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Review

A comparison of herpes simplex virus type 1 and varicella-zoster virus latency and reactivation

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Herpes simplex virus type 1 (HSV-1; human herpesvirus 1) and varicella-zoster virus (VZV; human herpesvirus 3) are human neurotropic alphaherpesviruses that cause lifelong infections in ganglia. Following primary infection and establishment of latency, HSV-1 reactivation typically results in herpes labialis (cold sores), but can occur frequently elsewhere on the body at the site of primary infection (e.g. whitlow), particularly at the genitals. Rarely, HSV-1 reactivation can cause encephalitis; however, a third of the cases of HSV-1 encephalitis are associated with HSV-1 primary infection. Primary VZV infection causes varicella (chickenpox) following which latent virus may reactivate decades later to produce herpes zoster (shingles), as well as an increasingly recognized number of subacute, acute and chronic neurological conditions. Following primary infection, both viruses establish a latent infection in neuronal cells in human peripheral ganglia. However, the detailed mechanisms of viral latency and reactivation have yet to be unravelled. In both cases latent viral DNA exists in an 'end-less' state where the ends of the virus genome are joined to form structures consistent with unit length episomes and concatemers, from which viral gene transcription is restricted. In latently infected ganglia, the most abundantly detected HSV-1 RNAs are the spliced products originating from the primary latency associated transcript (LAT). This primary LAT is an 8.3 kb unstable transcript from which two stable (1.5 and 2.0 kb) introns are spliced. Transcripts mapping to 12 VZV genes have been detected in human ganglia removed at autopsy; however, it is difficult to ascribe these as transcripts present during latent infection as early-stage virus reactivation may have transpired in the post-mortem time period in the ganglia. Nonetheless, low-level transcription of VZV ORF63 has been repeatedly detected in multiple ganglia removed as close to death as possible. There is increasing evidence that HSV-1 and VZV latency is epigenetically regulated. *In vitro* models that permit pathway analysis and identification of both epigenetic modulations and global transcriptional mechanisms of HSV-1 and VZV latency hold much promise for our future understanding in this complex area. This review summarizes the molecular biology of HSV-1 and VZV latency and reactivation, and also presents future directions for study.

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Introduction

Herpes simplex virus type 1 (HSV-1; human herpesvirus 1) and varicella-zoster virus (VZV; human herpesvirus 3) are human neurotropic alphaherpesviruses usually acquired early in life. Primary HSV-1 infection is usually localized and may be asymptomatic, although it can produce a more widespread systemic infection in neonates and immunocompromised adults, whilst primary VZV infection is systemic and results in childhood varicella (chickenpox). During primary infection, both viruses gain access to neurons most likely through retrograde transport from the site of cutaneous lesion (Topp *et al.*, 1994). Whilst there is no conclusive evidence showing virus accessing ganglia via retrograde axonal transport from cutaneous lesions in humans, retrograde axonal transport is supported by infectious virus in cutaneous vesicles (Grose & Brunel, 1978), virus-infected neurons in the region (Chen *et al.*, 2004), and HSV-1 and VZV retrograde axonal transport *in vitro* (Antinone & Smith, 2010; Markus *et al.*, 2011). VZV is

also thought to enter ganglia haematogenously. Evidence for this route of ganglionic infection is provided from the study of simian varicella virus (SVV), the monkey counterpart of human VZV (White *et al.*, 2001). Primary SVV infection results in viraemia (Dueland *et al.*, 1992); however, SVV DNA is present in monkey ganglia prior to rash (Mahalingam *et al.*, 2001; Ouwendijk *et al.*, 2012c), indicating virus can infect ganglia during the viraemic stage of infection. This finding was recently confirmed and extended by the demonstration that SVV infects memory T-cells prior to rash and virus DNA is present in neurons, in close proximity to memory T-cells (Ouwendijk *et al.*, 2013). In addition, VZV-infected T-cells, most likely memory T-cells (Ku *et al.*, 2004), can transfer virus to engrafted human dorsal root ganglia in a model of VZV pathogenesis using severe combined immunodeficient (SCID) mice (Zerboni *et al.*, 2005).

In either case (retrograde axonal transport for HSV-1 and VZV or infected memory T-cells for VZV), virus enters the neuron and latency is established. Virus DNA replication in the neuron is not a strict prerequisite for the establishment of latency (Caudill *et al.*, 1986; Steiner *et al.*, 1990); however, virus replication in the ganglion may increase the overall proportion of latently infected neurons (Yang *et al.*, 2000; Nicoll & Efstathiou, 2013) through a process called 'round-trip infection'. In round-trip infection, virus replicates in the neuron, travels anterograde to the skin and produces fresh lesions. Virus within the new skin lesions travels back to the ganglia to infect more neurons (Simmons & Nash, 1985; Imai *et al.*, 2009). The outcome of virus infection, in part, may depend upon the specific class of neurons infected. For example, during acute infection of mouse trigeminal ganglia, HSV-1 can be found in both $A5^+$ [nerve growth factor (NGF)-responsive neurons that project Aδ and C fibres into laminae I and II (outer) dorsal horn] and $KH10^+$ [small-diameter neurons that project C fibres to laminae II (inner) dorsal horn], but during latency HSV-1 is present more in $A5^+$ than $KH10^+$ neurons, thus indicating the virus preferentially establishes latency in $A5^+$ neurons (Yang *et al.*, 2000; Bertke *et al.*, 2011).

Both viruses can be latent in the same ganglion (Cohrs *et al.*, 2000), in neighbouring neurons (Cohrs *et al.*, 2005) and even in the same neuronal cell (Theil *et al.*, 2003b). Both viruses can reactivate to produce disease (clinical reactivation) or without producing disease (asymptomatic shedding). Clinical reactivation of HSV can occur repeatedly and mostly in the young, whereas clinical VZV reactivation typically occurs once per individual and predominantly in 25 % of the elderly. Although HSV-1 reactivation usually produces oral/labial lesions (cold sores), disease can also occur frequently elsewhere on the body at the site of primary infection (e.g. whitlow) and particularly at the genitals. Rarely, HSV-1 reactivation can cause encephalitis; however, a third of the cases of HSV-1 encephalitis are associated with HSV-1 primary infection (Whitley & Gnann, 2002). Importantly, HSV-1 is the most common cause of blindness due to infection in developed countries (Liesegang *et al.*, 1989). VZV reactivation from latency typically causes herpes zoster (shingles), which is a painful vesicular skin eruption occurring in a dermatomal distribution, and this may be followed by severe, chronic pain called post-herpetic neuralgia, which can be highly refractory to treatment (reviewed by Gilden *et al.,* 2013). VZV reactivation resulting in zoster may also be followed by meningoencephalitis, cranial nerve palsies, zoster paresis, vasculopathy or multiple ocular disorders. It is also recognized that VZV reactivation from latency may cause acute (Gilden *et al.*, 2015), subacute (Birlea *et al.*, 2011) or chronic (Morita *et al.*, 2003) neurological conditions in the absence of clinical herpes zoster. Furthermore, it has a been shown recently that VZV reactivation can be associated with a chronic brain lesion that may be misdiagnosed as a low-grade tumour (Halling *et al.*, 2014). It should be appreciated that primary VZV infection (varicella) may itself be complicated by several neurological conditions, such as encephalitis, myelitis and polyradiculitis (Gibbons *et al.,* 1956). Clinical aspects of virus reactivation show differences between HSV-1 and VZV. Whilst HSV-1 reactivation is often recurrent and occurs in a younger population, VZV reactivation is seldom recurrent and more prevalent in the elderly (reviewed by Kennedy & Steiner, 1994). These differences may indicate that the mechanism by which HSV-1 and VZV maintain latency differs; however, current studies suggest these differences may result from the basic biology of the viruses.

A major limitation concerning this review stems from the systems used to study virus latency. Guinea pigs (Scriba, 1975), mice (Stevens & Cook, 1971) and rabbits (Laibson & Kibrick, 1966; Stevens *et al.*, 1972; Martin *et al.*, 1977; Nesburn *et al.*, 1977) have been used to understand HSV-1 latency and reactivation, but whilst many small-animal models for VZV latency have been explored, to date, none has fully recapitulated human disease. As a consequence, human ganglia removed at autopsy have been used to investigate VZV latency (Kennedy & Cohrs, 2010). Studies using cadaveric ganglia are fraught with the same difficulty inherent in studying the human population from which they are obtained. Added to this are the difficulties specific to molecular analysis of tissue undergoing normal decomposition following death. As a result, more is known about HSV-1 latency than VZV latency. This review will summarize work on both viruses, and focus on the molecular biology of latency, reactivation and control of virus reactivation via immune surveillance by resident T-cells. HSV-1 data were obtained from animal models of a human virus, whilst VZV data were obtained from human tissue removed following death.

