latency and reactivation. One approach is to characterize the viral transcriptome, which has the potential to identify viral genes that can be used as subunit vaccine components; targets for novel therapeutics or to generate rationally attenuated vaccine strains.

To identify viral genes that impact VZV acute infection, latency and reactivation, several studies characterized differences in transcriptional levels of VZV open reading frames (ORFs) between lytic and latent infection. Although VZV acute transcription has not been investigated in the natural host, all 68 unique VZV ORFs are expressed in virus-infected cells in culture (Cohrs *et al*, 2003b; Kennedy *et al*, 2005; Nagel *et al*, 2009). VZV transcription in latently infected human sensory ganglia has been examined by various methods including Northern blot hybridization, cDNA libraries, in situ hybridization (ISH), RT-PCR, quantitative and multiplex PCR. The core transcripts detected in these studies include ORFs 4, 21, 29, 62, 63, and 66 (Cohrs *et al*, 1996; Cohrs *et al*, 1995; Cohrs and Gilden, 2007; Cohrs *et al*, 2003a; Cohrs *et al*, 2000; Kennedy *et al*, 2000; Kennedy *et al*, 2001; Nagel *et al*, 2011). The results from these studies demonstrate that VZV latency is associated with the expression of a limited number of viral genes and unlike other α -herpesviruses, VZV is not known to encode latency-associated transcripts (LATs) (Cohen *et al*, 2007). One potential constraint of VZV transcriptional studies during latency is that the sensory ganglia examined are sometimes obtained from older individuals suffering from a number of co-morbidities such as cancer and sepsis, which could impact viral gene expression.

We developed a nonhuman primate (NHP) model where rhesus macaques (*Macaca mulatta*) are infected with the highly homologous simian varicella virus (SVV, *Cercopithecine herpesvirus 9*) (Messaoudi *et al*, 2009). The SVV and VZV genomes are collinear and share 70–75% DNA homology (Gray and Oakes, 1984; Gray *et al*, 2001; Pumphrey and Gray, 1992). Rhesus macaques infected with SVV display the hallmarks of VZV infection in humans: 1) appearance of generalized varicella, 2) development of cellular and humoral immunity with similar kinetics as those described in children during primary VZV infection, and 3) establishment of latency with limited transcriptional activity in sensory ganglia (Messaoudi *et al*, 2009). This model provides a unique opportunity to conduct a comprehensive analysis of the *in vivo* gene expression profiles during acute and latent infection in the same immune competent animal. Our analysis of the SVV transcriptome revealed that during acute infection all known SVV ORFs were expressed to detectable levels, although the relative amount was specific for each gene. We also found that latency is associated with a limited transcriptional profile and that the expression of SVV ORF 61 is the most prevalent. Identifying the viral genes expressed from different tissues at different times during infection will aid in the selection of immunological targets for vaccine strategies or therapeutic targets for drug development against VZV.

Materials and Methods

Ethics statement

All animals were housed at the Oregon National Primate Research Center and were handled in accordance with good animal practices as defined by all relevant national and/or local animal welfare bodies. Animal work was approved by the Oregon national Primate Research Center Institutional Animal Care and Use Committee (IACUC#0779).

Animals and samples

Rhesus macaques were infected intrabronchially with 4×10^5 PFU as previously described (Messaoudi *et al*, 2009). Peripheral blood mononuclear cells (PBMC), bronchial alveolar lavage cells (BAL) and sensory ganglia were collected from 5 juvenile (ages 3–5 years old) rhesus macaques (RM, *Macaca mulatta*) as previously described (Messaoudi *et al*, 2009). One million BAL cells were collected on 3 and 7 dpi and one million PBMC cells were collected on 10 dpi. Cell samples were treated with Trizol (Invitrogen, Carlsbad CA) and stored at -80°C until further processing. Animals were euthanized at >72 days post-infection. Sensory ganglia: trigeminal (TG), cervical, thoracic and lumbar-sacral dorsal root ganglia (DRG-C, DRG-T, and DRG-L/S respectively) were collected in Trizol and stored at -80°C until analysis. SVV viral loads in whole blood (WB), BAL and sensory ganglia were measured by quantitative real-time PCR (qPCR) using primers and probe specific for SVV ORF 21 as previously described (Messaoudi *et al*, 2009) (Supplementary Table 1).

