The establishment of a recombinant system for *Papiine alphaherpesvirus 2*

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Contents

1. Summary

Simplexviruses of primates are closely related neurotropic herpesviruses that establish lifelong latent infections. While neuropathogenic infections are uncommon in their respective natural hosts, zoonotic transmission of *Macacine alphaherpesvirus 1* (McHV-1, Herpes B virus) from macaques to humans is associated with severe encephalitis and high fatality rates. The closely related *Cercopithecine alphaherpesvirus 2* (CeHV-2) and *Papiine alphaherpesvirus 2* (PaHV-2) have not been reported to be pathogenic in humans. The reasons underlying the differential pathogenicity are unclear, in part due to a lack of recombinant systems which allows analysis of mutant viruses. The goal of this thesis was to generate a recombinant system for PaHV-2.

For the generation of recombinant PaHV-2 a combination of fosmid- and transformation associated recombination (TAR)-based cloning approaches was employed. Restriction digest indicated that the genome of the recombinant PaHV2 was intact and the recombinant virus replicated with the same efficiency as uncloned virus in the cell line Vero76. In order to study the viral cell tropism and neutralization sensitivity, recombineering was applied to generate viruses carrying reporter genes. These studies revealed that PaHV2 but not the closely related CeHV-2 replicated efficiently in macaque cell lines. Sera from adult hamahydras baboons inhibited PaHV-2 infection while sera from infant animals (<1 year) did not, suggesting that primary infection occurs after the first year of life. Finally, PaHV-2 was sensitive to the antivirals acyclovir, ganciclovir and cidofovir, but not foscarnet, in line with published work. In summary, the first recombinant system for PaHV-2 was developed in the present study and will be instrumental for the identification of pathogenicity determinants of primate simplex viruses.

2. Zusammenfassung

Die Simplexviren der Primaten sind eng verwandte, neurotrope Herpesviren, die lebenslange latente Infektionen in den Neuronen ihrer Wirte verursachen. Während neuropathogene Infektionen bei ihren natürlichen Wirten ungewöhnlich sind, ist die zoonotische Übertragung des *Macacine alphaherpesvirus 1* (McHV-1, Herpes-B-Virus) von Makaken auf Menschen mit schwerer Enzephalitis und einer hohen Letalität verbunden. Die eng verwandten Viren *Chercopithecine alphaherpesvirus 2* (CeHV-2) und *Papiine alphaherpesvirus 2* (PaHV-2) wurden nicht als pathogen für den Menschen beschrieben. Die Gründe für die unterschiedliche Neurovirulenz sind unbekannt, unter anderem da rekombinantee Systeme fehlen, mit denen Virus-Mutaten hergestelt werden können. Das Ziel dieser Arbeit war es ein rekombinantes System für PaHV-2 zu entwickeln.

Die Entwicklung eines rekombinanten Systems für PaHV-2 erforderte eine Kombination aus Fosmid- und transformationsassoziierter Rekombination (TAR) basierten. Klonierungsansätzen. Eine Restriktionsanalyse zeigte, dass das rekombinante virale Genom intakt war und das rekombinante Virus replizierte mit vergleichbarer Effizienz wie das nicht-klonierte Virus in der Zelllinie Vero-76. Für die Analyse von Zelltropismus und Neutralisation wurden mit Hilfe von *Recombineering* Reporterviren hergestellt. Diese Arbeiten zeigten, dass sich PaHV-2 aber nicht CeHV-2 efficient in Zelllinien von Rhesusaffen vermehrt und dass Seren von erwachsenen Mantelpavianen die PaHV-2-Infektion hemmen, während Seren von jungen Tieren (<1 Jahr) inaktiv sind. Dies weist darauf hin, dass die Primärinfektionen nach dem ersten Lebensjahr erfolgt. Schließlich wurde PaHV-2 durch Virostatika wie Aciclovir, Ganciclovir, und Cidofovir, aber nicht durch Foscarnet gehemmt. Zusammenfassend wurde in dieser Studie zum ersten Mal ein

rekombinantes System für PaHV-2 entwickelt. Dieses System kann wesentlich zur Identifizierung von Pathogenitätsdeterminanten von Simplexviren von Primaten beitragen.

3. Introduction

3.1. Alphaherpesviruses

Alphaherpesviruses are enveloped DNA viruses with epithelial and mucosal sites of active replication (Roizman & Whitley, 2013). Their genomes contain unique long and unique short (UL and US respectively) regions each flanked by inverted repeat regions. Most alphaherpesviruses establish lifelong latency in neurons, with a few non-neuronal exceptions such as Marek's disease virus (MDV; *Gallid alphaherpesvirus 2*, GaHV-2). They are ubiquitous worldwide with three species infecting humans and dozens infecting other hosts (Davison, 2010). Apart from a direct impact on human health, they also have an indirect impact by infection of veterinary species involved in food production or kept as companion animals (Loncoman et al., 2017).

3.2. Taxonomy

According to The International Committee on Taxonomy of Viruses (ICTV), Herpesviruses are grouped into a single order (*Herpesvirales*) and three families (*Alloherpesviridae*, *Malacoherpesviridae*, and *Herpesviridae*). Based on their biological properties, the family of *Herpesviridae* is divided again into three subfamilies (*Alphaherpesvirinae*, *Bethaherpesvirinae*, and *Gammaherpesvirinae*).

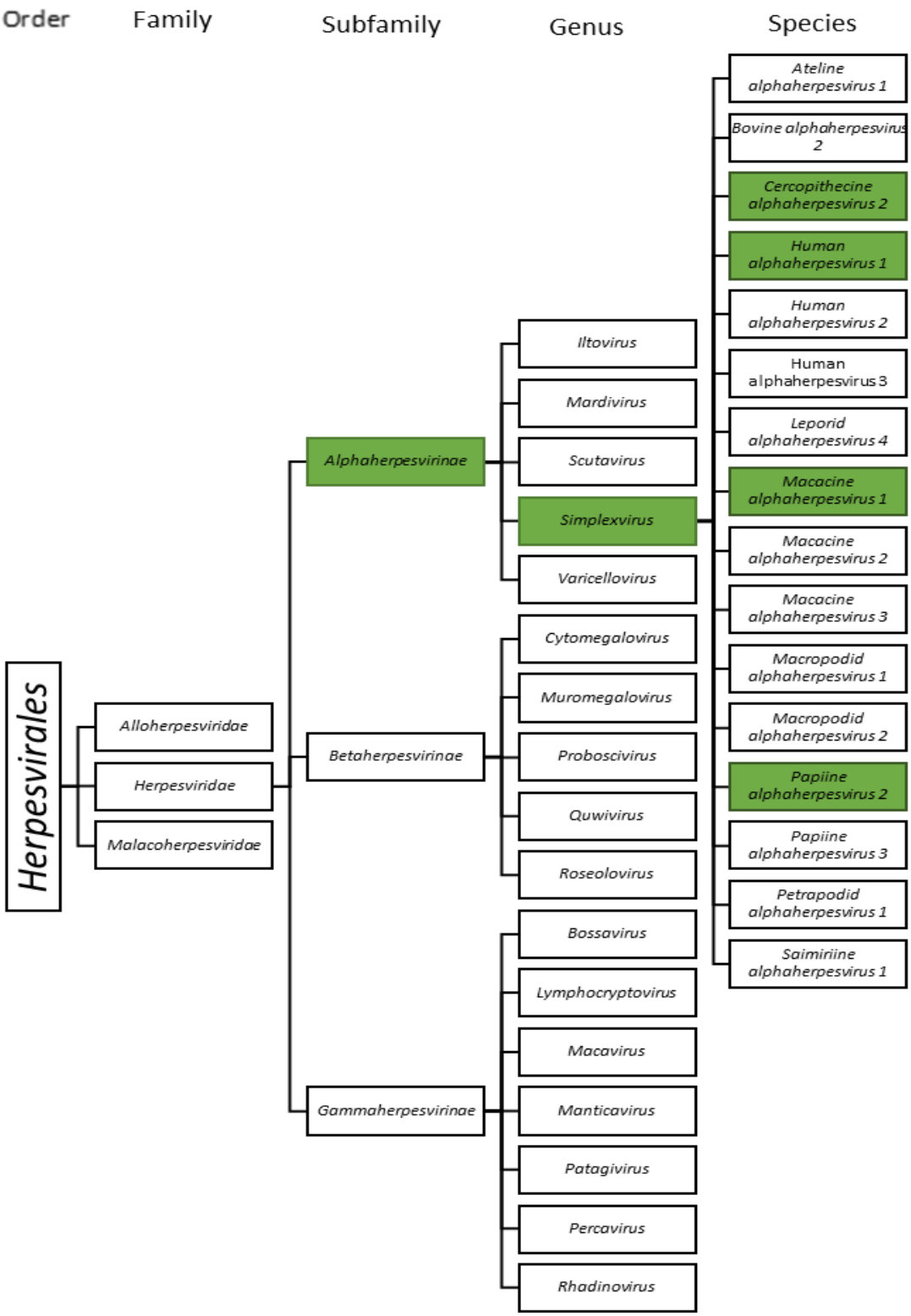

Figure 1. Herpesviruses taxonomy. The main focus on this report is on the simplexvirus genus of the *Alphaherpesvirinae* subfamily. Viruses analysed in the present study are marked in green [The data was taken from ITCV (2021)].