Basic biology of HSV-1 and VZV

In most tissue culture cells, HSV-1 is an efficient virus whose productive (lytic) replication cycle culminates in 8–12 h with release of 10^3–10^4 virus particles per cell with a particle : infectious virus ratio ranging from 10 : 1 to 100 : 1 (Dargan *et al.*, 1995; Döhner *et al.*, 2006; Suspène

et al., 2011). At the single-cell level, the VZV lytic replication cycle culminates in 9–12 h (Reichelt *et al.*, 2009), but it can take 3–5 days for the entire culture to show extensive VZV-induced syncytia (Grose & Brunel, 1978) and cell death either by lysis or apoptosis (Brazeau *et al.*, 2010; Pugazhenthi *et al.*, 2011). Whilst cell-free VZV preparations have been reported, virus yield remains extremely low: 10^5 p.f.u. per 2×10^7 human malignant melanoma cells (Grose *et al.*, 1979) and 1.6×10^6 p.f.u. per 5×10^6 human retinal pigment epithelial cells (Schmidt-Chanasit *et al.*, 2008). VZV in tissue culture is essentially cell-associated with a particle : infectious virus ratio of $4 \times 10^4 : 1$ (Carpenter *et al.*, 2009), but VZV in skin vesicles is cell-free (Takahashi *et al.*, 2009; Grose *et al.*, 2013).

Complete DNA sequences are available for both HSV-1 and VZV, and annotated examples of the virus genomes are available on the National Center for Biotechnology Information server. Both genomes are composites of two unique DNA segments [unique long (U_L) and unique short (U_S)] each bound by regions of inverted repeats [repeat long (R_L) and repeat short (R_S)] (Fig. 1). An interesting consequence of this DNA arrangement is that the long and short DNA segments with their attendant terminal repeats can rearrange to yield four isomeric configurations (prototype, inversion of U_L, inversion of U_S, inversion of both U_L and U_S). Equimolar concentrations of all four HSV-1 isomers are packaged into the virion (Roizman, 1979), whilst only two isomers (both U_S inversions) predominate in packaged VZV (Kinchington *et al.*, 1985). The drastic reduction of U_L inversions in VZV DNA within infectious virus particles is attributed to a DNA sequence uniquely located at the extreme left-end of the virus U_L DNA that is responsible for cleavage of genome-size DNA during packaging (Kaufer *et al.*, 2010). As this sequence is not present at the extreme right-end of VZV U_L DNA, the single cleavage site does not permit U_L inversions to be packaged into infectious virus particles.

The current annotated version of the 152 kbp HSV-1 genome contains 77 genes, three of which [infected cell protein 34.5 (ICP34.5/RL1), ICP0/RL2 and immediate-early protein of 175 Da (IE175/RS1)] are located in repeated regions of the virus genome (McGeoch *et al.*, 1986, 1988; Perry & McGeoch, 1988) (Fig. 1). An additional 11 HSV-1 genes were detected experimentally resulting in the most

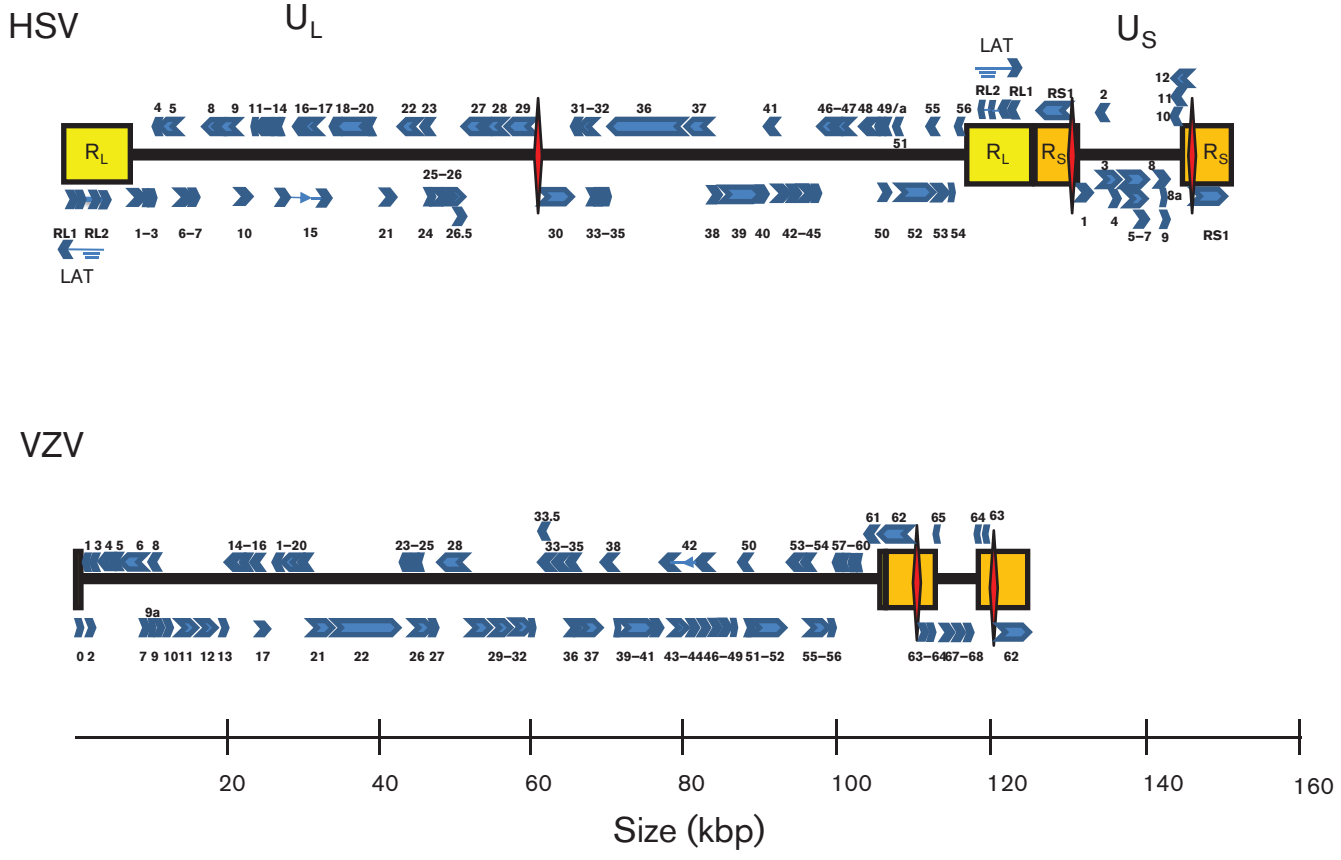

Fig. 1. HSV and VZV genomes. The HSV and VZV ORFs are represented by blue arrows on the genome maps. The diamond-shaped red lines denote the origin of DNA replication. Both the genomes have a unique long U_L and a unique short U_S region flanked by repeat regions (R_L and R_S). The VZV genome is ~30 kbp smaller than the HSV genome. The primary HSV-1 latency associated transcript (LAT) along with the two (nested) introns is shown.

current list of 85 protein-encoding HSV-1 genes (reviewed by Roizman *et al.*, 2013). In addition to these protein-coding transcripts, seven HSV-1 transcripts have been detected which do not encode proteins, but function as non-coding RNAs. The most recognized is the family of latency associated transcripts (LATs) whose association with latency is under extensive investigation and will be described in more detail below. Using the 52 polyadenylation addition signals found in the HSV-1 genome to facilitate construction of long-oligo-based arrays, transcripts mapping to all 52 virus targets were quantified (Stingley *et al.*, 2000). Recently, transcripts mapping to HSV-1 genes have been quantified by individual SYBR Green-based quantitative PCR analysis of virus-infected Vero cells (Garvey *et al.*, 2014).

The original annotation of the 124 884 bp VZV genome identified 70 virus genes and included ORF42/45 spliced into a single unit as well as ORF62/70, 63/69 and 64/68 located in repeated regions of the virus genome (Davison & Scott, 1986). Later, three VZV genes were experimentally identified: ORF0 (Kemble *et al.*, 2000), ORF9A (Ross *et al.*, 1997) and ORF33.5 (Preston *et al.*, 1997) (Fig. 1). This annotation represents the most current understanding of the VZV proteome, and has formed the basis for analyses of VZV transcripts in mRNA for infected tissue culture cells by PCR-based arrays (Cohrs *et al.*, 2003b), long-oligonucleotide-based arrays (Kennedy *et al.*, 2005), multiplex PCR (Nagel *et al.*, 2009) and next-generation-based deep sequencing (Baird *et al.*, 2014a; Jones *et al.*, 2014). Transcripts mapping to all annotated VZV ORFs have been detected in virus-infected cells, but evidence from PCR-based VZV transcription arrays, Northern blot analysis, cDNA PCR (Cohrs *et al.*, 2003b) and strand-specific cDNA deep sequencing (Baird *et al.*, 2014a) suggests that unannotated VZV genes remain.

The VZV genome, at 83 % the size of the HSV-1 genome, is a compact unit of information that contains, on average, 332 nt between annotated genes. This small intergenic distance combined with almost equal distribution of DNA strand usage (37 genes map to the prototype strand and 34 genes map to the complementary strand) suggests that typical next-generation sequencing information without source strand identification (Jones *et al.*, 2014) provides less information than next-generation sequencing in which mRNA source strand identification is maintained (Baird *et al.*, 2014a). Taken together, our understanding of the HSV-1 and VZV transcriptomes, although significant, is not yet complete.