RNA isolation and amplification

Total RNA was isolated from acutely infected PBMC and BAL cells and latently infected sensory ganglia from infected rhesus macaques using the Trizol method according to the manufacturer's protocol. First strand cDNA was synthesized from 1 μg of total RNA using 0.5 μM T7 oligo(dT)-T7 primer and 250 U SuperScript III reverse transcriptase (Invitrogen). Double-stranded cDNA was generated by the addition of second-strand buffer (Invitrogen) as per the manufacturer's instruction. Double-stranded cDNA was purified using phenol-chloroform-isoamyl alcohol extraction and concentrated in Amicon Ultra centrifugal filters (Millipore, Billerica MA) before being amplified using the T7 MEGascript Kit (Ambion, Austin TX) according to the manufacturer's method. The amplified RNA (aRNA) was purified using the RNeasy Mini Kit (Qiagen, Valencia CA) using the manufacturer's protocol. Five μg of aRNA was reverse transcribed with 350 U SuperScript III reverse transcriptase in the presence of 9 μg of random primers (Invitrogen). Double-stranded cDNA and second round aRNA was generated as described above and repeated for a total of three rounds of RNA amplification.

Quantitative real-time reverse transcriptase PCR (RT-qPCR) detection of SVV gene expression

RT-qPCR was used to quantify SVV gene expression. Single-stranded cDNA was generated from third round aRNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City CA) following the manufacturer's protocol. The cDNA was analyzed by RT-qPCR using primer and probe sets specific for each ORF in the SVV genome designed using Primer Express software (Applied Biosystems). A list of the primer and probe sequences used for this study are listed in Supplementary Table 1. RT-qPCR was performed using specific TaqMan probes and 2 $\times$ Gene Expression Master Mix (Applied Biosystems) or Maxima Probe/ROX qPCR Master Mix (2 $\times$) (Fermentas, Glen Burnie MD). Following an initial 10 minute 95°C step, 40 cycles of 15 sec at 95°C and 1 minute at 60°C were completed using a StepOnePlus RT-qPCR machine (Applied Biosystems). Plasmids containing each target amplicon or synthesized amplicons were used as quantification standards. A positive signal in our analysis required that any given SVV gene express at least 100 copies in 1 μg of total RNA. Copy numbers are reported as the average of triplicate RT-qPCR analysis for each sample and are within 25% standard deviation for the population. The reported average copy numbers are not absolute but relative to our sample set.

RESULTS

SVV infection and viremia

Five juvenile rhesus macaques (RM) were infected intrabronchially with 4×10^5 PFU. All RMs displayed the hallmarks of SVV infection including the development of varicella and viremia that resolved approximately 21 days post-infection (dpi). All RMs developed varicella by 10 dpi and a qualitative assessment of rash for each animal during acute SVV infection showed that the severity varied between animals with RM 25043 displaying the greatest number of skin lesions followed by RM 25339, RM 20226 and RM 24953, and lastly RM 24943 (Table 1). SVV establishes latency within the sensory ganglia, and as early as two months following varicella, SVV DNA can be found exclusively in ganglia and not in other tissues (Grinfeld and Kennedy, 2007; Mahalingam *et al.*, 1991; Messaoudi *et al.*, 2009; Ou *et al.*, 2007). Thus, our SVV latency analysis was investigated in sensory ganglia collected greater than 72 dpi. To follow the SVV viral load during acute infection we analyzed both whole blood and bronchial alveolar lavage (BAL) samples. DNA was extracted from a portion of both whole blood and BAL cells collected from each RM to measure viral genome levels by quantitative real-time PCR (qPCR) using a primer and probe set specific for SVV ORF 21. Table 1 shows the genome copies per μg of DNA in BAL at 3 and 7 dpi and in whole blood at 10 dpi. Peak SVV DNA levels are usually detected in BAL between 3 and 7 dpi and in whole blood between 7 and 10 dpi. However, a sufficient number of PBMCs to complete the transcriptional analysis were only available at 10 dpi. Viral DNA loads were significantly higher and peaked earlier during acute infection (10^2 – 10^5 copies/ μg) in BAL than in whole blood (10–100 copies/ μg), likely reflecting the route of infection. SVV viral loads in the BAL correlated with varicella severity during acute infection with RM 25043 and RM 25339 displaying the most extensive rash and the highest viral loads (Table 1). To assess the latent viral burden in the same SVV infected RMs, a portion of the sensory ganglia collected at day 73 (RM 24953 and 25043), day 77 (RM 24943 and 25339) or day 178 (RM 20226) post-infection was used for SVV DNA analysis. Table 1 lists the genome copy number per μg DNA for the trigeminal ganglia (TG), cervical dorsal root ganglia (DRG-C), thoracic dorsal root ganglia (DRG-T), and lumbar/sacral dorsal root ganglia (DRG-L/S). All of the sensory ganglia tested were positive for SVV DNA except in one animal (RM 25043) where one of four ganglia was positive for SVV DNA. The tissue sections used for analysis of SVV DNA viral loads were not the same tissue sections used for transcriptional analysis consequently RM 25043 does show SVV gene expression during latency in all sensory ganglia (Figure 2A).