There are nine unique human herpesviruses that have been widely explored, *Human alphaherpesvirus 1* (HHV-1; herpes simplex type 1, HSV-1), *Human alphaherpesvirus 2* (HHV-2, herpes simplex type 2, HSV-2), *Human alphaherpesvirus 3* (HHV-3; Varicella-Zoster virus, VZV), *Human betaherpesvirus 5* (HHV-5; human cytomegalovirus, HCMV), *Human betaherpesvirus 6A* (HHV-6A), *Human betaherpesvirus 6B* (HHV-6B), *Human betaherpesvirus 7* (HHV-7), *Human gammaherpesvirus 4* (HHV-4; Epstein-Barr-virus, EBV) and *Human gammaherpesvirus 8* (HHV-8; Kaposi's sarcoma-associated herpesvirus, KSHV), Two of them, HHV-4 and HHV-8, are oncogenic (Mesri et al., 2010; Raab-Traub, 2012). The focus of this thesis is on simplexviruses (subfamily *Alphaherpesvirinae*) such as HSV-1, *Cercopithecine alphaerpesvirus* 2 (CeHV-2; simian agent 8, SA-8), *Macaccine alphaherpesvirus 1* (McHV-1; herpes B virus, BV), and *Papiine alphaherpesvirus 2* (PaHV-2; herpesvirus papio 2, HVP-2) (Fig. 1).

3.3. Simplexviruses

Primate simplexviruses are herpesviruses that extensively coevolved with their respective host species (McGeoch & Cook, 1994; Wertheim et al., 2014). As a consequence, they share a common genome structure, which is essentially collinear with HSV-1, the best characterized and referenced herpesvirus species. Several simplexviruses from non-human primates (NHP) have been characterized, including PaHV-2 (Perelygina et al., 2003), *Panine alphaherpesvirus 3* (PaHV-3; chimpanzee herpesvirus) (Luebcke et al., 2006), CeHV-2 (Malherbe & Strickland-Cholmley, 1969), and the human pathogenic alphaherpesvirus McHV-1 (Sabin & Wright, 1934).

The comparison of HSV-1, HSV-2, McHV-1, CeHV-2, and PaHV-2 genomes showed that all their genes are homologous and collinear (Barnett et al., 1992; Dolan et al., 1998; Everett & Fenwick, 1990; McGeoch et al., 1991; Perelygina et al., 2003; Tyler et al., 2005; Tyler & Severini, 2006). The only notable difference is that, among the simplexviruses, only HSV-1 and HSV-2 have the RL1 (ICP34.5) open reading frame. This gene is thought to be highly important for HSV neuropathogenesis (Chou et al., 1990). It possesses two genetically separable functions, namely neurovirulence in mice (Chou et al., 1990) and inhibition of the protein kinase R (PKR) system of the host cell (Chou et al., 1995). The interferon-induced PKR system shuts down the protein synthesis of the host cell in response to the presence of double-stranded viral RNA, and constitutes an important innate defense against the viral infection. Because PKR inhibition appears to be required for proper HSV-1 replication in cell culture, the absence of RL1 could imply a significant divergence in the pathogenetic mechanism of simian simplexviruses (Chou et al., 1995; Clemens, 1997; Clemens & Elia, 1997). The simplexvirus infection of non-human primates (NHP) and HSV-1 infection of humans exhibit a similar biology. These viruses are usually transmitted through exposure to bodily secretions or genital contact and hijack biological systems of the host cell, including transcription, to express proteins which are required for viral DNA replication and production of progeny virions. During primary infection, the viruses can infect sensory neurons and reach neuronal ganglia, where a lifelong latency can be established. In the latent stage, the viruses are able to persist lifelong without producing infectious particles. However, under conditions of stress or immuno-suppression, the viruses can reactivate from neurons, establish transient replication,

followed by shedding of infectious viruses (Huff & Barry, 2003; Keeble et al., 1958; Smith, 2012; Weigler, 1992).

Among the simplexviruses of NHP, only McHV-1, with monkeys of the genus *Macaca* as the natural host, is known to be pathogenic for humans. Natural infection of macaques is usually apathogenic, with few exceptions (Eberle & Jones-Engel, 2017), while humans can develop severe and frequently fatal encephalomyelitis in the absence of treatment (Davidson & Hummeler, 1960; Loomis et al., 1981; Palmer, 1987; Thompson et al., 2000; Weigler, 1992). However, asymptomatic McHV-1 infections have also been reported (Coulibaly et al., 2004). Conversely, despite having 85% identity with McHV-1 on the genomic level, no transmission of either CeHV-2 or PaHV-2 to humans has been reported.

3.3.1. Virion structure

The simplexvirus virion consists of about 40 proteins, and 10 of which are glycosylated (Kukhanova et al., 2014). The virion core contains large double-stranded DNA (dsDNA) which is tightly wrapped as a toroid. The DNA is packaged into a highly stable icosahedral capsid which is covered by tegument proteins followed by an outer lipid bilayer envelope membrane containing glycoprotein spikes on its surface. The virion diameter varies from 120 to 260 nm (Fig. 2).

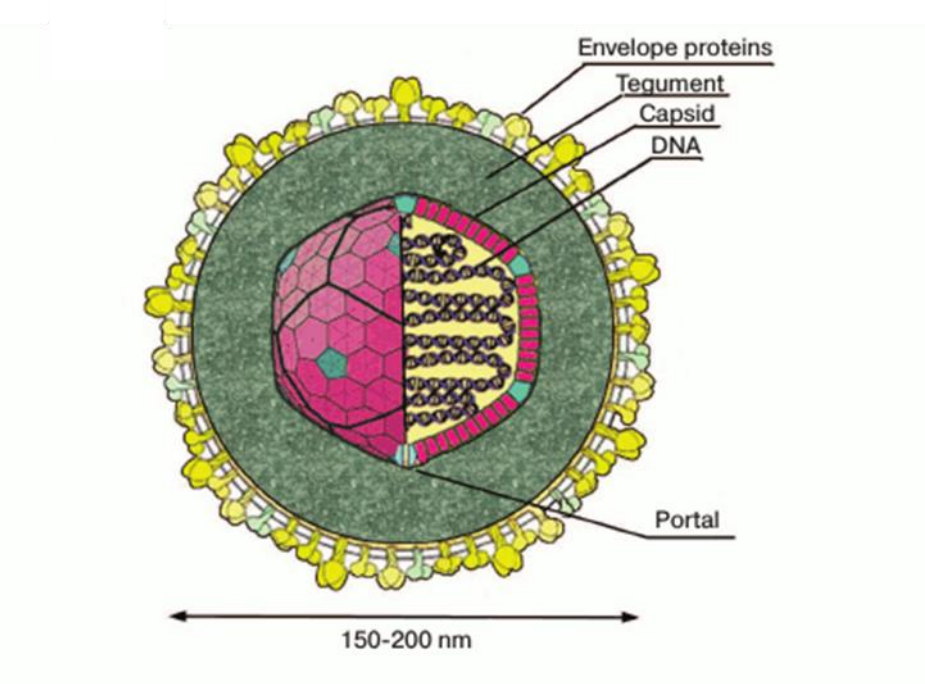

Figure 2. The structure of Human alphaherpesvirus type 1 (HHV-1) virion, as a representative of Simplexviruses. Virions have a spherical shape. The genomic dsDNA is surrounded by capsid proteins and an outer lipid bilayer envelope containing glycoprotein spikes [image was taken from (Kukhanova et al., 2014)]

The tegument consists of roughly 25 proteins, a number of them taking part in capsid transport to the nucleus and other organelles (UL36, UL37, ICP0) (Radtke et al., 2010) or viral DNA entry into the nucleus (VP1-2, UL36) (Jovasevic et al., 2008). Another tegument protein, known as VP16 (virion protein 16) or alpha-gene-transactivating factor (α-TIF) and encoded by the UL48 gene, stimulates the transcription of immediate early (IE) genes during the initial stage of viral infection. In addition, in the late stage, it is involved in the virion assembly and maturation process (Ace et al., 1989; Zhang et al., 2016). The other tegument proteins have also important functions, such as to suppress the biosynthesis of cellular proteins, and to degrade the mRNAs (VHS, UL41) (Barzilai et al., 2006). Finally, the tegument also contains RNA-binding proteins US11, UL47, and UL49 which bind viral and cellular transcripts packaged in the virion (Donnelly et al., 2007).