Virus transcription during productive infection in tissue culture is highly orchestrated

The first wave of HSV-1 transcription is initiated when tegument proteins, delivered to the cytoplasm upon fusion of the virus envelope with the cell membrane, reach the nucleus. The tegument is an amorphous protein-rich region located between the virus-DNA-containing capsid and the virus envelope. Virus proteins that facilitate virus gene transcription are amongst the many tegument proteins. HSV-1 tegument contains virion protein 16 (VP16, also known as UL48 and α-TIF) (Zhu & Courtney, 1994) and immediate-early proteins ICP0 (Yao & Courtney, 1992) and ICP4 (Bibor-Hardy & Sakr, 1989). Following virus entry into most, if not all cell types, but especially neurons cultured from human or rat dorsal root ganglia, HSV-1 VP16/UL48, an integral part of the virus tegument (Elliott *et al.*, 1995), is shed from the nucleocapsid as it undergoes transaxonal retrograde transit (Antinone & Smith, 2010; Hafezi *et al.*, 2012). Free VP16/UL48 in the axon and cytosol (Aggarwal *et al.*, 2012) associates with host cell factor (HCF)-1 through a conserved tetrapeptide motif found in most basic leucine-zipper proteins (Freiman & Herr, 1997). The cytoplasmic VP16/UL48 : HCF-1 complex may translocate to the nucleus (Kristie *et al.*, 1999) where it associates with octamer transcription factor (Oct)-1, a site-specific POU domain transcription factor that binds to TAATGA(A/G)AT sequences located in promoters of HSV-1 immediate-early genes, to form the VP16/UL48-induced complex (VIC) (Liu *et al.*, 1999). At low m.o.i., VIC-dependent transcription of immediate-early virus genes is required to initiate productive infection (Luciano & Wilson, 2002). High m.o.i. or virus infection under stressful conditions (infection in the presence of *N*′,*N*′-hexamethylene bisacetamide) overcomes the requirement for VIC (McFarlane *et al.*, 1992; Nogueira *et al.*, 2004). Interestingly, HSV-1 contains two additional tegument proteins [UL46 (VP11/12) and UL47 (VP13/14)] that map 3′ to VP16/UL48 and regulate TIF-activity; UL47/VP13/14 increases VP16/UL48 activity up to 20-fold, whilst UL46/VP11/12 downregulates this increased transactivation (McKnight *et al.*, 1987). It should be noted that protein-free HSV-1 and VZV DNA is infectious when properly introduced into cells, albeit at low efficiencies, indicating that no virus protein is absolutely required to produce progeny virus.

The VZV orthologue to HSV-1 VP16/UL48 is VZV ORF10, a tegument protein not required for VZV growth *in vitro* (Cohen & Seidel, 1994). Along with VZV ORF10, ORF4, 62 and 63 are transcriptional transactivators, and all are present in the virus tegument (Kinchington *et al.*, 1992, 1995), but the mechanism by which VZV transcription is initiated upon infection is yet to be determined. Whilst blocking IE62 incorporation into progeny virions by modifying specific IE62 phosphorylations does not impede virus replication (Erazo *et al.*, 2008), and transfected VZV DNA is infectious (Zhang *et al.*, 2008), analysis of early events in virus infection requires high-titre virus inoculum. To date, sufficient amounts of cell-free VZV have not been produced and our understanding of virus gene regulation during productive infection is best known for HSV-1-infected cells.

Fate of incoming DNA

Entry of HSV-1 or VZV DNA into the nucleus triggers an innate anti-DNA response provided by the assembly of a host protein complex collectively termed promyelocytic leukaemia (PML) protein nuclear bodies (Everett & Maul, 1994) or nuclear domains (ND10) (Maul *et al.*, 1993). PML/ND10 are typically located in interchromosomal spaces and are involved in a number of cell functions including maintaining DNA integrity and regulating transcription (Tavalai & Stamminger, 2009), but are located at sites where virus DNA replication ultimately occurs (Maul *et al.*, 1996; Everett & Murray, 2005). Whilst the full range of functions and modes of action of this multiprotein assembly are yet to be determined, various components are important in silencing transcription of incoming virus DNA. Speckled protein of 100 kDa (Sp100) specifically binds hypomethylated CpG islands (Isaac *et al.*, 2006). The Sequence Manipulation Suite, a web-based tool designed to analyse DNA (Stothard, 2000), indicates that HSV-1 DNA with 68.3 (G+C)% has 147 856 CpG islands and VZV DNA, with a lower 46.2 (G+C)%, has 22 833 CpG islands. Chemical analysis of replicating HSV-1 DNA identifies 5-methylcytosine only transiently present [4–9 h post-infection (p.i.)] in total DNA extracted from infected cells, but not in DNA extracted from purified virus nucleocapsids (Sharma & Biswal, 1977). Analysis of HSV-1 DNA radiolabelled *in vivo* with L-[methyl-^{3}H]methionine (Low *et al.*, 1969), or by restriction endonuclease digestion (Dressler *et al.*, 1987) or DNA sequencing following bisulfite treatment (Kubat *et al.*, 2004a), indicates no detectable methylation at CpG islands in HSV-1 DNA during productive or latent infections. Thus, HSV-1 and, by extension, VZV DNA with their large number of unmethylated CpG islands are prime targets for Sp100.

Once targeted to virus DNA through Sp100 binding to hypomethylated CpG islands, HDaxx, an essential multifunctional PML/ND10 component, suppresses transcription by recruiting histone-modifying enzymes, histone deacetylase 1 and 2 (Lukashchuk & Everett, 2010). PML/ND10-dependent gene silencing is through the formation of heterochromatin structures on promoters (Newhart *et al.*, 2012). This results in reduced virus yield if not counteracted by virus-encoded proteins, ICP0/RL2 for HSV-1 and ORF61 for VZV (Everett *et al.*, 2006, 2010), or inactivation of histone deacetylase by phosphorylation by virus serine/threonine kinase, US3 for HSV-1 and ORF66p for VZV (Walters *et al.*, 2010). In HSV-1-infected cells ICP0/RL2 induces ubiquitin-dependent degradation of Sp100 as well as PML.1 [one of six PML isoforms (Cuchet-Lourenço *et al.*, 2012)] and the scaffold protein organizing PML/ND10 assembly (Gu & Roizman, 2003). Along with disarming the intrinsic antivirus activity supplied by PML/ND10, ICP0/RL2 dampens the host innate immune response to HSV-1 infection (reviewed by Lanfranca *et al.*, 2014). For example, production of antiviral type 1 IFNs is blocked by ICP0/RL2. ICP0/RL2 in the nucleus targets the host pattern-recognition receptor, IFN-inducible protein 16 (IFI16), for proteasomal degradation, which suppresses recognition of incoming HSV-1 dsDNA, ultimately inhibiting phosphorylation and nuclear localization of regulatory factor 3 and transcriptional activation of IFN-stimulated genes (ISGs) (Orzalli *et al.*, 2012). In addition to its nuclear function, cytoplasmic ICP0/RL2 promotes HSV-1 replication by blocking ISG expression through a pathway that involves ubiquitination, but not proteasomal degradation, as well as through a pathway that is independent of protein ubiquitination altogether (Taylor *et al.*, 2014). In addition to ICP0/RL2-dependent enhancement of virus growth through degradation of ND10 protein domains, HSV-1-encoded microRNA (miRNA)-H1 targets α-thalassemia/mental retardation syndrome X-linked (ATRX) protein, a critical ND10 component involved in transcription regulation, which also functions to remodel chromatin (Jurak *et al.*, 2012). Whilst ATRX is degraded in cells infected with HSV-1 lacking miRNA-H1 through an ICP0/RL2 pathway, it is clear that HSV-1 has redundant mechanisms to disarm host intrinsic antiviral defences. However, VZV, with a much smaller U_L repeat region, does not contain sequence homologous to HSV-1 miRNA-H1. Indeed, miRNA deep-sequencing results failed to find any evidence of VZV miRNAs (Umbach *et al.*, 2009). Nonetheless, VZV does dissociate ND10 structures following virus infection. VZV immediate-early protein IE61 also counteracts PML/ND10 function through a different mechanism. PML/ND10s are disrupted in VZV-infected cells, but PML is not degraded and Sp100 levels are only slightly diminished (Kyratsous & Silverstein, 2009). Whilst large PML/ND10 structures are decreased in size following VZV infection, PML colocalizes with VZV nucleocapsids where they form 'PML cages' (Reichelt *et al.*, 2011). PML cages encasing newly assembled VZV nucleocapsids are seen in experimentally infected human neurons (Reichelt *et al.*, 2011), suggesting the intrinsic antivirus activity of PML/ND10 function at two distinct levels: silencing transcription and physically isolating developing nucleocapsids.

Initially, cellular histones rapidly assemble on incoming, protein-free virus DNA, forming unstable, mobile nucleosomes that are post-translationally modified to silence transcription (Liang *et al.*, 2009; Lacasse & Schang, 2010). Recruitment of lysine-specific demethylase (LSD)-1 to the specific promoters through interaction with VP16/UL48 assists in removal of repressive histone markings, thus facilitating transcription (Gu *et al.*, 2005; Du *et al.*, 2010; Zhou *et al.*, 2013). Loss of complex formation by VP16/UL48 mutation and decreased virus transcription in cells treated with LSD-1 inhibitors substantiate the mode of VP16/UL48 action early during virus infection (Ottosen *et al.*, 2006; Liang *et al.*, 2009).