SVV gene transcription profiles during acute infection in rhesus macaques

We monitored *in vivo* SVV gene expression in 5 infected RMs; the tissues available for transcriptional analysis during the acute phase of infection are listed in Table 2. In order to assess the entire SVV transcriptional profile for each sample and since we are only able to collect a limited number of cells from each animal, RNA was subjected to three rounds of amplification. Amplified RNA was reverse transcribed into cDNA and subjected to quantitative real time PCR (RT-qPCR) using primer and probe sets specific for all 69 unique SVV ORFs (Supplementary Table 1). Before addressing the entire transcriptome for each sample, the total RNA was assessed by measuring the average copy number for the reference gene glutathione synthetase (GSS) by RT-qPCR. GSS levels were equivalent among the samples tested (data not shown). The RNA amplification process can produce bias in the results by over- or under-representing RNA transcripts compared to the starting material. Therefore, we compared the average copy number for a subset of SVV genes by RT-qPCR in the original and amplified sample and the relative abundance did not differ significantly (ORFs 4, 21, and 61; data not shown). Our analysis does not include the poly(A) negative fraction of RNA.

To characterize the acute SVV gene expression profiles *in vivo*, we analyzed BAL cells at 3 and 7 dpi and PBMC at 10 dpi. Our results demonstrate that SVV gene transcription is evident as early as 3 dpi in the BAL with 71% of SVV ORFs expressed at detectable levels (Figure 1A). The highest expressed SVV ORFs at 3 dpi in BAL include ORF 57, ORF 41, ORF 55 and ORF 63 representing viral proteins whose functions are unknown, structural, DNA synthesis and transcriptional activation respectively. The number and abundance of SVV genes expressed increases from day 3 to day 7 post-infection in the BAL at which point transcripts associated with every SVV ORF are detected at different intensities between animals but with similar profiles (Figure 1 A–C). The viral ORFs highly expressed and in common to all three animals during acute infection at 7 dpi in BAL encode a transcriptional activator (ORF 63) and virion structural proteins (ORFs 49 and 41). Other ORFs highly expressed in 2 of 3 animals include ORF 23 (capsid protein), ORF 53 (gamma-1 protein, unknown), and ORF 65 (tegument phosphoprotein). Table 3 lists the relative copy numbers for all SVV ORFs in the 3 animals tested at 3 and 7 dpi.

The transcriptional profile at 7 dpi in the BAL for RM 25339 (Figure 1B) is comparable to RM 25043 (Figure 1A). All SVV genes are detected with the exception of ORF 22, a tegument protein detected in RM 25043 at less than 400 copies per μg of total RNA, which is not detected in RM 25339 (Table 3). RM 24953 (Figure 1C) on the other hand, displays decreased viral gene transcription at 7 dpi in the BAL, both in abundance and the number of SVV viral ORFs detected. The transcriptional profile of RM 24953 at 7 dpi is reminiscent of RM 25043 at 3 dpi in the BAL. Interestingly, the viral load detected in the BAL at 7 dpi and severity of varicella at 10 dpi of RM 24953 was lower than that of RM 25043 or RM 25339 (Table 1) suggesting a quicker resolution of the acute SVV infection in this animal, which may have affected the levels of viral transcription in the BAL. In summary, with the exception of ORF 22, we were able to detect all known SVV genes during acute infection in the BAL at 7 dpi in at least 2 of 3 rhesus macaques examined.

Our previous data suggested that SVV viral loads peaked in the blood at a later time post-infection than in the BAL (10 vs. 7 dpi), most likely due to the route of infection (intrabronchial), thus we analyzed SVV gene expression in isolated PBMCs collected at 10 dpi for animals 20226, 24943 and 25339. In contrast to our observations in the BAL, only 18 SVV ORFs were detected in PBMCs at 10 dpi in the three animals tested. ORF 32 (phosphoprotein) and ORF 54 (viral DNA cleavage/packaging) were detected in all three PBMC samples (Table 4). In agreement with acute infection in the BAL, ORFs 23, 41 and 63 were amongst the most highly expressed viral ORFs in PBMCs at 10 dpi in 2 of 3 animals.

SVV gene transcription profiles during latent infection of rhesus macaques

Lastly, we investigated SVV gene expression during latency (>72 dpi) in sensory ganglia (TG, DRG-C, DRG-T and DRG-L/S) isolated from the same 5 animals described above. The latently infected sensory ganglia available for transcriptional analysis for each RM are listed in Table 2. As predicted, SVV gene transcription is highly restricted during latency (Figure 2). Only 12 SVV ORFs were detected in all 16 sensory ganglia tested and the average copy number for any individual ORF did not exceed 4,000 copies per μg of total RNA (Table 5). ORF 61 was the most prevalent transcript detected during latent infection, present in at least one sensory ganglia in all animals tested and present in 10 of 16 sensory ganglia total. We also detected ORF B in 4 of 16 ganglia; ORFs A and 65 in 3 of 16 ganglia; ORF 68 in 2 of 16 ganglia; and ORFs 4, 10, 55, 63, 64, 66, and 67 in 1 of 16 latently infected sensory ganglia. Previous studies reported that the viral transactivator ORF 61 also expresses an antisense transcript in the ganglia of latently infected NHPs that is hypothesized to be the equivalent of the herpes simplex virus type-1 (HSV-1) latency-associated transcript (LAT) (Ou *et al.*, 2007). Follow up studies have shown that the