The capsid shell consists of four essential proteins: VP5 (encoded by UL19 gene) as the major capsid protein, VP26 (encoded by UL35 gene) as an accessory protein, and finally UL18 and UL38 genes encoding VP23 and VP19C proteins respectively. In addition, the capsid also contains the UL6 protein, which forms a portal on the vertex of one of the pentamers (fivefold symmetry axes), through which the viral genome is packaged into the capsid (Brown & Newcomb, 2011). There are three different types of capsids that can be isolated from infected cells, A-capsids, B-capsids, and C-capsids. A-capsids, also known as pro-capsids, do not have both scaffold proteins and viral DNA, while B-capsids only contain scaffold but not DNA. Only C-capsids contain both scaffold and the viral genome (Gibson & Roizman, 1972; Sheaffer et al., 2001).

The outer envelope of the virion consists of a lipid bilayer harbouring a number of glycoproteins gB, gC, gD, gE, gG, the gH-gL heterodimer, gI, gJ, gK, and gM, along with two unglycosylated membrane proteins (UL20 and US9). Additionally, the lipid bilayer is derived from cellular membranes acquired during viral egress by exocytosis (Chowdhury et al., 2013).

3.3.2. Genomic organization

Simplexviruses have a common genomic organization. It consists of large double stranded DNA (dsDNA) which contains 150-160 kilo-base pairs (kb) and very high G+C content (>70 %) (Brown, 2007). It is tightly packaged in a linear form and circularized upon entering the nucleus of the host cell for replication of the genome. The genome is divided into long and short unique regions (U_L and U_S, respectively), each flanked by tandem and inverted repeat sequences (TR_L-IR_L and TR_S-IR_S), respectively. These large repeat regions present a distinct problem in cloning and sequencing (De Bustos et al., 2016).

Many alphaherpesvirus genomes contain regions of high G+C content and highly repetitive areas, such as short tandem repeated sequences, also known as short sequence repeats (SSRs) and variable number of tandem repeats (VNTRs). In HSV-1, the G+C content tends to be even higher within these repeats compared to VZV (Szpara et al., 2014; Szpara et al., 2011) which leads to secondary structures such as stem-loops and G-quadruplexes that decrease the overall yield of sequence data in these areas and further complicate the assembly of these high G+C repetitive regions. Since the genome contains inverted repeat sequences, the U_L and the U_S unit can be recombined to generate four different linear genome isomers (Mocarski & Roizman, 1982) (Fig. 3).

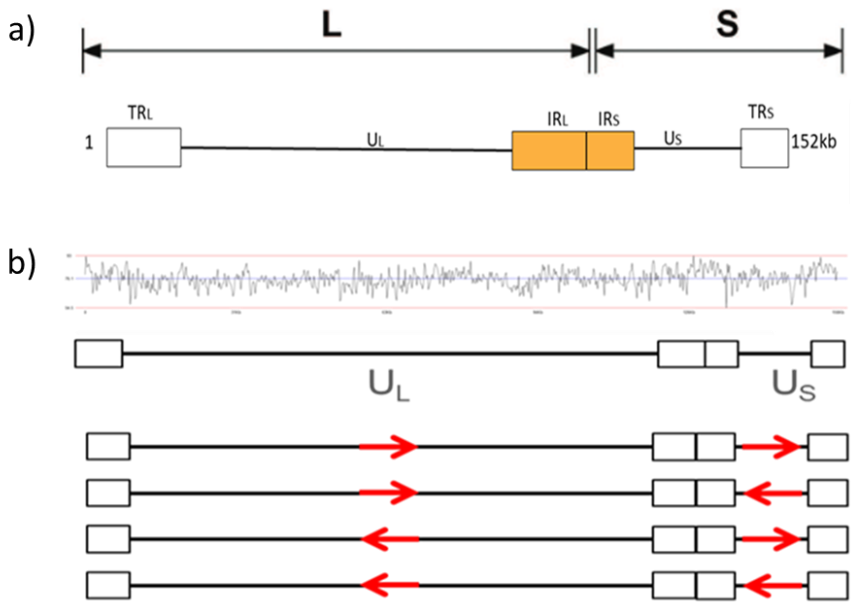

Figure 3. (a) The structure of the simplexvirus genome. The unique long region (U_L) is flanked by repeat sequences (TR_L and IR_S respectively), the short one (U_S) is flanked by IR_S and TR_S at the U_L–U_S junction and at the U_L terminus (Kukhanova et al., 2014). (b) The illustration of four different linear genomic isomers which are generated by the recombination between inverted repeat sequences both in the UL and US separately. A graph of the GC-content along the genome is shown on top.

The packaged herpesvirus genomes are linear, formed by site specific cleavage from complex concatemeric intermediates which consist of two conserved areas named pac1 and pac2. These areas are conserved in McHV-1, HSV-1 and HSV-2 (McVoy et al., 1998; McVoy et al., 2000). In addition, according to the sequences of the genes of the short region, they are also conserved and collinear with McHV-1.

3.3.3. Replication cycle

There are several discrete stages in the herpesvirus life cycle: viral entry, transport to the nucleus, viral gene expression, viral DNA replication, viral assembly, and capsid maturation. Infections starts with viral entry into the cell via endocytosis. The interaction of surface glycoproteins of the virus with specific cellular surface receptors plays an important role. Attachment of the virion to the cell surface is mediated by viral glycoproteins C (gC) and B (gB), which interact with cell surface glycosaminoglycans, especially heparin sulfate (Fan et al., 2018). The virus particle will undergo fusion of the viral outer envelope with cellular membranes which is mediated by four glycoproteins, gD, gB, and the heterodimer gH/gL (Aravantinou et al., 2017; Fan et al., 2017). Glycoprotein gD binds to three types of receptors: nectin-1 and nectin-2, herpes virus entry mediator (HVEM), and 3-*0*-sulfated heparin sulfate (3-*0*-S-HS). The latter is produced by 3-*0*-sulfotransferases 2-7 (3-*0*ST), which makes them promising therapeutic targets for antiherpetic drugs research (Baldwin et al., 2013).

The N-terminal domain of gD interacts with cellular receptors. This interaction will induce the release of its C-terminal domain to and activates the gB and gH/gL complex to induce membrane fusion. However, the C-terminal domain of

gD is inhibited when it is not coupled to the ligand (Arii et al., 2009; Baldwin et al., 2013).

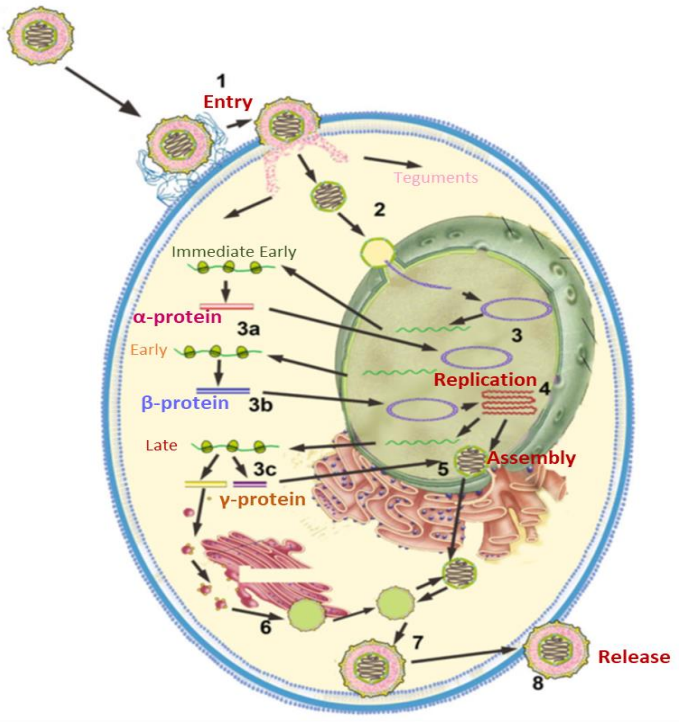

Figure 4. Simplexvirus life cycle: 1) virion attachment to the cellular receptors and entry into the cell; 2) transport of the virion to the nucleus while some tegument proteins remain in the cytoplasm; 3) viral gene expression: a) immediate early (IE), (b) early , and (c) late (L) proteins; 4) viral DNA replication; 5) nucleocapsid assembly; 6) capsid maturation; 7) primary envelope formation; and 8) viral progeny release (Kukhanova et al., 2014).

After entering the host cell, the viral particle with the capsid still coated with tegument proteins, is transported to the nucleus. The viral genome is released through the nuclear pore into the nucleus, where transcription, replication of the viral genome, and assembly of new progeny capsids takes place (Fig. 4). During infection, the host nucleus is reorganized: its size is increased, the nucleolus (Calle et al., 2008) and nuclear domain-10 (ND-10) (Everett et al., 1998; Rivera-Molina et al., 2013) subdomains are disrupted, cellular chromatin is condensed and in the late steps of infection, the latter and the nuclear lamina are destructed (Simpson-Holley

et al., 2005). Key cellular processes – transcription (Jenkins & Spencer, 2001), splicing of the cellular RNA (Hardy & Sandri-Goldin, 1994), protein biosynthesis and cellular response to infection (Neumann et al., 1997) – are also blocked. All these steps increase the efficiency of viral genome replication and transcription.