Along with activating transcription, immediate-early proteins also inactivate host immune response and modify the host transcription apparatus to recognize virus promoters. One host antivirus response dampened by the virus is the presentation of virus peptides through the

MHC class I complex on the surface of the infected cell. HSV-1 immediate-early protein ICP47 binds to the TAP heterodimer complex, thereby preventing MHC class I-associated peptide presentation (Hill *et al.*, 1995; Jugovic *et al.*, 1998). HSV-1 ICP4 is an essential HSV-1 immediate-early protein involved in reprogramming the host transcription apparatus (Kristie & Roizman, 1986). Binding of HSV-1 ICP4 to host transcription factor IIA and mediator stabilizes the preinitiation complex on HSV-1 gene promoters, resulting in induction of HSV-1 gene transcription (Zabierowski & DeLuca, 2008; Lester & DeLuca, 2011). ICP4 also functions to repress transcription of immediate-early and early virus genes. Site-specific binding of ICP4 and recruitment of TATA-binding protein (TBP) and transcription factor IIB to virus promoters in the neighbourhood of the TATA box represses transcription (Kuddus *et al.*, 1995) of ICP0 (Lium *et al.*, 1996) and LAT (Farrell *et al.*, 1994). Interestingly, ICP4's repression of specific virus gene transcription may be mediated through association of mediator with an intact kinase domain (Wagner & DeLuca, 2013). HSV-1 immediate-early protein ICP22 targets RNA polymerase II activity to virus promoters through altered phosphorylation of the C-terminal domain of the large polymerase subunit (Poffenberger *et al.*, 1993; Rice *et al.*, 1995; Lin *et al.*, 2010). Most HSV-1 genes are not spliced and HSV-1 immediate-early protein ICP27 binds unspliced mRNA facilitating nuclear export and translation of low-abundance HSV-1 mRNA amongst excess cellular transcripts (Sandri-Goldin, 1998; Cheung *et al.*, 2000). In summary, HSV-1 immediate-early proteins efficiently inactivate host antivirus defence, commandeer the transcription apparatus and establish an environment conducive to continued virus gene expression. VZV also downregulates cell surface expression of MHC class I, but the accumulation of MHC class I molecules in Golgi compartments is attributed to the virus protein kinase encoded by VZV ORF66 (Abendroth *et al.*, 2001; Eisfeld *et al.*, 2007; Verweij *et al.*, 2011).

When sufficient immediate-early proteins accumulate, a switch in promoter usage takes place and early virus genes are transcribed. The 11 HSV-1 early genes encode enzymes that replicate virus DNA in two stages: first by θ replication (Severini *et al.*, 1994) followed by a σ (rolling circle) mechanism (Skaliter *et al.*, 1996). Transcription of the third class of HSV-1 genes reaches its peak after virus DNA replication has begun (Wagner *et al.*, 1998). This class of virus genes is divided into leaky-late or strict-late depending on if their transcription is reduced (leaky-late) or not detected (strict-late) when virus DNA replication is inhibited. The 64 late HSV-1 genes (22 leaky-late, 23 strict-late and 19 undetermined-late subclass) encode components of the virion, proteins directing assembly of the virus particle within specific regions of the cell and proteins that regulate cell fusion along with release of the infectious particle (reviewed by Roizman *et al.*, 2013). The temporal cascade of HSV-1 gene expression results in the ordered assembly and release of thousands of infectious virus particles.

Although the ordered process of virus gene transcription is probably also present during VZV infection (Cohrs *et al.*, 2003b; Kennedy *et al.*, 2005), classification of individual VZV genes into temporal groups cannot be experimentally verified due to the lack of high-titre VZV preparations. Therefore, most VZV genes are classified by their homology to HSV-1 genes. However, experimental evidence from single-cell analysis has allowed the classification of time of expression for a few specific VZV genes. Accordingly, VZV ORF61 and 62 transcripts are first detected 1 h p.i. (Reichelt *et al.*, 2009), and are designated immediate-early genes (reviewed by Cohen, 2010); VZV ORF63, previously characterized as immediate-early (Debrus *et al.*, 1995), is first detected at 4 h p.i.; VZV ORF29 is first detected at 4–6 h p.i. and is classified as an early protein; and VZV gE, previously assumed to be a late gene product (Montalvo *et al.*, 1985; reviewed by Grose 1990) is most likely leaky-late due to its accumulation prior to virus DNA replication (Reichelt *et al.*, 2009). The multiple immediate-early genes identified in HSV-1, VZV and bovine herpesvirus type 1 (BHV-1), an alphaherpesvirus of cattle (van Santen, 1991), contrast with the single immediate-early protein identified in the alphaherpesviruses, pseudorabies virus (Everett & Dunlop, 1984) and equine herpesvirus type 1 (Gray *et al.*, 1987; Caughman *et al.*, 1988). Nonetheless, all alphaherpesviruses encode an ICP4 equivalent whose major function is to initiate transcription from the virus genome and for VZV this is the only gene with TAATGARAT elements (Abmayr *et al.*, 1985; Smith *et al.*, 1992; Fraefel *et al.*, 1994). It is therefore not clear how other VZV immediate-early protein transcription is initiated.

During latency, HSV-1 and VZV gene transcription is restricted

The virus DNA burden in human neurons removed at autopsy is extremely low, requiring real-time PCR analysis for reliable quantification. When calculated per 10^5 ganglionic cells, the virus genome copy number for HSV-1 DNA in human trigeminal ganglia is 2902 ± 1082 ($n=15$) (Pevenstein *et al.*, 1999), 3043 ± 2738 ($n=12$) (Cohrs *et al.*, 2000) and 2017 ± 5778 ($n=12$) (Vrabec & Alford, 2004); for VZV, the virus genome copy number in human trigeminal ganglia is 258 ± 38 ($n=15$) (Pevenstein *et al.*, 1999) and $9497 \pm 13\,079$ ($n=17$) (Cohrs *et al.*, 2000). These results underscore the variability encountered when examining autopsy tissue obtained from a random sample of the local population independent of age, sex, time of initial virus infection and number of re-exposures to the virus.

In human trigeminal ganglia, HSV-1 (Efstathiou *et al.*, 1986) and VZV (Clarke *et al.*, 1995) DNA persists in a non-integrated form, most likely as 'end-less' (circular) episomes of

genomic unit length or genome concatemers which are, for the most part, transcriptionally silent. The only HSV-1 gene transcripts abundantly detected during latency maps antisense to ICP0/RL2 within the R_L (Stevens *et al.*, 1988). The most abundant LATs are introns spliced from the primary 8.3 kb LAT transcript (Zwaagstra *et al.*, 1990). Due to a non-canonical splice site junction, the 1.5 kb HSV-1 LAT remains as a stable lariat in the neuronal nucleus with a half-life of 24 h (Spivack *et al.*, 1991; Wu *et al.*, 1996; Thomas *et al.*, 2002; Ng *et al.*, 2004). LAT-containing neurons are occasionally surrounded by $CD8^+$T-cells (Theil *et al.*, 2003a; Verjans *et al.*, 2007) that are primed in the periphery (Held *et al.*, 2012) and LAT functions in part to protect neurons from granzyme B-induced apoptosis (Allen *et al.*, 2011; Jiang *et al.*, 2011). LAT, although dispensable to the establishment of latency, does play an active role in silencing HSV-1 lytic gene promoters during establishment of latency in part by facilitating methylation of lysine 9 on histone H3, a post-translational modification indicative of heterochromatin structures (Wang *et al.*, 2005). Specifically, promoters for HSV-1 genes expressed during lytic infection (ICP4, ICP27 and ICP8) are associated with H3K9 and H3K27 trimethylations in ganglia of latently infected mice, and these post-translational modifications indicative of facultative heterochromatin are reduced when latency is established with HSV-1 containing mutations precluding LAT transcription (Amelio *et al.*, 2006a; Cliffe *et al.*, 2009; Kwiatkowski *et al.*, 2009). Interestingly, LAT-dependent heterochromatin formation ($H3K27me^3$) on lytic virus promoters during establishment of latency – a phenomenon that is virus strain and infection model-specific – may involve modifications of host polycomb repressor complex group 2 protein (PRC-2) as Suz12, a normal PRC-2 component, is not detected on latent HSV-1 DNA during silencing of the virus genome (Kwiatkowski *et al.*, 2009; Cliffe *et al.*, 2013).

HSV-1 encodes 16 miRNA identified by deep-sequencing studies of virus-infected cells; 11 of these miRNAs are encoded within or near to the LAT gene (Jurak *et al.*, 2010) with seven detected in latently infected mouse or human ganglia (Umbach *et al.*, 2009; Held *et al.*, 2011; Kramer *et al.*, 2011). Three HSV-1 miRNAs are antisense to, and reduce expression of, virus genes involved in the initiation of virus gene transcription (ICP4), neurovirulence (ICP34.5) and inactivation of host antiviral pathways (ICP0/RL2) (Umbach *et al.*, 2008; Flores *et al.*, 2013). Mutations within the LAT promoter that reduce LAT transcription accumulation of miRNA mapping to the LAT primary transcript are not essential in regulating acute infection or establishment of latency (Kramer *et al.*, 2011). Consequently, other miRNAs may still have a role in the establishment or maintenance of latency. Interestingly, a newly identified HSV-1 miRNA, miRNA27, located in the non-coding region of HSV-1 ICP0/RL2, targets the 3′ UTR of Kelch-like human family member 24 (KLHL24) (Wu *et al.*, 2013). As overexpression of the KLHL24 transcription factor reduces virus yield, its inhibition by HSV-1 miRNA27 increases acute virus infection clearly demonstrates that HSV-1 miRNAs cannot only target virus genes, but also host genes to the better facilitate virus infection. The findings that HSV-1 LAT-encoded miRNA functions to maintain latency is supported by studies that show steady-state levels of HSV-1 miRNA decrease as the virus reactivates (Du *et al.*, 2011, 2012, 2013). The effect of miRNA on HSV-1 gene expression is not limited to virus-encoded miRNA. Recently, a neuronal-specific miRNA (miR-138) has been shown to target sequences within HSV-1 ICP0/RF2, reducing ICP0 expression and thus expression of most lytic virus genes (Pan *et al.*, 2014). In addition, two small (961 and 36 nt) HSV-1 RNAs reduce expression of HSV-1 ICP4, productive virus yields and cold shock-induced apoptosis (Shen *et al.*, 2009). Recently, these two HSV-1 small non-coding RNAs were shown to increase neuronal expression of herpesvirus entry mediator (HVEM), an HSV-1 receptor protein in the tumour necrosis factor receptor superfamily that regulates cellular homeostasis (Allen *et al.*, 2014). Whilst the functions of LAT are not absolutely required for latent infection or reactivation (Javier *et al.*, 1988; Leib *et al.*, 1989), the original findings that LAT facilitates virus reactivation from latency still holds true (Perng *et al.*, 2000; Inman *et al.*, 2001).