frequency of the antisense transcripts greatly exceeds that of the sense transcripts in latently infected ganglia but not in infected Vero cells (Messaoudi *et al*, 2009; Ou *et al*, 2007). Our transcriptional analysis does not differentiate between the sense and antisense transcripts of ORF 61 but does support the previous data and suggests that the expression of ORF 61 plays an important role during the establishment or maintenance of SVV latency.

Discussion

VZV and SVV are two closely related α -herpesviruses that cause varicella in humans and nonhuman primates, respectively. VZV and SVV have the smallest genomes of the herpesvirus family. VZV encodes 68 ORFs, 3 novel ORFs and 3 ORF duplications (Cohen *et al*, 2007; Kemble *et al*, 2000; McMillan *et al*, 1997; Ross *et al*, 1997). Similarly, SVV encodes 72 ORFs with 69 unique ORFs and 3 ORFs duplicated in the terminal repeat sequence (TRS). The SVV and VZV genomes differ at the left terminus where SVV encodes ORF A that is absent from VZV and SVV lacks VZV ORF 2. ORF B of SVV is homologous to VZV ORF S/L. In this study, we examined SVV gene expression during acute and latent infection in rhesus macaques inoculated intrabronchially in order to identify genes that could play a critical role in the establishment and/or maintenance of latency. To the best of our knowledge, this is the first study to characterize the SVV transcriptome during acute and latent infection in tissues obtained from the same animal.

A comprehensive analysis of the *in vitro* acute SVV transcriptome using macroarrays showed that all SVV ORFs were expressed at 3 dpi in Vero cells and ORF 9 was the most abundant SVV transcript detected followed by ORFs 32, 63 and 23 (Deitch *et al*, 2005). Likewise, our acute transcriptional analysis detected all SVV ORFs in BAL samples between 3 and 7 dpi. More specifically, in rhesus macaque 25043 (the animal with the highest overall gene expression) ORFs 49 and 41 were the most abundant transcripts in the BAL at 7 dpi, followed by ORFs 63 and 23. The top 4 ORFs detected in RM 25339 at 7 dpi, include ORF 41, ORF 63, ORF 53, and ORF 7. Similarly, in BAL cells from RM 24953 at 7dpi, which displayed a decreased transcriptional profile, SVV ORFs 41, 49, 63 and 53 were also the most abundantly expressed. However, in contrast to the *in vitro* macroarray study, ORF 32 was in the top ten highest expressing transcripts but ORF 9 was not in the top 20 though the relative expression exceeded 2×10^5 copies. The difference in ORF 9 expression is most likely due to the cell type that SVV infected *in vivo* (lymphocytes and lung epithelial cells) versus the *in vitro* study, which utilized Veros (kidney epithelial cell line), which are interferon-deficient and a different cellular response to viral infection could modulate SVV gene expression.

Previous studies examined SVV gene expression in skin, lung, liver, spleen, and thoracic dorsal root ganglia samples harvested at 10–11 dpi in acutely infected St. Kitts vervet monkeys (*Cercopithecus aethiops*). Using RT-PCR, the study focused on ORFs 31, 36, 62, 63 and 68, which were detected in all tissues including the ganglia (Gray *et al*, 2002). Our analysis of PBMCs collected 10 dpi detected only 18 viral genes. ORFs 32 and 54 were detected in all three samples studied and ORFs 23, 30, 41, and 63 were detected in 2 of 3 samples. ORF 23 (capsid), ORF 41 (capsid) and ORF 63 (transcriptional activator) were the most abundantly expressed genes in PBMC during acute infection. The differences between our observations and those of this earlier study could be due to differences in SVV pathogenesis. Vervet monkeys are highly susceptible to SVV infection, whereas rhesus macaques experience very low levels of SVV replication in peripheral blood (Soike *et al*, 1984). Moreover, we examined purified PBMC whereas this earlier study looked at spleen tissue and it is likely that viral gene expression is cell-type specific (Streblow *et al*, 2007).