The viral mRNA is synthesized by the host cell RNA polymerase II with the participation of viral factors. Viral proteins regulate sequential transcriptional cascades (immediate early-IE, early-E, and late-L genes) and a series of posttranslational modifications. Transcription of IE gene requires the tegument protein VP16 (Mackem & Roizman, 1982), which is a transcriptional activator. VP16 is also a late tegument protein that further participates in the assembly and maturation of nucleocapsid in the cytoplasm. Of six IE genes (ICP0, ICP4, ICP22, ICP27, ICP47, and US1.5), only ICP47 is not involved in activating E gene transcription. Proteins encoded by E genes are involved in the activation of viral replication machinery, regulation of nucleotide metabolism (e.g. thymidine kinase-TK UL23), activation of L genes, and also suppression of IE genes. Some viral proteins, like thymidine kinase (UL23), ribonucleotide reductase (UL39, UL40), modulate the nucleotide metabolism and are essential for the viral DNA synthesis because the expression of the corresponding host cell enzymes is downregulated in infected cells (Weller & Coen, 2012).

After start of the viral DNA replication, the expression of late L genes, especially those encoding capsid proteins, increases, leading to the assembly of new progeny virions. The capsid assembly and viral genome packaging occur in the nucleus followed by nucleocapsid egress from the nucleus by budding through the nuclear membrane. With the participation of UL31 and UL34 proteins, the capsid is transported from the nucleus to the cytoplasm where the virion maturation and outer

shell formation occurs (Arii, 2021; Johnson & Baines, 2011; Mettenleiter, 2016). The release of the virion from the cells by exocytosis accomplishes the viral reproductive cycle (Pasdeloup et al., 2013).

In lytically infected skin tissue simplexviruses gain access to sensory nerve endings, where they establish latent infection (Hafezi et al., 2012; Smith, 2012). During infection, envelope and tegument proteins are shed and only the capsids are transported to the nuclei of the sensory neurons, residing in neuronal ganglia such as the trigeminus ganglia. As a result, important lytic regulators, such as the tegument protein VP16, are not transported to the nucleus. As a consequence, a latent infection is established where cells bear a circular form of the viral genome, and RNA is transcribed mainly from one region leading to the latency associated transcripts (LATs). This LAT region is involved in maintaining the latent state (Nicoll et al., 2016). However, the viral genome retains its ability to reactivate. The mechanism of reactivation is incompletely understood, but involves cellular stress signalling, which converts the transcriptionally inactive chromatin covering the simplexvirus genome into a more active and accessible form (Cliffe et al., 2015). As an example, HSV-1 is detected only in neurons and ganglia that directly stimulate the epithelium that is prone to infection (Webre et al., 2012).

3.3.4 Biology of Simplexviruses

3.3.4.1. Human simplexviruses (HSV-1, HSV-2)

The World Health Organization (WHO) stated that 40-90% of the human population is infected by herpesviruses (Kukhanova et al., 2014; Kuny & Szpara, 2021). Among those viruses, three are members of the subfamily

Alphaherpesvirinae: HSV-1, HSV-2 and VZV, of which HSV-1 is the most prevalent and well explored (Xu et al., 2002).

Primary infection with HSV-1 is usually associated with vesicles or sores on the lips. Additionally, genital herpes, keratitis and peripheral skin lesions may appear, particularly in immunocompromised hosts (Denes et al., 2018). HSV-2 infection is normally associated with genital skin blisters (Groves, 2016; Magdaleno-Tapial et al., 2020). The outer genital lesions (thigh, finger, eye, buttocks and groin) may also occur during infection (Corey and Wald, 2008). Sores generally occur a few days after the primary infection, and reappear more or less regularly later in life in a substantial percentage of those infected. The blisters contain a large number of viral particles and are thought to play a central role in viral transmission. The symptoms may be increased by host conditions, such as the degree of the immune response. The viruses persist in nerve cells for a long period before being transferred to the mucosa via axons. The frequency and severity of the recurrent infections tend to decrease over time. Although the genital form is less likely to result in recurring blisters, the virus can still be transmitted through the mucosa (Fatahzadeh & Schwartz, 2007; Groves, 2016; Magdaleno-Tapial et al., 2020).

The most common symptom of an active infection is mucosal ulcers, although HSV-1 and HSV-2 can also cause cutaneous lesions, particularly around the nails of fingers and toes, a condition known as herpetic whitlow (Clark, 2003), and both viruses can also infect the eyes, producing keratitis which can lead to blindness (Farooq & Shukla, 2012). They may target the brain, causing encephalitis or meningitis, due to their affinity for neurons and epithelial cells (Rozenberg et al., 2011). Herpes simplex encephalitis (HSE) is caused by genetic defects, particularly in TRL3-interferon (IFN) and IFN-responsive pathways (Zhang et al., 2013). It could

be due to stressors in the host, resulting in a reduction in immunological surveillance (Fatahzadeh & Schwartz, 2007).

According to the analysis of genome sequences, HSV-1 is closely related to HSV-2 (Dolan et al., 1998; Dolan et al., 1992; McGeoch et al., 1991), with McHV-1 (Perelygina et al., 2003), PaHV-2 and CeHV-2 (Tyler et al., 2005) being more distant. This is also due to the RL1 which is only available in HSVs, but not in other simplexviruses.

3.3.4.2. *Cercopithecine alphaherpesvirus 2* (CeHV2; Simian Agent 8, SA8)

The *Cercopithecine alphaherpesvirus 2* (CeHV2, Simian agent 8, SA8) was first discovered in the brain tissue of an African green monkey and is only found in this species (Malherbe & Strickland-Cholmley, 1969). Clinical symptoms are not detected upon infection of the natural host. However, infection with CeHV-2 in baboons cause a disease that is very similar to genital herpes in humans (caused by HSV-2) (Martino et al., 1998). It is largely spread by sexual contact, and primary infection is typically asymptomatic but can occasionally be associated with oral and/or genital diseases (Levin et al., 1988; Martino et al., 1998). New-born monkeys can be infected by this virus, which causes more severe disease in females than males (Martino et al., 1998), and severe infections of new-born monkeys resembling human neonatal herpes have also been documented (Brack et al., 1985; Eichberg et al., 1976). Furthermore, infection in African green monkeys is frequently asymptomatic, and the potential for zoonotic transmission is unknown (Eichberg et al., 1976).

CeHV-2 genomic length, 150 kb, is close to HSV-1 and HSV-2 (152 kb and 155kb respectively) and all of the genes are homologous and collinear with McHV-

1 including the absence of the RL1 (ICP34.5) open reading frame which is involved in neurovirulence in HSVs (Chou et al., 1995; Chou et al., 1990; Tyler et al., 2005; Whitley et al., 1993; Whitley & Roizman, 2001). The U_L and U_S sequences are separated by a pair of long and short inverted repeats (Eberle et al., 1993). The genome is similar to that of other simplexviruses and exhibits the largest G+C content (>76%) (Tyler et al., 2005).

CeHV-2 shares 83% DNA homology with McHV-1 (Perelygina et al., 2003), 64.1% with HSV-1 (Dolan et al., 1992), and 68.8%with HSV-2 (Dolan et al., 1998). Due to the high similarities to McHV-1, CeHV-2 has been suggested as an alternative antigen for diagnosing of McHV-1 seropositivity (Malherbe & Strickland-Cholmley, 1969; Takano et al., 2001). Because CeHV-2 and PaHV-2 are closely related (Eberle et al., 1995), both viruses were originally identified as CeHV-2, leading to some confusion in early publications (Malherbe & Strickland-Cholmley, 1969).

3.3.4.3. Macaccine alphaherpesviruses (McHV)

*Macaccine alphaherpesvirus*es (McHVs) have *Macaque spp*. as their natural host. According to the phylogenetic analysis of viruses isolated from different species of macaques, they are divided into three species: *Macacine alphaherpesvirus 1* (McHV-1) isolated from rhesus macaque (*Macaca mulatta*), bonnet macaque (*M. radiata*), and japanese macaque (*M. fuscata*); *Macacine alphaherpesvirus 2* (McHV-2) isolated from lion-tailed macaque (*M. silenus*), and *Macacine alphaherpesvirus 3* (McHV-3) isolated from pig-tailed macaque (*M. nemestrina*) (Fig. 1) (ICTV, 2021). Information about zoonotic transmission and pathogenicity of these viruses is not available except for McHV-1, which is also termed herpes B virus. The transmission of McHV-1 from macaques to other non-

human primates has been documented. The majority of them, such as Debrazza's monkey (*Cercopithecus negletus*), a patas monkey (*Erythrocebus patas*), and a black and white colobus monkey (*Colobus spp*.) (Loomis et al., 1981; Thompson et al., 2000; Wilson et al., 1990), died from McHV-1 infection. In cell culture, McHV-1 exhibits a broad host tropism and can infect a large variety of cell lines (Hilliard et al., 1987; Kubicek et al., 1973).