VZV gene expression in human ganglia containing VZV DNA is also highly restricted (Cohrs *et al.*, 1992). Although VZV is present in latent form in human ganglia (Kennedy *et al.*, 1998), especially the trigeminal ganglion (Mahalingam *et al.*, 1992), it is not practical to obtain such tissues except at post-mortem. The ganglia undoubtedly undergo changes following death, such as hypoxia (Sawtell & Thompson, 2004; reviewed by Wilson & Mohr, 2012), that may alter transcription of the resident virus genome. Transcripts mapping to 12 VZV genes have now been identified in human ganglia removed at autopsy (Cohrs *et al.*, 1996, 2003a, b; Kennedy *et al.*, 2000; Nagel *et al.*, 2011). Interestingly, the nearer to death human trigeminal ganglia are obtained, the fewer VZV transcripts are detected (Ouwendijk *et al.*, 2012b). The number of VZV transcripts detected in human ganglia may, therefore, reflect post-mortem events rather than the latent state. By contrast, removal of human ganglia that contain HSV-1 DNA can be seen as similar to removal of mice ganglia that also contain HSV-1 DNA, in the sense that explants of both human and mice ganglia can reactivate resident HSV-1 (Baringer & Swoveland, 1973; Lewis *et al.*, 1982; Birmanns *et al.*, 1993). Whilst VZV reactivation in explants of human ganglia remains elusive (Plotkin *et al.*, 1977), it is reasonable to assume that this could be achieved, when the appropriate experimental conditions that favour this process are discovered. There is one report of VZV reactivation from human ganglia that had been removed 1–5 days post-mortem (Saitoh *et al.*, 2013), but this observation has not been confirmed. Furthermore, only minimal amounts of VZV DNA replication, presumably an event preceding virus reactivation, have

been demonstrated within this time frame (Azarkh *et al.*, 2012).

Of the VZV genes whose transcripts have been detected in human ganglia, only VZV IE63 transcripts have been found in trigeminal ganglia removed ≤ 9 h post-mortem (Ouwendijk *et al.*, 2012b). VZV IE63 is a 278 aa protein present in the virus tegument (Kinchington *et al.*, 1995; Sadzot-Delvaux *et al.*, 1998). During productive infection, the highly phosphorylated protein (Mueller *et al.*, 2010) is predominantly located in the nucleus (Mueller *et al.*, 2009). In tissue culture cells, VZV IE63 represses transcription (Di Valentin *et al.*, 2005), most likely through histone modification (Ambagala *et al.*, 2009), as well as reducing translation in response to IFN induction (Ambagala & Cohen, 2007); however, in human ganglia the presence of VZV IE63 is yet to be confirmed, but may function to inhibit VZV-induced apoptosis (Hood *et al.*, 2006; Pugazhenthi *et al.*, 2011).

VZV IE63-encoded protein has been detected in ganglionic neurons removed at autopsy, although this is not a common event (Mahalingam *et al.,* 1996; Kennedy *et al.*, 2000; Zerboni *et al.*, 2010). Whether IE63 and other VZV proteins are expressed in human ganglia is an active area of investigation. Multiple VZV proteins have been reported in human ganglia removed at autopsy (Lungu *et al.*, 1998; Cohrs *et al.*, 2003b; Theil *et al.*, 2003b; Grinfeld & Kennedy, 2004). However, a more recent study reported that ascites-derived murine and rabbit antibodies raised against VZV proteins contain endogenous antibodies that react with human blood type A antigens in human neurons and that apparent VZV-specific neuronal staining is strongly associated with blood type A (Zerboni *et al.,* 2012). Thus, animal-derived antibodies should first be screened for anti-blood type A reactivity to avoid misidentification of viral proteins in neurons of individuals who are blood type A. Ascites-derived mAbs also contain endogenous antibodies that react with blood group A1-associated antigens present in neurons in snap-frozen human ganglia (Ouwendijk *et al.*, 2012a). Whilst such cross-reactivity may not explain VZV protein staining detected in ganglionic tissues obtained from individuals with different blood groups, the extent of VZV protein expression in human neurons clearly remains controversial.

Alphaherpesvirus gene transcription is deregulated during virus reactivation

As *in vitro* cultures of latently infected neurons are an ideal system to test pathways involved in virus reactivation, a brief description of available models is warranted. HSV-1 becomes latent in both rabbit and mouse trigeminal ganglia following infection of scarified cornea; however, species-specific differences have been demonstrated (Perng *et al.*, 2001). Most notable is the spontaneous reactivation with virus recovered in tears that occurs significantly more often in latently infected rabbits than in mice (Gebhardt & Halford, 2005). HSV-1 does reactivate from latently infected mice trigeminal ganglia, but the rate is extremely low and ganglia must be dissociated for virus isolation (Margolis *et al.*, 2007) or the latently infected mouse treated by hypothermia (Sawtell & Thompson, 1992). Both rabbits and mice have advanced our understanding of the biology of HSV-1 latency (reviewed by Webre *et al.*, 2012), but *in vitro* studies predominantly use rodent ganglia. Although a rodent model of VZV latency has been studied following footpad inoculation, a significant limitation of this system is that the virus does not reactivate, and the virus is located in both neuronal and non-neuronal cells, which do not reflect the human situation (Kennedy *et al.*, 2001). There have also been other small-animal models of VZV latency, including mice (Wroblewska *et al.*, 1993) and guinea pigs (Myers & Connelly, 1992). Whilst a mouse model of VZV latency has not be obtained, recent work suggests guinea pigs may hold promise as a model for VZV latency.

An *in vitro* model of VZV latency has been developed using isolated enteric neurons obtained from adult guinea pigs and fetal mice (Gershon *et al.*, 2008). VZV latency is established in cultures of neurons obtained from the small intestine and bowel of these animals following infection with cell-free VZV. Not only are some viral genes expressed in the infected cultures, but also reactivation is induced, albeit non-physiologically, by expressing VZV ORF61 protein or HSV ICP0 in the infected neurons. Studies in human tissues found VZV in 88 % of samples of human intestine. Subsequent studies have confirmed and extended these findings, and show that both WT and vaccine-type (vOka) VZV can establish non-productive infections in enteric neurons in the human gut (Chen *et al.*, 2011). This portends the potential importance of using this *in vitro* system to model VZV infection of human neurons (Gershon *et al.*, 2012).

Recent advances have been made in the development of an *in vitro* model of VZV latency. Terminally differentiated human neurons, derived either from induced pluripotent stem cells or human embryonic stem cells, are maintained in culture for at least 2 weeks following VZV infection. The results can be summarized as follows: infection of neurons with cell-associated VZV or high-titre cell-free VZV leads to productive virus infection and neuronal cell death, but neurons infected with cell-free virus at low m.o.i. survive and show no discernible cytopathic effect (Dukhovny *et al.*, 2012; Sloutskin *et al.*, 2013; Yu *et al.*, 2013). Although, the non-permissive virus-infected neurons survive for at least 2 weeks in culture, abundant virus transcripts and proteins are present as well as many empty capsids (capsids lacking DNA) (Grose *et al.*, 2013). RNA sequence analysis (RNA-seq) revealed that the VZV transcriptome in non-productively infected neurons is similar to that seen in productively infected human lung fibroblasts, but unlike VZV-infected human lung fibroblasts, VZV DNA does not accumulate in the virus-infected neuronal culture (Baird *et al.*, 2014a, b). These results are

similar to quiescent HSV-1 in neurons obtained from adult murine trigeminal ganglia wherein RNA-seq data suggest transcription from multiple regions of the virus genome in addition to LAT (Harkness *et al.*, 2014). As the neurons can be maintained in compartmented microfluidic chambers that orientate neurite outgrowth, infection of axons with fluorescently tagged virus permits direct viewing of retrograde transport to neuronal cell bodies (Markus *et al.*, 2011; Grigoryan *et al.*, 2012). These near-pure (>95 % βIII-tubulin expressing) neuronal cultures provide an excellent opportunity to observe the outcome of VZV infection, such as induction of host anti-apoptotic genes (Markus *et al.*, 2014), although latency (long-term presence of VZV DNA with limited transcription) has not yet been obtained. An interesting alternative is the long-term cultivation of human neuronal stem cells in suspension culture at low gravity (Goodwin *et al.*, 2013). These three-dimensional arrays can be maintained for >3 months following low m.o.i. VZV infection during which time virus DNA replicates, genes are transcribed and very low (<50 p.f.u.) cell-free virus is sporadically released into the tissue culture fluid. However, in these 'tissue-like assemblies' VZV is not latent, but present in a quiescent state from which infectious virus recrudescence can occur. Taken together, *in vitro* cultures containing human neurons are being used to investigate VZV infection and hopefully continued work will provide a model to study quiescent VZV in culture.