VZV acute transcriptional analysis has only been conducted in tissue culture to date. An *in vitro* macroarray study analyzed VZV lytic infection of BSC-1 cells and found that expression of transcripts associated with all VZV genes increased from 1 to 3 dpi but the relative abundance did not change. The 4 most abundant VZV ORFs were 9, 64, 33, and 49 (Cohrs *et al.*, 2003b). VZV gene expression was also investigated by microarray in two additional cell lines, human melanoma (MeWo) and human astroglial (SVG) cells at 72 hpi (Kennedy *et al.*, 2005). The results demonstrated that VZV transcriptional profiles differed between these two cell types and from BSC-1 cells. 68 of 71 VZV ORFs were detected in MeWo cells and the most abundant ORFs were 57, 9, 49, 58, 48, and 69 while only 20 ORFs were detected in SVG cells and the most abundant ORFs were 24, 68, 61, 13, 32, and 53. The differences in copy numbers and ORFs detected could be due to the virus strains used and/or the cell types infected. For example, the VZV macroarray study used the Ellen strain of VZV in BSC-1, an African green monkey kidney cell line, whereas the microarray study utilized the Dumas strain of VZV in a melanoma cell line and an astroglial cell line. Interestingly, the SVV and VZV *in vitro* transcriptional studies using macroarrays identified ORF 9 as one of the most highly expressed genes (Cohrs *et al.*, 2003b; Deitch *et al.*, 2005). Both of the studies utilized cell lines derived from African Green monkeys (Vero and BSC-1), which may influence the viral transcriptional program. In contrast, ORF 9 was not amongst the most abundant transcripts detected *in vivo* in the BAL or PBMC in our rhesus macaque model. This result emphasizes the value of not only studying the virus in single cell-types but also in the context of the host.

Expression of SVV ORFs 21, 28, 29, 31, 61, 62, and 63 was characterized *in vivo* during latency by RT-PCR in liver, lung, trigeminal, cervical, and lumbar ganglia obtained from St. Kitts vervet monkeys (Ou *et al.*, 2007). Only SVV ORF 21 and ORF 61 sense and ORF 61 antisense transcripts were detected in sensory ganglia. The expression of SVV ORFs was not detected in either the lung or liver during latency. Another study using RNA ISH did not detect either SVV ORFs 21 or 63 in the ganglia of an African Green monkey two months post-infection (Grinfeld and Kennedy, 2007). As reported in our current study we detected high levels of ORF 61 transcripts, but we did not detect ORF 21 above 100 copies, our established limit of detection. We also detected ORF 63 during latency, though only in one sample. The lack of expression of ORF 21 in our latently infected sensory could be due to differences in the monkey species (St. Kitts vervet or African green versus rhesus macaque) or the duration of latency. Animals in our study were euthanized at day 73 (RM 24953 and 25043), 77 (RM 24943 and 25339) or 178 (RM 20226) post-infection, the St. Kitts vervet monkeys in Ou *et al.* were euthanized at day 108 or 119 post-infection and in the Grinfeld *et al.* study, the African Green monkey was analyzed 2 months post-infection. Our rhesus macaques clear acute SVV infection between days 17 and 21 for a latency period of at least 2 months. While there are differences in the duration of latent infection between the studies, we propose that one of the major differences in SVV latent transcription are due to the nonhuman primate species utilized.

The most frequently detected gene in our analysis during latency was SVV ORF 61. SVV ORF 61 transactivates its own promoter as well as the promoters of immediate early gene ORF 62, early gene ORF 28 and 29, and late gene ORF 68 in transfected Vero cells (Gray *et al.*, 2007). During latency a latency-associated transcript (LAT) that is antisense to SVV ORF 61 was found to be expressed primarily in sensory ganglia (Ou *et al.*, 2007). Both ORF 61 and LAT transcripts were detected in ganglia, but ORF 61 sense transcripts were detected most often during acute infection whereas LAT antisense transcripts were more abundant during latent infection (Ou *et al.*, 2007). SVV ORF 61 is nonessential for replication *in vitro* (Gray *et al.*, 2007) but its role *in vivo* remains to be determined. Together with the previous data and our current analysis, the data strongly suggests that ORF 61 plays an important role in the establishment and/or maintenance of SVV latency.

ORF B was the next most frequently detected transcript in 4 of 16 sensory ganglia collected from 5 SVV infected rhesus macaques. ORF B is the homolog to VZV ORF S/L, which was recently determined to play a role in viral DNA cleavage (Kaufer *et al*, 2010). SVV ORF A and 65 were detected in 3 of 16 sensory ganglia. SVV ORF A is a truncation of SVV ORF 4 but has not been shown to transactivate viral genes like ORF 4 and has no homolog in VZV (Gray, 2010). SVV ORF 65 is a tegument protein and its VZV homolog was shown to be dispensable for viral replication in cell culture and *in vivo* (Cohen *et al*, 2001; Niizuma *et al*, 2003; Zhang *et al*, 2010). ORF 68 or glycoprotein E (gE) was detected in 2 of 16 ganglia and the VZV homolog is essential for VZV replication *in vitro* (Mallory *et al*, 1998; Zhang *et al*, 2010). The gE protein is involved in cell-to-cell spread and VZV infectivity (Ali *et al*, 2009; Zerboni *et al*, 2011) as well as immune evasion by interfering with complement activation and antibody-dependent cell-mediated cytotoxicity (ADCC) (Litwin *et al*, 1992).