McHV-1 virions exhibit the typical structure of an alphaherpesvirus virion, packaging 157 kb of linear genomic DNA. The entire genome of McHV-1 has been sequenced (Ohsawa et al., 2003; Ohsawa et al., 2002; Perelygina et al., 2003), and all McHV-1 genomes have a very high G+C content (>76%) and a genomic structure that is comparable to HSV-2 and CeHV-2 (Eberle et al., 2017). McHV-1 shares a significant degree of genetic similarity with HSV-1 and HSV-2 with the absence of the RL1 (ICP34.5) open reading frame (Perelygina et al., 2003; Tyler & Severini, 2006). McHV-1 usually does not cause severe disease in its natural host (Eberle & Jones-Engel, 2018; Eberle et al., 2017; Huff & Barry, 2003). However, if the virus is transmitted to humans, it can cause encephalomyelitis with a 70% case-fatality rate if not treated (Eberle & Jones-Engel, 2018).

Human McHV-1 infections are rare, according to The Center for Disease Control and Prevention (CDC). Only about 50 cases have been reported since the discovery of the virus, with 21 of the afflicted patients dying (CDC, 2019). The initial infection was typically caused by monkey bites or scratches or exposure of broken skin to body fluids from an infected monkey. After body fluid from an infected monkey splattered into her eye, a researcher died of McHV-1 infection in 1997 (CDC, 1998). As a result of the high biosafety requirements, only few studies have been conducted with McHV-1. Because of its severe neuropathogenicity, McHV-1

is classified as a biosafety level (BSL) 4 pathogen in the United Kingdom and the United States of America and as a BSL 3 pathogen in Germany. In order to avoid work in BSL3 or BSL4 laboratories, some studies used BSL 2 herpesviruses as models to investigate McHV-1. These model viruses include HSV-1, HSV-2, and CeHV-2. However, it has been reported that PaHV-2 is genetically and antigenically more closely related to McHV-1 than HSVs and CeHV-2 (Black & Eberle, 1997; Eberle et al., 1995; Ohsawa et al., 2002; Perelygina et al., 2003) and might thus be the model of choice.

3.3.4.4. *Papiine alphaherpesvirus 2* (PaHV-2; Herpesvirus papio 2, HVP-2)

Papiine alphaherpesvirus 2, (PaHV2; Herpesvirus Papio 2, HVP2) was discovered in baboons (*Papio* spp.) (Levin et al., 1988) in which it behaves similarly to McHV in macaques and HSV in humans (Elmore & Eberle, 2008). The genomic arrangement is identical to that of other simplexviruses, with inverted repeat sequences flanking each of the distinct long and short segments (U_L and U_S respectively) (Tyler & Severini, 2006). PaHV-2 is genetically similar to McHV-1 and also HSVs (Elmore & Eberle, 2008; Engel et al., 2002; Focher et al., 2007; Tyler & Severini, 2006) but lacks the RL1 (ICP34.5) open reading frame (Tyler et al., 2005; Tyler & Severini, 2006). PaHV-2 and McHV-1 are antigenically so similar that PaHV-2 has been utilized as a substitute antigen for McHV-1 serology diagnostics (Ohsawa et al., 1999; S. Tanaka et al., 2004; Yamamoto et al., 2005).

In a murine model, researchers discovered two groups of PaHV-2 strains with different pathogenicity, one apathogenic and the other neurovirulent (Rogers et al., 2003; Rogers et al., 2006) The difference in pathogenicity between both groups was linked to the UL39 gene (encoding ribonucleotide reductase large subunit) which is indispensable for PaHV2 neuropathogenesis in mice. The UL39 gene also plays an

important role in the viral replication in non-dividing cells, such as neurons. It is also associated with anti-apoptotic and anti-necroptotic responses, as well as interferon response regulation. (Black et al., 2014). Several PaHV-2 isolates are highly neurovirulent in mice, and their pathophysiology closely resembles that of McHV-1 in mice (Ritchey et al., 2005; Rogers et al., 2006). However, PaHV-2 infection of humans has never been detected, despite the virus's resemblance to McHV-1. As a result, PaHV-2 is classified as a BSL 2 pathogen.

3.4. Recombinant systems

The information generated from the genomic sequences will be of enormous use if it can be converted into functional data, which will require dissection and modification of the viral genome using genetic recombinant technology. Genetic recombination is a process by which a molecule of nucleic acid is broken and then joined to a different DNA molecule. There are two popular methods for mutagenizing DNA, random transposon and site-directed mutagenesis. Site-directed mutagenesis is an *in vitro* method of introducing a mutation into ds-DNA plasmid using specially designed oligonucleotide primers (Kunkel, 1985). Transposon mutagenesis, on the other hand, produces a wide range of mutants. Since the mutagenesis is random, determining the mutation site requires sequencing or PCR analysis.

Recombinant systems for viruses allow the easy introduction of changes into the viral genome before rescuing the virus. Herpesviruses, particularly simplexviruses, have been studied using a variety of approaches. First, virus can be generated from a group of overlapping cosmids, as shown for HSV-1 (Cunningham & Davison, 1993). More recently fosmids, cosmids with a low-copy F-factor, have been employed for PRV, MDV and CeHV-2 (Chukhno et al., 2019; Li et al., 2016; Zhou et al., 2018). Second, herpesviral genomes have been cloned and modified

using bacterial artificial chromosomes (BACs) (Meseda et al., 2004; Messerle et al., 1997; Tanaka et al., 2003). Finally, the genomes of herpesviruses and other viruses have also been cloned and assembled via transformation-associated recombination in yeast (Oldfield et al., 2017; Thi Nhu Thao et al., 2020; Vashee et al., 2017). An advantage of all these recombinant methods is that they do not require multiple rounds of plaque purification, because all reconstituted viruses are recombinant (contain the gene modification), allowing these techniques to be widely used particularly in herpesvirus research.

3.4.1. Cosmids and Fosmids

Cosmids are plasmids with bacteriophage λ DNA cohesive sites (cos sites) (Collins & Hohn, 1978; Cronan, 2003). A cosmid vector mainly contains an origin of replication (ori), a selection marker such as an antibiotic resistance gene, a cos site, and multiple cloning sites (MCS) for inserting up to 40 kb foreign DNA fragment (Fig.5). Since the herpesvirus genomic DNA is large, a series of overlapping cosmid clones is required to cover the entire viral genome (Liao et al., 2021). Therefore in 1988, recombinant PRV (pseudo rabies virus) mutants were successfully produced utilizing five cosmid clones containing overlapping DNA fragments of the PRV genome after cotransfection into permissive cells and and subsequent recombination (Cunningham & Davison, 1993).

To apply cosmid cloning, the herpesvirus genome was partially digested with different restriction enzymes to produce overlapping viral DNA fragments that were then cloned into cosmid vectors. To construct the modified/recombinant viral cosmid, the gene of interest was deleted, mutated, or inserted. To reassemble the mutant virus, restriction enzyme digestion was used to release all viral DNA fragments from the cosmids, which were then co-transfected into permissive cells

to generate the recombinant viruses. Since originally introduced, this technique has been utilized to generate a significant number of mutants of HSV-1, EBV, VZV, equine herpesvirus (EHV), MDV and herpesvirus turkey (HVT) to examine gene function (Cohen & Seidel, 1993; Cunningham & Davison, 1993; Lindenmaier & Bauer, 1994; Nicolson et al., 1994; Reddy et al., 2002; Reilly & Silva, 1993; Tomkinson et al., 1993).

Cosmid clones are occasionally unstable (Horsburgh et al., 1999) and may lose the inserted DNA which could indicate sequences that are harmful to *E. coli*, particularly at high copy numbers (Redenbach et al., 1996). Another limitation of this method is the handling of large DNA fragments, which may result in unwanted mutations and genome rearrangements in the resulting recombinant viruses. As a consequence, multiple recombination events are required to reconstitute the full-length viral genome in cells. Additionally, due to the nature of the approach, it is difficult to generate revertant viruses, which are crucial to rule out the possibility that the altered phenotype is attributable to other undesired mutations. The use of entire genome sequencing, on the other hand, could eliminate the necessity for the generation of revertant viral (Liao et al., 2021).