Many models of HSV-1 latency and reactivation have been described, but the following have come to recent attention. The initial work was pioneered by Christine Wilcox, who was the first to develop an *in vitro* model of HSV-1 quiescence/latency and reactivation in neurons (Wilcox & Johnson, 1987). Sympathetic ganglia are removed from the E21 rat, dissociated and cultured in medium containing 5-fluoro-2′-deoxyuridine to inhibit dividing, non-neuronal cells. Within 5 days, surviving neurons adhere to collagen-coated tissue culture plates where they form networks. The differentiated neurons are infected with HSV-1 and maintained for 1 week in NGF-supplemented medium containing acyclovir to inhibit HSV-1 DNA replication. Upon acyclovir removal, cultures are monitored for ~1 week. During this time, low-level spontaneous reactivation occurs that is increased following specific treatments (Kobayashi *et al.*, 2012a). This model, pioneered by the Wilcox laboratory (Wilcox & Johnson, 1987), has been used to show HSV-1 reactivation is induced following removal of NGF (Wilcox & Johnson, 1988; Wilcox *et al.*, 1990). More recently, NGF-dependent maintenance of latency has been linked to phosphatidylinositol 3-kinase signalling through Akt (protein kinase B) (Camarena *et al.*, 2010). Specifically, constitutive mammalian target of rapamycin (mTOR) complex 1 (mTORC-1)-mediated hyperphosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP) translational repressor maintains cap-dependent translation, and NGF removal inactivates mTORC-1, blocks 4E-BP phosphorylation, suppresses cap-dependent mRNA translation and induces HSV-1 reactivation. It is important to note that NGF signalling originates in axons and terminates at the nucleus (Kobayashi *et al.*, 2012b), thus forming an exquisite path to communicate stress signals from the environment to neuronal nuclei where latent HSV-1 resides. A significant refinement of the Wilcox model is the culturing of adult trigeminal ganglia instead of E21 rat sympathetic ganglia (Bertke *et al.*, 2011). The neuronal subtypes present in the adult mouse trigeminal cultures reflect those seen in adult mice as opposed to neonatal mice, and are currently employed to determine the host and virus components that function to restrict HSV-1 and HSV-2 (human herpesvirus 2) latency to specific neuronal subtypes (Bertke *et al.*, 2013).

In addition to the second rodent model of HSV-1 latency and reactivation (discussed below) is the rabbit model pioneered by the Hill lab (Shimomura *et al.*, 1983). HSV-1 latent infection in rabbit trigeminal ganglia is established following ocular infection and virus reactivation is experimentally induced with epinephrine. As this is the only animal model in which infectious HSV-1 is shed in tears, as noted in humans undergoing stress (Kaufman *et al.*, 2005), it is of use to analyse the molecular events regulating virus reactivation (Kang *et al.*, 2003; Danaher *et al.*, 2005).

An additional model system to study HSV-1 latency is the establishment of latency in rodent trigeminal ganglia followed by reactivation *in vivo* or removal of latently infected tissue and initiation of reactivation in the explant cultures. HSV-1 latency is established in outbred Swiss Webster mouse trigeminal ganglia following infection of scarified cornea. Reactivation is initiated by transient thermal stress applied to moderately restrained animals until their core body temperature equilibrates to a 43 °C water bath followed by controlled return to 35.8–37.4 °C to minimize hypothermia (Sawtell & Thompson, 1992; Sawtell, 1997). Abundant HSV-1 antigen and release of infectious virions are seen in latently infected trigeminal ganglia 24 h following thermal stress. Using this model and contextual analysis (a method of quantifying nucleic acids within single cells in the context of intact tissue), a 2.3 kbp fragment in HSV-1 LAT was deemed sufficient to establish, maintain and promote reactivation of latent HSV-1 (Sawtell *et al.*, 1998). Latent virus copy number was related to the frequency of virus reactivation (Sawtell, 1998; Thompson & Sawtell, 2000) and HSV-1 VP16/UL48 was determined to be required for expression of HSV-1 proteins early during reactivation (Thompson *et al.*, 2009; Sawtell *et al.*, 2011), whilst ICP0/RL2 was required to produce infectious progeny virus (Thompson & Sawtell, 2006).

Explanted trigeminal cultures undergo stress (Sawtell & Thompson, 2004), most likely from lack of axonal NGF signalling due to axotomy (Kobayashi *et al.*, 2012b). However, carefully controlled experiments that take these confounding factors into account have unveiled early molecular events leading to an initial round of virus

transcription from the previously silenced genome during latency. The initial step of HSV-1 reactivation involves transcription from the silenced DNA and does not follow the same well-ordered process of gene transcription that occurs during productive infection (Du *et al.*, 2011; Kim *et al.*, 2012). Presumably, this is due to the absence of high levels of VP16/UL48 (Batterson & Roizman, 1983), the tegument-associated, site-specific DNA-binding protein that guides Oct-1 and HCF-1 to promoters of immediate-early virus proteins. Reactivation from latency is a series of events that ultimately culminate in release of progeny virions and may be viewed as a dynamic continuum. This continuum may contain critical checkpoints that prevent reactivation or may involve a programme that, once initiated, may be irrevocable. Various investigators have identified events during reactivation as an aid in describing the critical events that lead to virus release. Using transient thermal stress to initiate HSV-1 reactivation, virus reactivation is divided into two stages: exit from latency and production of infectious virions (Thompson & Sawtell, 2006). Exit from latency occurs in response to external stimuli and results in release of gene repression with transcription from the latent virus DNA – an event named 'animation of the latent virus genome' (Penkert & Kalejta, 2011). An interesting consequence of animation is the possibility that expression of virus proteins during this early stage of virus reactivation may be recognized by sentinel HSV-1-specific CD8 T-cells resident in the ganglion (Knickelbein *et al.*, 2008). CD8 T-cells found in trigeminal ganglia removed at autopsy from humans and latently infected mouse trigeminal ganglia recognize different HSV-1 glycoprotein B (gB) epitopes. These HSV-1 gB-specific CD8 T-cells initiate degradation of ICP4, the major immediate-early HSV-1 transactivator, through a granzyme B-dependent mechanism (Knickelbein *et al.*, 2008). As a consequence continued virus gene expression is halted, latency is re-established and the neuron returns to the native (viable) state (St Leger & Hendricks, 2011). This scenario is currently debated. Indeed, a recent study of 12 human trigeminal ganglia did not find evidence of HSV-1 gB-specific CD8 T-cells, but did identify resident CD8 T-cells that recognize two immediate-early HSV-1 proteins, three early proteins and eight late virus proteins (van Velzen *et al.*, 2013). In addition, latency is not only maintained through the dynamic interplay of non-lytic CD8 T-cells as highlighted above, but also includes roles for IFN-γ produced by these immune cells and NK-cells (reviewed by Bigley, 2014).

Animation of the latent virus genome may also function as a checkpoint to transit into reactivation leading to release of infectious virus or to halt reactivation and re-enter latency (Kim *et al.*, 2012). For the first $\sim$24 h after explantation, VP16/UL48 (and probably other virus proteins) is made during HSV-1 animation, but the protein is sequestered in the cytoplasm. Following this period, VP16/UL48 translocates to the nucleus and reactivation continues with the classical cascade of virus gene expression culminating in the assembly of progeny virions, transport to axon termini and release of infectious virus particles (Kim *et al.*, 2012). However, if the initial stress factor is removed, animation may not be committed to assembly of virus, but may be reversed, resulting in resilencing of the virus genome and re-establishment of latent infection. Therefore, therapeutic intervention to mitigate disease caused by virus reactivation can target either stage of reactivation by reversing animation of the latent genome or blocking spread of released virus. The later stage of reactivation (release of infectious virus resulting in disease) is best studied in small-animal models with an intact immune system. It should be noted that animation of the latent virus genome as an initial response to the trigger for reactivation is a new concept and not universally accepted. The key component of animation is generalized transcriptional derepression – a concept supported by Du *et al.* (2011), Kobayashi *et al.* (2012b) and Kim *et al.* (2012), where initial virus gene transcription does not coincide with any specific kinetic class. However, in these studies, progeny virus first appears 48 h post-reactivation stimuli (Kim *et al.*, 2012) – a finding in contrast to Pesola *et al.* (2005) who demonstrated a complete round of virus production, from latency to release of infectious HSV-1, occurred within 14 h post-explantation. Pesola *et al.* (2005) suggests that the generalized transcriptional deregulation seen early during reactivation (animation) may result from a first round of virus growth in reactivated neurons followed by a new round of virus infection. Clearly, more work must be undertaken to differentiate events that transpire during virus reactivation from events that result from subsequent rounds of virus infections (Tal-Singer *et al.*, 1997).