SVV ORFs 4, 10, 55, 63, 64, 66, and 67 were only detected in 1 of 16 latent ganglia. SVV ORF 4 is a transcriptional activator and the homolog in VZV is an essential gene for virus replication (Cohen *et al*, 2007). SVV ORF 10 is a viral transactivator and virion tegument protein. The VZV homolog was shown to be dispensable for growth in culture (MeWo) but a deletion virus showed a replication defect in skin organ cultures (Zhang *et al*, 2010). VZV and SVV ORF 55 have homology to HSV UL5, which is a component of the helicase-primase complex and is essential for viral DNA synthesis (Zhu and Weller, 1988; Zhu and Weller, 1992). SVV ORF 63/70 is a viral transcriptional activator and its homolog in VZV is a tegument protein and at least one gene copy is required for viral replication (Sommer *et al*, 2001). VZV ORF 63 is the most frequently detected transcript in latently infected human ganglia (Azarkh *et al*, 2010; Kennedy and Cohrs, 2010) and is involved in the inhibition of the interferon- α induced antiviral response (Ambagala and Cohen, 2007). Furthermore, VZV ORF 63 was shown to inhibit apoptosis in primary human neurons thus promoting cell survival (Hood *et al*, 2006). VZV ORF 64/69 is a tegument phosphoprotein that is not essential for viral replication in culture but is associated with abnormal plaque phenotype (Sommer *et al*, 2001). VZV ORF 66 is a protein kinase (Eisfeld *et al*, 2006; Kinchington *et al*, 2000) that phosphorylates ORF 62, the main transactivator preventing nuclear accumulation, thus providing a potential mechanism to help maintain viral latency. VZV ORF 66 is also shown to be important for VZV infection of lymphocytes (Moffat *et al*, 1998; Schaap *et al*, 2005; Soong *et al*, 2000) and to play a role in MHC-I downregulation (Abendroth *et al*, 2001; Eisfeld *et al*, 2007). Finally, SVV ORF 67 or glycoprotein I (gI) is the binding partner for gE and deletion of gI in VZV results in small plaque phenotype and is avirulent in a skin xenograft model (Mallory *et al*, 1997; Oliver *et al*, 2011). Studying the role of these SVV ORFs *in vivo* in the rhesus macaque model will yield critical insight into viral pathogenesis and latency.

The following observations highlight some differences between the transcriptional profiles observed during SVV and VZV latency. VZV ORF 63 is the most frequent and abundant transcript detected during latency in sensory ganglia (Cohrs and Gilden, 2007). A recent study characterized the entire VZV transcriptome in latently infected human trigeminal ganglia using a multiplex RT-PCR approach (Nagel *et al*, 2011; Nagel *et al*, 2009). In agreement with previous analyses, this study detected VZV ORFs 4, 29, 40, 62 and 63 and ORF 63 was the most prevalent transcript. Moreover, this approach also detected ORFs 11, 41, 43, 57 and 68, which were not previously reported, but not ORFs 18, 21, and 66 previously detected in latently infected ganglia. ORF 66 has been detected in latently infected human ganglia by RT-dependent nested PCR, ISH and real-time PCR (Cohrs and Gilden, 2007; Cohrs *et al*, 2003a). Similar to the VZV latency analyses, our study detected SVV ORFs 4, 63, 66 and 68 in latent sensory ganglia albeit in very few ganglia. The VZV transcriptional studies did not detect ORF 61 transcripts during latency. This difference could be due to host factors influencing latency or different mechanisms employed by SVV

and VZV to establish and/or maintain latency. VZV ORF 61 is expressed early during infection of human fibroblasts (Reichelt *et al*, 2009) and is important for VZV lytic replication *in vitro*. Specifically, in transient expression assays ORF 61 enhanced VZV infectivity (Moriuchi *et al*, 1992). While deleting a large portion of ORF 61 impaired VZV replication in cell culture (Cohen and Nguyen, 1998), total ORF 61 deletion resulted in complete loss of VZV replication (Zhang *et al*, 2010). Interestingly, both VZV ORF 21 and 61 were dispensable for the establishment of latency in the cotton rat model (Sato *et al*, 2003; Xia *et al*, 2003). These observations differ from what is observed in SVV where ORF 61 deletion has minimal effect on SVV replication *in vitro* (Gray *et al*, 2007) although the effect of VZV ORF 61 loss in the natural host remains unknown.