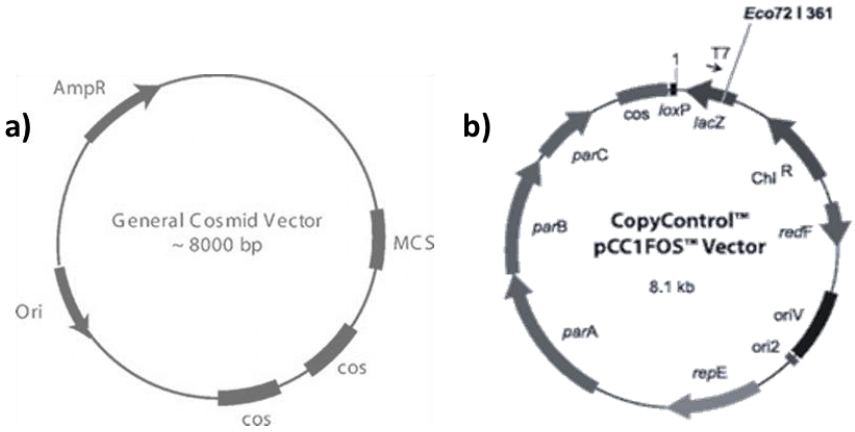

Figure 5. (a) The general scheme of a cosmid vector and (b) copy control pCC1FOS vector which commonly used to generate fosmids library [taken from (DiLella & Woo, 1987; Wild et al., 2002)

Fosmids are cosmids which have a low copy F factor origin. Fosmids were utilized to generate stable libraries from complex genomes, since they were reported to have high structural stability and to effectively conserve human DNA even after 100 generations of bacterial growth (Magrini et al., 2004; L. Zhang et al., 2007). A fosmid library is constructed by extracting the target organism's genomic DNA, creating randomly sheared genomic DNA fragments and cloning them into fosmid vectors (Moon & Magor, 2004). As a result, generating infectious clones of large DNA viruses, such as herpesviruses, based on a fosmid library will allow for more efficient viral genome editing with a high level of biosafety, as only parts of the genome are handled at a time (Chukhno et al., 2019; Zhou et al., 2018).

Fosmids containing viral genome segments should be stable due to the low copy number in bacteria but suffer from low DNA yields. To combine single-copy cloning with the advantages of high DNA yields, vectors were constructed containing a high-copy oriV which leads to amplification in an *E. coli* host strain expressing *trfA* under the control of araC-P_{ABD} regulatory region (Cunningham & Davison, 1993; Kim et al., 2003; Zhou et al., 2018). Accordingly, in the absence of inducer, clones can be kept in single copy for stability, or induced to high copy by adding *L*-arabinose to the growth medium to switch on the *trfA* expression, which subsequently activates oriV, resulting in up to 100 plasmid copies per cell (Martinez et al., 2007; Martinez et al., 2010).

Fosmid-based cloning has been applied to generate recombinant virus, particularly herpesvirus such as MDV, PRV, and CeHV-2, by introducing the overlapping fosmids clones to the permissive cells (Fig. 6) (Chukhno et al., 2019; Cui et al., 2005; Cui et al., 2004; Li et al., 2016; Liu et al., 2016; Zhou et al., 2018).

In addition to thistechnology being straighforward, the overlapping fosmid method avoids the use of eukaryotic selection markers that may interfere with virus propagation (Tischer et al., 2007).

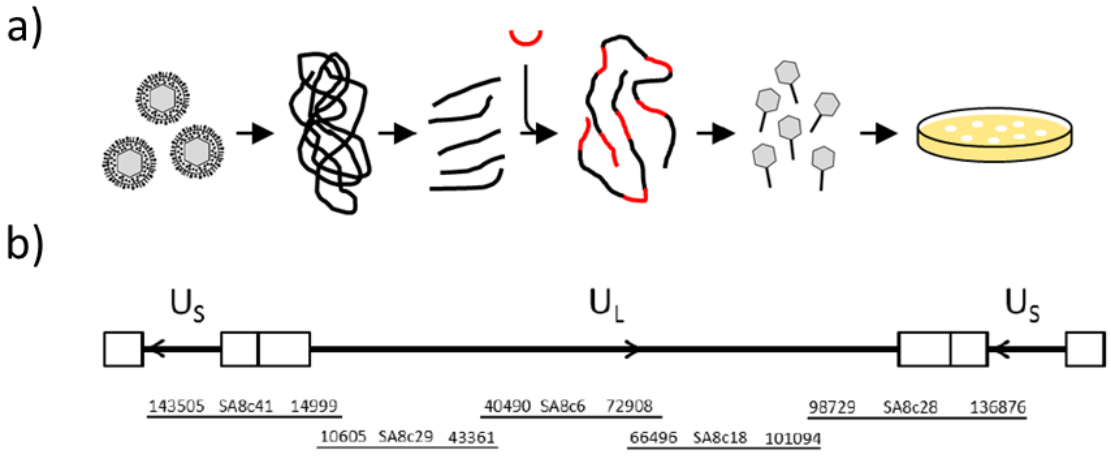

Figure 6. Cloning and characterization of a fosmid library. (a) Scheme of herpesvirus genome cloning. Viral DNA was isolated from virus particles, fragmented by shearing, end-repaired and size fractionated on an agarose gel. Fractionated fragments were ligated into pCC1FOS (red) fosmid vector and packaged into phage lambda particles, which were subsequently utilized to transduce cells of E. coli strain EPI300. Colony PCR, end-sequencing, and restriction digest test were used to characterize each coloniy. (**b**) An illustration of fosmid map in which all characterized fosmid clones are located based on their position (Chukhno et al., 2019)

Taking advantage of the fosmid-based approaches, herpesviruses carrying reporter genes have been generated (Chukhno et al., 2019; Rahman Siregar et al., 2022). Reporter genes were inserted into fosmid clones via recombineering which relies on homologous recombination in bacteria expressing λ red recombinase (Weigler, 1992; Yu et al., 2000). For this, fosmids were introduced into E. coli GS1783 (Tischer et al., 2010), which expressed phage recombinases after heat induction and I-SceI under arabinose control (Tischer et al., 2006).

3.4.2. Bacterial Artificial Chromosomes (BACs)

Bacterial artificial chromosomes (BACs) are plasmid vectors based on the fertility (F-) factor that replicate consistently in low copy numbers (Hosoda et al., 1990; Shizuya et al., 1992). BACs are a potent technology for carrying the entire genomes or genes with flanking regulatory DNA to provide all signals for correct spatiotemporal gene expression due to their huge insert capacity (Antoch et al., 1997; Jessen et al., 1998; Valjent et al., 2009). Thus, BACs containing full-length genes in their original chromosomal arrangement are becoming a popular tool for investigating genome structure and function, and they offer an interesting alternative to conventional vector systems (Nagel et al., 2014).

Cloning whole viral genomes into BACs has substantially aided mutagenesis of herpesviruses due to the large size of their genomic DNA. Because their copy number is controlled at only 1-2 copies per bacterial cell, they reliably maintain large fragments of DNA (Borst et al., 2007; Kelley et al., 1999; Messerle et al., 1997; Smith & Enquist, 1999). Because of their low copy number and relative ease and precision in creating mutants, BACs are particularly beneficial for several herpesviruses. Low copy number reduces the likelihood of illegitimate inter- and intramolecular recombination between repetitive sequences found throughout the herpesviral genomes. It also allows fast and well-established mutagenesis in *E. coli*, such as RecA-mediated homologous recombination, Red recombination, and transposon-based mutagenesis (Adler et al., 2003; Brune et al., 2000; Ruzsics & Koszinowski, 2008; Wagner & Koszinowski, 2004). Overall, viral BACs in *E. coli* can be more stable than natural or conventional viral mutants (Warden et al., 2011).

Viral BACs are constructed by introducing a BAC cassette into a viral genome. A typical BAC cassette contains an origin of replication (e.g., *oriS*), genes

required for BAC replication (such as *repE*), and genes that control the partition of plasmids to daughter cells (such as *sopA* and *sopB*). In order to select only bacterial colonies bearing the BAC herpesvirus genome of interest, an antibiotic resistance marker (such as chloramphenicol) is included within the BAC cassette. The BAC cassette needs to be flanked by 500–1000 homologous base pairs to the target sequence into which it will be inserted. Finally, a BAC cassette should additionally carry a selectable marker for eukaryotic cells (such as GFP, beta-galactosidase, antibiotic resistance genes, or metabolic genes) in order to select BAC-containing recombinant virus in eukaryotic cells. Importantly, two loxP sites are frequently added at both ends of the BAC sequence allowing the BAC cassette to be removed from recombinant viruses.