Latent HSV-1 DNA is partitioned into distinct regions of the neuronal nucleus where virus gene transcription is governed, in part, by specific post-translational acetylation and methylation of bound histones. Actively transcribed (euchromatic) virus DNA regions contain nucleosomes with histone protein H3 post-translationally modified by acetylation at lysines 9 and 14 (H3K9ac/14ac) and dimethyation at lysine 4 ($H3K4me^2$) (Kubat *et al.*, 2004b; Neumann *et al.*, 2007). Similarly, $H3K9me^2$, $H3K9me^3$ and $H3K27me^3$ post-translational modifications are present in nucleosomes found on promoters of transcriptionally silent (heterochromatic) virus genes (Cliffe & Knipe, 2008; Cliffe *et al.*, 2009). The boundary between euchromatin and heterochromatin is maintained through the action of the CCCTC-binding factor protein (CTCF) (Amelio *et al.*, 2006b; Chen *et al.*, 2007) – a site-specific DNA-binding protein that blocks spread of heterochromatic modifications onto regions of euchromatin, blocks enhancer-dependent gene transcription and functions along with cohesin to maintain DNA loops (reviewed by Gaszner & Felsenfeld, 2006; Hou *et al.*, 2008; Rubio *et al.*, 2008; Didych *et al.*, 2012).

During stress, CTCF is released from the latent virus genome, enhancer activity is restored and the immediate-

early ICP0/RL2 gene is transcribed (Bloom *et al.*, 2010; Ertel *et al.*, 2012). Along with ICP0/RL2, transcription of multiple early and late virus genes is also initiated (Du *et al.*, 2011; Kim *et al.*, 2012). This initial generalized deregulation of gene transcription (animation) does not require *de novo* viral protein synthesis. How latent virus DNA quickly undergoes generalized transcriptional deregulation resulting in transcription of multiple immediate-early, early and late genes without prior protein or virus DNA synthesis is unknown, but may involve RNA polymerase II recruitment at CTCF/cohesin complexes (Chernukhin *et al.*, 2007; Wada *et al.*, 2009; Taslim *et al.*, 2012). Interestingly, CTCF-directed cohesin rings can tether DNA strands present on different chromosomes for coordinated transcription (Kagey *et al.*, 2010). Whilst not yet demonstrated in alphaherpesviruses, stress-induced dissociation of CTCF/cohesin leading to release of paused RNA polymerase II in early stages of latent Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) reactivation has been documented (Kang & Lieberman, 2011; Chen *et al.*, 2012). Overall, epigenetic regulation of latent HSV-1 gene transcription is an exquisite mechanism through which virus can undergo rapid animation in response to cellular stress, resulting in transcription of multiple genes independent of *de novo* protein synthesis. It must be noted that much work has yet to be done to determine if similar epigenetic regulation of the latent VZV genome exists. Indeed, the predicted number and sites of CTCF-binding are significantly different between HSV-1 and VZV DNAs (Amelio *et al.*, 2006b).

Whilst animation and generalized HSV-1 transcription is independent of VP16/UL48, progression of reactivation through virus DNA replication and assembly of infectious virus is dependent upon VP16/UL48 (Thompson *et al.*, 2009; Sawtell *et al.*, 2011). During generalized transcription deregulation, VP16/UL48 accumulates first in the cytoplasm and then translocates to the nucleus. Nuclear VP16/UL48 facilitates heterochromatic to euchromatic modifications at immediate-early virus gene promoters leading to the orchestrated cascade of virus gene expression seen in productively infected tissue culture cells (Kim *et al.*, 2012). Thus, HSV-1 reactivation can now be viewed as a three-step process: (1) animation of latent virus genome: generalized transcription deregulation with accumulation of proteins of all kinetic classes that map throughout the virus genome; (2) exit from latency: ordered programme of virus transcription that maintains the classical immediate-early, early and late class structure; critically dependent on the function of HSV-1 VP16 (Sawtell *et al.*, 2011); and (3) production and release of infectious virus.

Superimposed on epigenetic modifications induced early during virus reactivation are the cellular pathways altered in response to the reactivation stimulus (reviewed by Wilson & Mohr, 2012). For example, hypoxia (Kobayashi *et al.*, 2012b) or dexamethasone treatment induces HSV-1 (Du *et al.*, 2012) and BHV-1 reactivation (da Silva *et al.*, 2012; Workman *et al.*, 2012), respectively. Both treatments induce multicomponent pathways that result in significant gene induction. Whilst hypoxia-induced reactivation hinges upon mTOR activity (Kobayashi *et al.*, 2012b), glucocorticoid treatment initiates apoptosis and reactivation of HSV-1 (Du *et al.*, 2012) as well as specific cellular transactivators that induce critical BHV-1 gene expression (da Silva *et al.*, 2012; Workman *et al.*, 2012). Whilst care must be exercised when comparing animal (SVV and BHV-1) to human alphaherpesviruses, the information obtained from each model can be useful in directing experiments to test hypotheses in the human system, and continued progression of virus reactivation from generalized to ordered transcription seems to require *de novo* protein synthesis and components from both the cell and the virus.

Whilst VZV infection of guinea pig enteric neurons (Chen *et al.*, 2011; Gan *et al.*, 2014) is an exciting new system to study virus–neuron interactions, studies with human ganglionic tissue have provided an opportunity to analyse the virus in its natural setting once death of the host is taken into account. Nevertheless, there are benefits to using human ganglia: (1) VZV latency is studied in its native environment, (2) latency reflects protein complexes on the virus genome that have been stabilized over multiple decades, (3) tissue acquisition from incidents of sudden death and exclusion of immunosuppressed individuals helps to ensure the results reflect the natural state of virus DNA as best as possible, and (4) optimized ganglion dissociation and maintenance medium keeps neurons in close proximity to their supporting satellite cells whilst bathed in medium that maintains viability. The major disadvantages to using human ganglia are that the tissue comes from deceased individuals with the associated death-related changes that must be factored into all analyses. In addition, humans represent an outbred heterogeneous population, and the dose of initial virus infection and number/duration of subsequent infections are also uncontrolled. However, these negative features must not eliminate the study of virus reactivation from autopsy-derived human ganglia as HSV-1 reactivates and infectious virus is released from cultured human trigeminal ganglia removed at autopsy (Bastian *et al.*, 1972; Baringer & Swoveland, 1973; Lewis *et al.*, 1982). Whilst prospective studies with humans are not feasible, in-depth analyses involving numerous autopsies has permitted key discoveries. For example, as with HSV-1, VZV appears to undergo initial generalized transcription deregulation. When ganglia are removed within 9 h of death, only VZV ORF63 transcripts are detected, albeit at low abundance and not in all samples, but when the time between death and autopsy is $\geq$9 h, transcripts mapping to 11 additional VZV ORFs are detected (Nagel *et al.*, 2011; Ouwendijk *et al.*, 2012b), again not in all samples or at the same abundance. These VZV genes include members of immediate-early, early and late kinetic classes, but do not include genes essential for virus DNA replication (ORF6, 16, 51, 62 and 55). It is tempting to speculate that hypoxia – a condition present in death and known to induce HSV-1

reactivation – may also be key to VZV reactivation, but experimental proof is lacking. In addition, in human ganglia removed >9 h post-mortem, euchromatic post-translational modifications (H3K9ac) on nucleosomes are present on actively transcribed VZV genes (Gary *et al.*, 2006).

Taken together, reactivation of latent HSV-1 and VZV can now be divided into identifiable stages (Fig. 2). During latency episomal virus DNA is coated with host protein complexes conferring epigenetic regulation of virus gene transcription with accumulation of LAT (HSV-1) and ORF63 (VZV) transcripts. Animation of latent HSV-1 DNA with global generalized transcription de-repression is the initial event in response to initiators of virus reactivation (e.g. death, explantation, stress, hypoxia, heat shock, NGF removal, dexamethasone, apoptosis). During animation, many virus genes located throughout the virus genome and members of all kinetic classes are transcribed independently of *de novo* protein or DNA synthesis. Whilst the global deregulation most probably results in