Our study furthers the understanding of lytic and latent gene transcription during SVV infection of rhesus macaques, a critical animal model of VZV infection. A detailed understanding of the molecular biology of SVV coupled with our robust animal model will aid the studies of VZV pathogenesis, antiviral therapy and vaccines. Future studies will characterize the role of these genes in the establishment and maintenance of latency *in vivo*, a critical step in the understanding of viral and host factors that modulate reactivation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SVV transcriptome in BAL from acutely infected animals. Total RNA was isolated from 1×10^6 BAL cells. RNA was amplified and analyzed by RT-qPCR using primer and probe sets specific for each viral ORF. Data shown are the average copy number for triplicate reactions per sample. A) RM 25043 (3 (lined) and 7 dpi (black) B) RM 25339 7 dpi (black) C) RM 24953 7 dpi (black).

Figure 2.

SVV transcriptome in latently infected sensory ganglia. Relative copy number from RT-qPCR analysis of SVV RNAs isolated from infected, trigeminal ganglia (TG), cervical dorsal root ganglia (DRG-C), thoracic dorsal root ganglia (DRG-T), lumbar/sacral dorsal root ganglia (DRG-L/S) at >72 dpi in A) RM 25043 B) RM 25339 C) RM 24953 D) RM 20226 E) RM 24943.

Table 1

Varicella severity and SVV DNA Viral Loads

		Animal ID					
Rash Severity *		RM 20226 + +	RM 24943 +	RM 24953 + +	RM 25043 + + + +	RM 25339 + + + +	
dpi	Sample	SVV Genomic copies per µg DNA					
3	BAL	N/A	142	5,951	2,164,348	371,314	
7	BAL	N/A	10,998	22,499	477,919	2,011,551	
10	WB	N/A	20	N/D	80	103	
>72	TG	448	90	164	N/D	47	
	DRG-C	49	89	143	N/D	56	
	DRG-T	101	79	175	60	51	
	DRG-L/S	233	67	60	N/D	50	

N/A = sample not available

N/D = viral DNA not detected

* += approximately 10 lesions

Table 2

Animal tissues evaluated for SVV transcriptome

Animal ID	Acute		Latent			
	BAL	PBMC	dpi	TG	DRG-C	DRG-T
RM 20226		10 dpi	178	+	+	+
RM 24943		10 dpi	77	+		+
RM 24953	7 dpi		73		+	+
RM 25043	3 & 7dpi		73	+	+	+
RM 25339	7 dpi	10 dpi	77	+		+

Table 3

Acute SVV gene expression in BAL^a

	3 dpi	7 dpi			
	Animal ID				
ORF	RM 25043	RM 25043	RM 25339	RM 25043	Putative function or function based on known VZV or HSV-1 homology ^b
A	14,088	443,745	49,015	1,286,901	Truncated homolog ORF4, unknown function
B	2,284	477,472	5,586	181,221	Homolog VZV ORF S/L: cytoplasmic viral protein, unknown function
1	30,000	2,414,229	22,473	708,613	Membrane protein
3	1,315	250,046	4,479	197,397	Virion assembly
4	40,405	548,791	50,842	130,030	Transcriptional activator, IE2
5	0	7,977	0	1,611	Glycoprotein K
6	0	187,663	0	21,501	Component of DNA helicase-primase complex
7	9,043	838,375	19,217	4,006,752	Virion phosphoprotein
8	264	10,075	291	5,618	DeoxyUTPase
9A	863	5,631	792	2,876	Glycoprotein N
9	3,756	235,216	11,652	331,421	Tegument protein
10	0	5,209	334	15,474	Transcriptional activator, tegument protein
11	5,877	392,104	29,906	309,148	Tegument protein
12	0	1,531	0	583	Tegument protein
13	7,169	570,414	17,425	324,323	Thymidylate synthetase
14	961	7,243	0	2,288	Glycoprotein C
15	5,497	555,897	49,594	776,823	Membrane protein
16	0	14,380	420	11,518	Associated with DNA polymerase
17	1,888	115,013	4,321	64,863	Host shutoff virion protein
18	9,626	599,385	4,886	203,973	Ribonucleotide reductase, small subunit
19	0	7,681	0	1,955	Ribonucleotide reductase, large subunit
20	1,346	18,847	2,185	7,169	Capsid protein
21	236	26,683	493	129,625	Tegument protein
22	0	344	0	0	Tegument protein