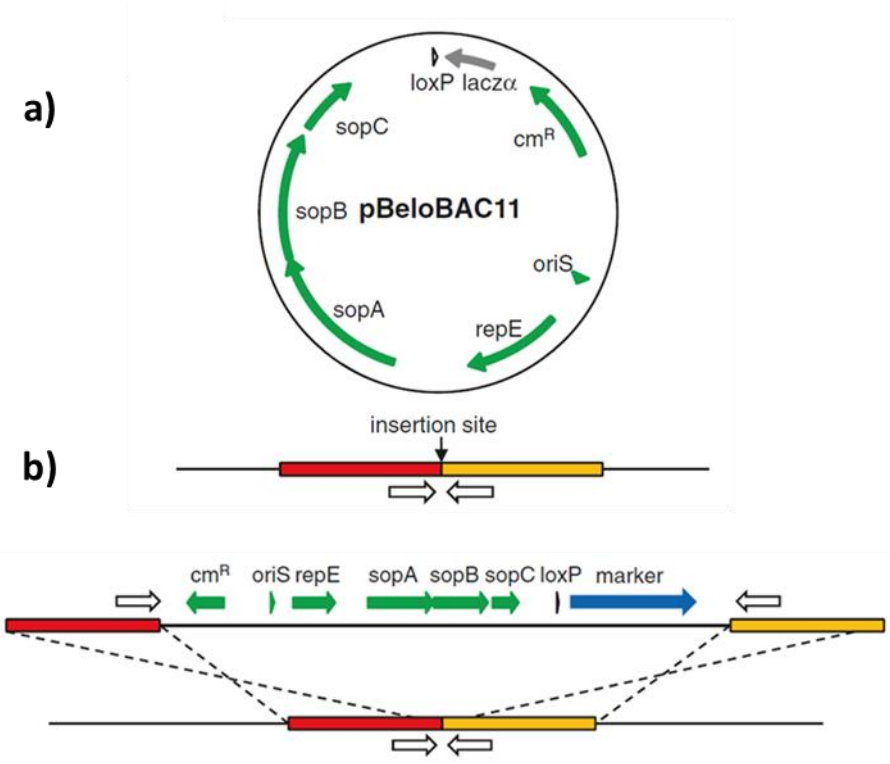

Figure 7. (a) Schematic representation of the bacterial artificial chromosome (BAC) cloning vector pBeloBAC11 and the potential insertion site on the targeted genome that is flanked by homology arms of about 2 kb (red and orange bars). The two white arrows exemplify two viral ORFs. (b) After linearization, the BAC vector inserts to the targeted genome by homologous recombination in permissive eukaryotic cells [Taken from (Nagel et al., 2014)].

One typical strategy for generating herpesvirus BACs is to employ homologous recombination to insert a BAC cassette into a specified region of the viral genome (Borenstein & Frenkel, 2009; Delecluse et al., 1998; Delecluse et al., 2001; Horsburgh et al., 1999; Messerle et al., 1997; Nagaike et al., 2004). The BAC cassette with flanked viral genomic sequences is linearized using restriction enzymes and cotransfected into viral permissive cells with purified viral genomic DNA. A ten kilobase BAC vector can be introduced into a non-essential region of a viral genome which has been deleted to accommodate the BAC cassette, therefore severe growth abnormalities on virus can be avoided (Wagner et al., 1999; Wussow et al., 2009).

Homologous recombination occurs in the cells, and a recombinant virus carrying a BAC vector is generated (Fig. 8a). The presence of a trait defined by a selectable marker is used for plaque purification. If the BAC cassette has a GFP expression cassette as a selectable marker, for instance, recombinant virus infection will result in green fluorescent plaques. The viral DNA containing the BAC cassette is extracted from infected cells and electroporated into an *E. coli* strain, such as DH10B. The fact that the herpesvirus genome circularizes during replication makes this step possible, despite the fact that herpesvirus DNA is large and difficult to transform into *E. coli*. The antibiotic resistance marker (e.g. chloramphenicol, as described above) present in the BAC cassette is used to select bacterial cells bearing viral BACs. If the viral BAC is stably maintained and can be replicated in *E. coli*, antibiotic-resistant colonies will be obtained. The viral BAC DNA is purified from *E. coli,* and, initially, restriction enzyme digestion and, sometimes, partial sequencing analyses were performed to confirm that the BAC harbouring virus is

free of major mutations (deletions) in the BAC DNA (Z. Zhang et al., 2007). Today BAC integrity can also be confirmed by next-generation sequencing.

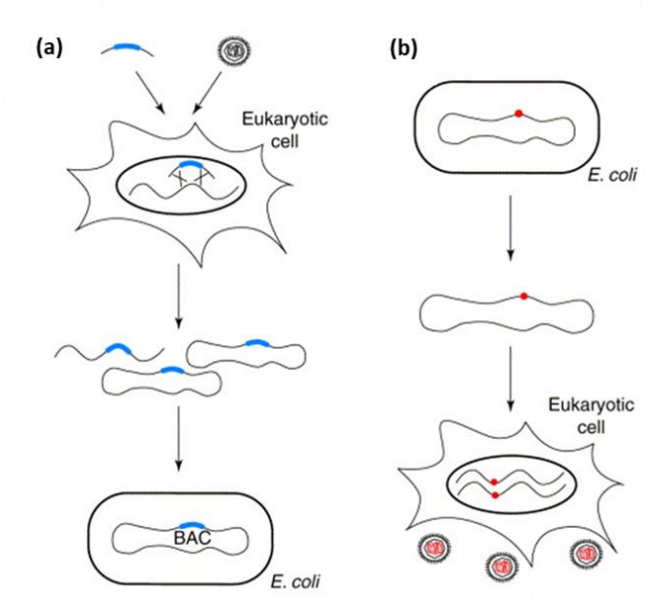

Figure 8. (a) Cloning a viral genome as a bacterial artificial chromosome (BAC). A mutant virus containing the BAC cassette (blue) is produced by homologous recombination in infected cells. Circular replication intermediates of the viral genome are isolated and transformed into *E. coli* to obtain a virus BAC. (b) BAC technology. The BAC-cloned viral genome can be engineered in *E. coli* to introduce a mutation (red). Transfection of a BAC containing the mutated viral genome leads directly to mutant viral progeny [Taken from (Brune et al., 2000)].

After confirmation of the integrity of the viral BAC, large amounts of the BAC-DNA can be isolated from bacterial cells and used for transfection into mammalian cells (Fig 8b). The development of a BAC system has limitations, despite its advantages in producing recombinants more efficiently than homologous recombination. Due to homologous recombination and many rounds of plaque purification, generating a recombinant BAC construct is time-consuming and labour-intensive (Gu et al., 2015; Guo et al., 2016).

3.4.3. Transformation Associated Recombination

The Transformation Associated Recombination (TAR) is a one-of-a-kind approach for isolating large fragments (up to roughly 300 kb) or complete genes from complex genomes in *Saccharomyces cerevisiae* as circular Yeast Artificial Chromosomes (YACs). This method has been used to clone big fragments of genes or gene clusters, as well as to characterize genome variants, mutational analysis of genes and filling the gaps between contigs in the genome (Kouprina et al., 2016).

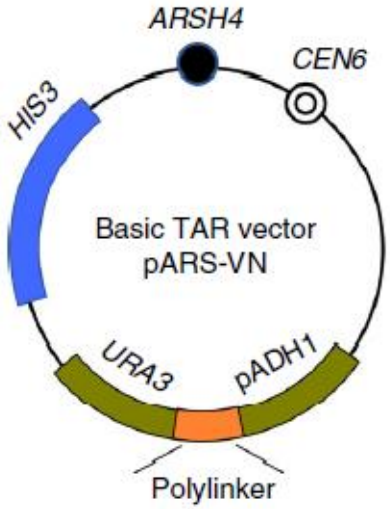

Figure 9. The scheme of the basic TAR vector pARS-VN. The vector contains ARS as an origin replication in yeast, CEN6 as a yeast centromere of two region-of-interest-specific targeting hooks, HIS3 and URA3 as a positive and negative selectable marker, respectively [Taken from (Kouprina et al., 2020)].

The original TAR cloning approach requires the presence of at least one autonomously replicating sequence (ARS) that can serve as the origin of replication in yeast in the cloned genomic DNA fragment (Theis & Newlon, 1997). Most mammalian genes can be identified using TAR cloning, since these sequences are abundant in mammalian DNA, with around one ARS-like region per 20–30 kb (Stinchcomb et al., 1980). The ARS frequency may be reduced in chromosomal areas with several repetitive elements (such as the centromere and telomere), GC-

rich regions with few ARS-like sequences, and relatively simple genomes, preventing their identification by the standard method.

The TAR vector also contains two region-of-interest-specific targeting hooks, a yeast centromere (*CEN6*), and yeast positive and negative selectable markers, *HIS3* and *URA3*, respectively (Fig 9). Negative genetic selection removes the background produced by vector recircularization during yeast transformation, which is caused by end-joining. URA3 is a hybrid gene containing the open reading frame of the *Saccharomyces cerevisiae URA3* gene and the promoter of the *Schizosaccharomyces pombe* ADH1 gene, which has specific spacing requirements for its function; the distance between the TATA element and the transcription initiation site must be less than 130 bp (Furter-Graves & Hall, 1990; Miret et al., 1998), as a greater distance causes transcription to initiate at an alternative site, inactivating URA3 expression. The specific spacing requirements allow selection against the recircularized vector. Hooks as small as 60 bp are possible (Noskov et al., 2001). The targeting hooks are inserted between the promoter and the open reading frame of the *URA3* gene in the TAR vector.