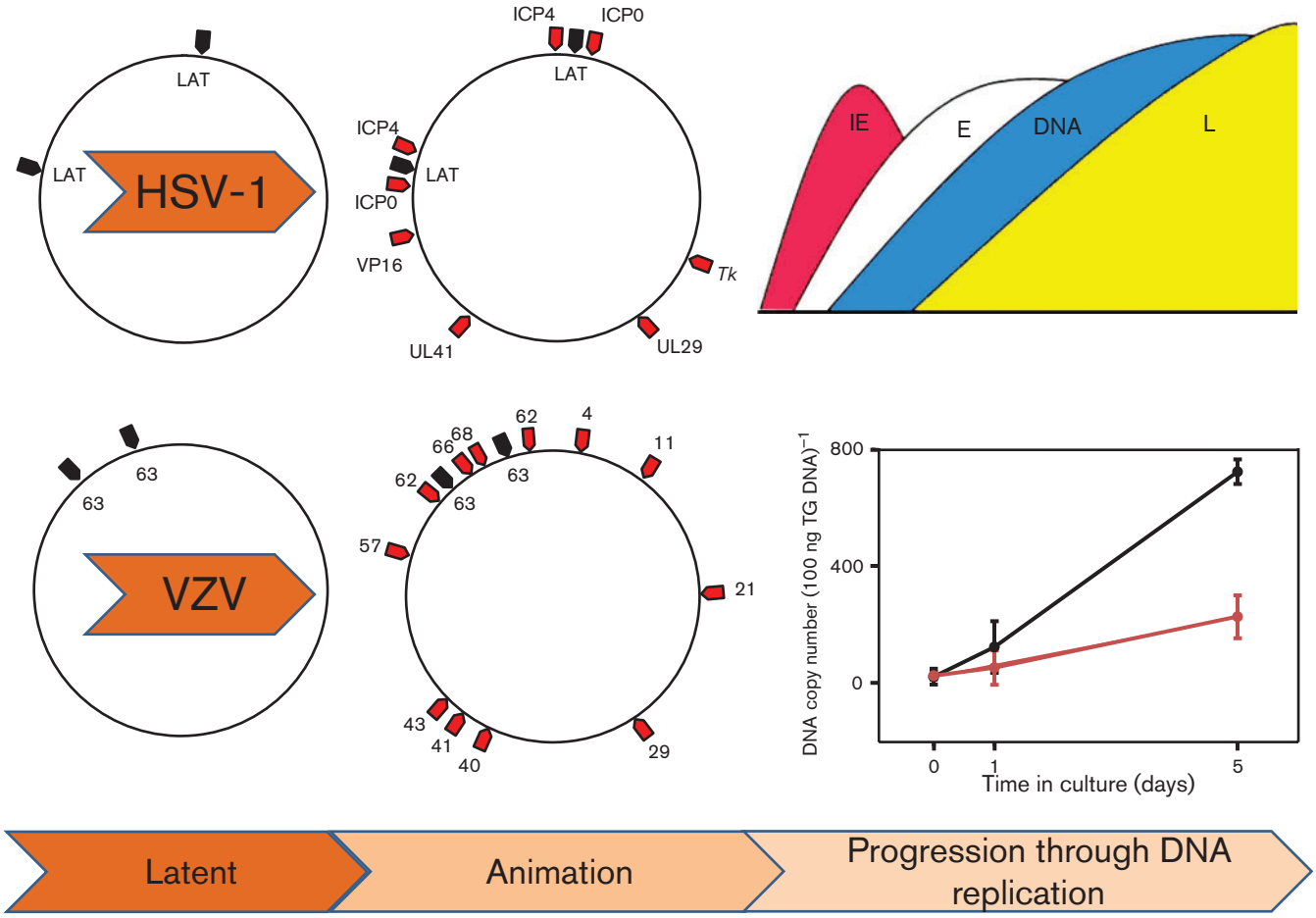

Fig. 2. Proposed pattern of alphaherpesvirus reactivation. HSV-1 DNA in latently infected murine trigeminal ganglia or VZV in human trigeminal ganglia removed ≤9 h post-mortem is episomal from which transcription is limited (LAT for HSV-1 and ORF63 for VZV). Following explant of latently infected HSV-1 mouse ganglia or human trigeminal ganglia >9 h post-mortem (VZV), virus gene transcription undergoes generalized deregulation (animation) characterized by transcription of multiple virus genes (only HSV-1 genes quantified <24 h post-reactivation are identified). Following animation, HSV-1 gene transcription undergoes a shift from generalized to organized transcription that orchestrates protein synthesis with DNA replication resulting in release of progeny infectious virus by 24 h for HSV-1. To date, VZV reactivation with release of infectious virus from human ganglia explants has not been demonstrated, but VZV DNA replication has been detected by quantitative PCR analysis of human ganglia explants (Azarkh *et al.*, 2012). Lower-right graph: *y*-axis, HSV-1 DNA copy number (black line) and VZV DNA copy number (red line); *x*-axis, time the human ganglia explants were incubated in optimized culture medium; data represent mean ± SEM for virus DNA quantification by real-time, TaqMan-based PCR. In these same human trigeminal explanted cultures, HSV-1 DNA replication is also seen (black line). Release of progeny infectious VZV has yet to be attained in explant cultures of human trigeminal ganglia; however, VZV DNA replication has been detected by 5 days in explanted human trigeminal ganglia cultures (red line). IE, Intermediate-early; E, early; L, late.

transcription of more HSV-1 genes than those identified in Fig. 2, only the genes whose transcripts were quantified <24 h post-reactivation were identified (Pesola *et al.*, 2005; Du *et al.*, 2011; Kim *et al.*, 2012). The VZV genes whose transcripts have been detected during animation are the result of PCR analysis for all annotated genes (Nagel *et al.*, 2011; Ouwendijk *et al.*, 2012b).

If stress continues, virus reactivation progresses through DNA replication requiring protein synthesis and follows the classical pattern (immediate-early, early, late) of virus gene expression, including accumulation of immediate-early virus proteins essential for orchestrated virus gene transcription. Whilst this stage occurs within 24 h for HSV-1, VZV DNA replication can be seen as late as 5 days in human trigeminal ganglionic explants (Azarkh *et al.*, 2012). Ultimately, virus components are assembled, transported through the nerve to the skin and complete virions are released with subsequent infection of neighbouring cells. Whilst the HSV-1 supporting data for this scenario has been presented in this review, there are many questions to be answered concerning VZV. For example, HSV-1 and VZV are present in the same ganglion, and possibly even within the same neuron, either following experimental infection (Sloutskin *et al.*, 2014) or in human ganglia removed at autopsy (Theil *et al.*, 2003b), but their pattern of reactivation is different. HSV-1 reactivation occurs earlier in life, often causing recurrent disease that is rapidly cleared with few lasting consequences. VZV reactivation occurs typically in the elderly, usually only once, and disease lasts longer with long-lasting consequences (e.g. postherpetic neuralgia) (reviewed by Mitchell *et al.*, 2003). In ganglionic explants, HSV-1 can be retrieved, but infectious VZV has not been obtained with the same protocols (Plotkin *et al.*, 1977). In addition, the molecular pathways resulting in reactivation of either virus must be divergent as HSV-1 and VZV reactivate in response to different stimuli (Steiner & Kennedy, 1993). Thus, the scenario developed for HSV-1 will be different in nuance to the scenario to be developed for VZV. It is hoped that this review will help set the stage for future studies by presenting a coherent outline of events to be examined.

Future perspective

A model of host-regulated control of alphaherpesvirus latency and reactivation allows a series of testable physical conditions. For example, does the density, distribution and modification of bound histones, insulator boundary elements and cohesin change during reactivation? If one considers the latent virus genome as chromatin containing a set of highly regulated gene loci, then a variety of protein components come into play. Stress-induced sequence-specific transcriptional activators may target virus promoters to activate viral gene expression. These same proteins initiate a normal response to the stimuli associated with virus reactivation and include responses to chemical- or temperature-induced stress, DNA damage or the action of autocrine/paracrine signals, such as NGF. Their affects are transient: translocation to promoters and quick deactivation by targeted destruction to downregulate the response.

A hypothetical model for the co-activation of transcription from a regulated host gene locus and a 'tethered' alphaherpesvirus genome would be bound by CTCF and closely associated cohesin/mediator complexes. RNA polymerase II mediator release following stress-induced cohesin dissociation would initiate transcription of virus genes independently of kinetic class, without *de novo* protein syntheses and without the requirement for virus-encoded transactivator proteins. Future studies involving *in vitro* cultures of latently infected neurons that combine pathway analysis with epigenetic modifications and global transcriptional determination (paired RNA-seq with chromatin immunoprecipitation sequencing) will shed much light on the enigma of alphaherpesvirus latency as it highlights potential therapeutic targets to mitigate disease – sometimes life-threatening – caused by virus reactivation.

A final important area for future studies is the increasing interest in subclinical or asymptomatic reactivation with shedding of virus as this may lead to interstrain recombination (Liljeqvist *et al.*, 2009), even though it does not interfere with clinical diagnosis (Leigh *et al.*, 2008). The detection rate for HSV-1 DNA in saliva was found to be $\sim$33 % on any given day when individuals were sequentially tested daily for 30 days and, in total, nearly all had detectable HSV-1 DNA in saliva for a duration of 1–3 days in the absence of disease (Kaufman *et al.*, 2005; Miller & Danaher, 2008). VZV DNA and infectious virus can also be detected in saliva from healthy individuals, although under the extreme mental, physical and physiological stress associated with spaceflight (Mehta *et al.*, 2004; Cohrs *et al.*, 2008). Closer to Earth, VZV DNA is present in saliva of $\sim$5 % of human immunodeficiency virus-positive individuals on highly active antiretroviral therapy treatment and only rarely (one of 53) in otherwise healthy individuals (Wang *et al.*, 2010). Of significant interest is that in all cases of HSV-1 and VZV shedding, no oral lesions were noticed. The biological significance of HSV-1 and VZV asymptomatic shedding is debatable, but may be another source of virus spread as has been shown in recent reports of asymptomatic HSV-2 shedding. Over the course of 30 consecutive samplings of 498 immunocompetent HSV-2-seropositive individuals, virus DNA was detected without genital lesions $\sim$10 % of the time (Tronstein *et al.*, 2011). Mathematical modelling of HSV-2 shedding data (frequency, duration, burden) has led to the interesting hypothesis that latent HSV-2 reactivation is nearly constant with the continued release of low-titre virus from neuronal termini (Schiffer *et al.*, 2009, 2014). If confirmed, our view of latency as a quiescent state of virus dormancy must be re-evaluated and perhaps animation of the virus DNA is an ongoing process.

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