	3 dpi	7 dpi			
	Animal ID				
ORF	RM 25043	RM 25043	RM 25339	RM 25043	Putative function or function based on known VZV or HSV-1 homology ^b
23	65,188	3,150,427	40,813	1,142,928	Capsid protein
24	0	11,424	193	9,122	Membrane phosphoprotein
25	123	21,968	276	12,848	Viral DNA cleavage/packaging
26	0	3,234	0	1,262	DNA cleavage/packaging
27	0	7,018	212	3,319	Nuclear phosphoprotein
28	1,238	109,302	0	180,589	DNA polymerase
29	956	30,469	1,166	334,241	Single-stranded DNA binding protein
30	182	7,539	1,556	3,090	Viral DNA cleavage/packaging
31	254	9,219	0	2,096	Glycoprotein B
32	22,533	1,338,433	38,645	1,139,522	Phosphoprotein
33	16,667	1,518,226	12,678	190,107	protease, capsid assembly protein
34	0	4,335	0	554	Viral DNA cleavage/packaging
35	6,832	91,870	0	2,800	Membrane protein
36	0	332	0	171	Thymidine kinase
37	1,148	122,694	2,781	70,765	Glycoprotein H
38	1,081	71,329	10,879	17,425	Virion protein
39	0	184	0	4,269	Envelope protein, viral egress
40	6	5,570	203	1,840	Major capsid protein
41	624,927	87,383,293	1,533,550	96,126,357	Capsid protein
42/45	1,905	192,439	6,478	86,900	Spliced product; viral terminase
43	1,148	51,669	0	6,073	Viral DNA cleavage/packaging
44	0	2,944	0	2,041	Virion protein
46	833	15,748	264	2,333	Tegument protein
47	556	245,474	0	1,776	Protein kinase
48	0	1,067	0	735	Deoxyribonuclease
49	24,761	346,248,208	1,384,850	2,616,008	Myristylated virion protein
50	10,457	709,187	18,789	453,678	Glycoprotein M

	3 dpi	7 dpi			
	Animal ID				
ORF	RM 25043	RM 25043	RM 25339	RM 25043	Putative function or function based on known VZV or HSV-1 homology ^b
51	0	26,328	2,396	64,474	Origin binding protein
52	754	16,212	0	7,370	Component of DNA helicase-primase complex
53	14,317	2,041	211,526	35,602,303	Gamma-1 protein
54	2,018	9,984	0	2,171	Viral DNA cleavage/packaging
55	300,878	850,146	3,324	462,147	Component of DNA helicase-primase complex
56	1,507	147,681	2,540	65,285	Gamma-2 protein
57	5,991,973	74,351	2,876	16,357	Nonessential VZV protein
58	0	2,384	0	14,325	Phosphoprotein
59	0	4,932	0	3,958	Uracil DNA glycosylase
60	0	14,528	0	2,201	Glycoprotein L
61	2,460	15,162	2,176	45,144	Transcriptional activator IE1 / latency-associated transcript
62	1,338	1,178	250	1,607	Transcriptional activator, IE3
63	198,530	6,824,892	224,147	47,283,036	Transcriptional activator, IE4
64	11,933	392,412	3,617	250,774	Tegument phosphoprotein
65	24,504	1,557,987	52,926	206,655	Tegument phosphoprotein
66	1,944	129,092	0	14,972	Protein kinase
67	9,220	453,634	5,600	149,243	Glycoprotein I
68	366	21,789	382	13,656	Glycoprotein E

^a relative copy number per µg total RNA

^b Gray, W.L. (2010). "Simian Varicella Virus: Molecular Virology." Curr Top Microbiol Immunol 342:291–308.

Table 4

SVV gene expression in PBMC at 10 dpi*

ORF	Animal ID		
	RM 20226	RM 24943	RM 25339
A	0	108	0
B	1,014	0	0
13	445	0	0
23	2,071	236	0
25	177	0	0
28	0	119	0
30	0	960	324
32	834	235	122
33	137	0	0
41	86,650	5,784	0
49	0	111	0
50	239	0	0
54	1,271	339	262
61	0	0	397
63	0	1,922	736
64	1,417	0	0
66	120	0	0
67	577	0	0

* relative copy number per µg total RNA

Table 5

SVV ORFs expressed in latently infected sensory ganglia

ORF	# samples positive/16	copy # ^a	Putative function or function based on known VZV or HSV-1 homology ^b
61	10	100–4,000	Transcriptional activator IE1 / latency-associated transcript
B	4	<500	Homolog VZV ORF S/L: cytoplasmic viral protein, unknown function
A	3	<400	Truncated homolog ORF4, unknown function
65	3	100–3,000	Tegument phosphoprotein
68	2	<400	Glycoprotein E
4	1	<600	Transcriptional activator, IE2
10	1	<300	Transcriptional activator, tegument protein
55	1	<300	Component of DNA helicase-primase complex
63	1	<1,500	Transcriptional activator, IE4
64	1	<1,200	Tegument phosphoprotein
66	1	<200	Protein kinase
67	1	<200	Glycoprotein I

^a relative copy number range / µg total RNA between 16 samples analyzed

^b Gray, W.L. (2010). "Simian Varicella Virus: Molecular Virology." *Curr Top Microbiol Immunol* 342:291–308.