Figure 10 depicts a general protocol for TAR cloning of a single copy gene from the whole genomic DNA. A specific endonuclease positioned between the hooks linearizes the TAR vector DNA, exposing the targeted regions. Before performing yeast transformation, genomic DNA can be processed with restriction enzymes or CRISPR/Cas9 endonuclease, if necessary, considerably increasing the yield of gene/region-positive TAR clones. When genomic DNA and linearized TAR vector are cotransformed into yeast cells, recombination between targeting sequences in the vector and targeted sequences in the genomic DNA fragment results in the establishment of the DNA fragment (or gene) as a circular

TAR/YAC/BAC molecule. TAR-isolated molecules containing a region of interest are subsequently transferred from yeast cells to bacterial cells by electroporation. The isolation of DNA from bacterial clones usually gives higher yields of the DNA, which will be used for further sequencing or functional analysis (Kouprina & Larionov, 2016). It is worth mentioning that even in the presence of an ARS-containing vector, cloning of large GC-rich bacterial DNA fragments can be problematic, and in this circumstance, only fragments of about 100kb can be recovered (Noskov et al., 2012).

TAR cloning has been used to assembled into a complete herpes simplex virus type 1 genome (Oldfield et al., 2017) and has recently been modified to directly clone a large HCMV genome (Vashee et al., 2017). This cloning strategy allows for easier genetic manipulation of primary isolates and opens the door to the development of new vaccines and vaccine vectors. In addition, TAR cloning can also be developed to generate synthetic viruses with unique features (Kouprina & Larionov, 2016).

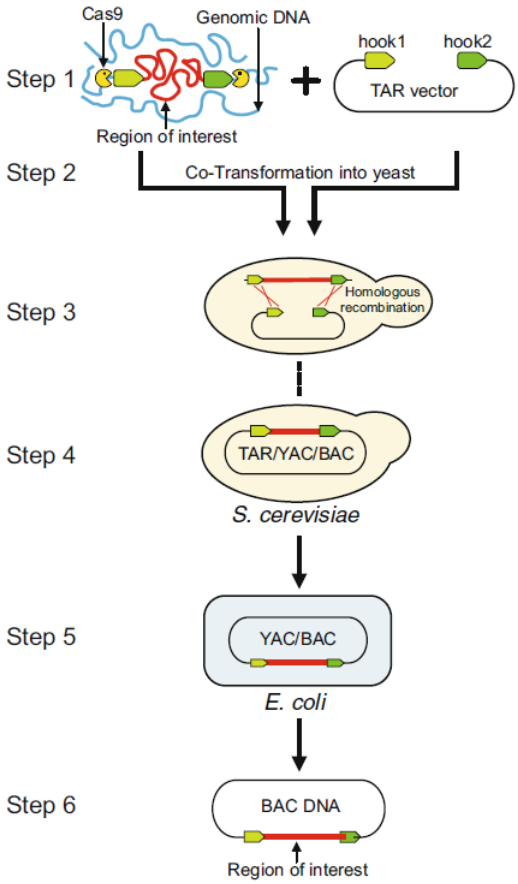

Figure 10. The TAR-based cloning protocols. 1st step: To clone a gene of interest from whole genomic DNA, a TAR vector with YAC or BAC cassettes, as well as two unique targeting sequences (hook1 and hook2 in green) corresponding to the 5′ and 3′ ends of a gene of interest, is utilized. It is possible to use hooks as small as 60 bp. One or both hook sequences could be unique, or common repeats. (for example, an Alu repeat for cloning from human genomic DNA). The TAR vector DNA is linearized by a particular endonuclease positioned between the hooks, exposing the targeted regions. Genomic DNA can be treated with CRISPR/Cas9 endonuclease before yeast transformation if necessary, dramatically increasing the yield of gene/region-positive TAR clones. Step 2: Genomic DNA and a linearized TAR vector are cotransformed into yeast Saccharomyces cerevisiae cells. 3rd and 4th steps: The fragment (or gene) is rescued as a circular TAR/YAC/BAC molecule after recombination between the vector and the genomic DNA fragment. Step 5: Electroporation of TAR-isolated molecules containing a region of interest from yeast to bacterial cells. Step 6: To isolate BAC DNA for further sequencing or functional studies, a traditional approach could be applied. [Taken from (Kouprina & Larionov, 2016)].

Despite being a relatively reliable and reproducible method, TAR cloning has limitations. To perform recombination, the hooks region of the TAR cloning vector must have at least 85% homology to the targeted sequence. Another issue is that if a target region contains repetitive homologous sequences, undesired recombinant products may emerge. To tackle this concern, contour-clamped homogenous electric field (CHEF) gel electrophoresis with sequence analysis can be applied to confirm the integrity of the recombinant target DNA. Because TAR cloning may capture larger DNA fragments, high-molecular-weight genomic DNA preparation is critical for cloning long genomic pieces. As a result, an optimized DNA preparation and its transfer to spheroplast yeast is vital to improving the integrity of large genomic fragments (Kouprina et al., 2020).

4. Aims

Macacine alphaherpesvirus 1 (McHV-1) but not the closely related primate simplexviruses Cercopithecine alphaherpesvirus 2 (CeHV-2) and Papiine alphaherpesvirus 2 (PaHV-2) is highly pathogenic in humans. The reason for this high pathogenicity is unknown. To identify determinants of McHV-1 pathogenicity on the molecular level, systems for the generation of recombinant primate simplexviruses are required.

The aim of this thesis was to establish a recombinant system for PaHV-2. For this, strategies were to be identified which allowed cloning of the entire PaHV-2 genome. Further, recombinant PaHV-2 was to be characterized for cell tropism, replication efficiency and susceptibility to antivirals and neutralizing antibodies. In order to facilitate these analyses, reporter genes were to be introduced into the viral genome using recombineering.

5. Publications

A fosmid-based system for the generation of recombinant *Cercopithecine alphaherpesvirus 2* encoding reporter genes.

(Viruses. November 2019)

https://doi.org/10.3390/v11111026

A recombinant system and viruses encoding reporter genes for *Papiine alphaherpesvirus 2*.

(Viruses, January 2022)

https://doi.org/10.3390/v14010091

5.1. First publication

Ekaterina Chukhno [1], Sabine Gärtner [1], **Abdul Rahman Siregar** [1,2], Alexander Mehr[1], MarieWende [1], Stoyan Petkov [3,4], Jasper Götting [5], Akshay Dhingra [5], Thomas Schulz [5], Stefan Pöhlmann [1,6] and Michael Winkler [1]*

A fosmid-based system for the generation of recombinant *Cercopithecine alphaherpesvirus 2* encoding reporter genes

(Viruses. November 2019)

https://doi.org/10.3390/v11111026

Individual contribution:

In the following paper, I conducted experiments for figure 4C: “Multicycle replication kinetics on cell lines derived from different species” showing strongly reduced replication in macaque cells.

viruses

MDPI

Article

A Fosmid-Based System for the Generation of Recombinant Cercopithecine Alphaherpesvirus 2 Encoding Reporter Genes

Ekaterina Chukhno [1], Sabine Gärtner [1], Abdul Rahman Siregar [1,2], Alexander Mehr [1], Marie Wende [1], Stoyan Petkov [3,4], Jasper Götting [5], Akshay Dhingra [5], Thomas Schulz [5], Stefan Pöhlmann [1,6] and Michael Winkler [1,*]

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Abstract: The transmission of Macacine alphaherpesvirus 1 (McHV-1) from macaques, the natural host, to humans causes encephalitis. In contrast, human infection with Cercopithecine alphaherpesvirus 2 (CeHV-2), a closely related alphaherpesvirus from African vervet monkeys and baboons, has not been reported and it is believed that CeHV-2 is apathogenic in humans. The reasons for the differential neurovirulence of McHV-1 and CeHV-2 have not been explored on a molecular level, in part due to the absence of systems for the production of recombinant viruses. Here, we report the generation of a fosmid-based system for rescue of recombinant CeHV-2. Moreover, we show that, in this system, recombineering can be used to equip CeHV-2 with reporter genes. The recombinant CeHV-2 viruses replicated with the same efficiency as uncloned, wt virus and allowed the identification of cell lines that are highly susceptible to CeHV-2 infection. Collectively, we report a system that allows rescue and genetic modification of CeHV-2 and likely other alphaherpesviruses. This system should aid future analysis of CeHV-2 biology.

Keywords: Cercopithecine alphaherpesvirus 2; fosmid; recombineering

1. Introduction

Herpesviruses are a large family of DNA viruses that usually establish a lifelong latency or persistence in their hosts. Herpes simplex virus 1 (HSV-1, human alphaherpesvirus 1) and other members of the genus *Simplexvirus*, subfamily *Alphaherpesvirinae*, establish latency in sensory neurons and have a conserved genome structure. Primate simplexviruses coevolved with their host species, resulting in codivergent trees for the viruses and their respective host species [1,2]. Despite this coevolution, these viruses are not species-specific, and cross-species transmission has been frequently observed. The transmission of Macacine alphaherpesvirus 1 (McHV-1, also termed herpes B virus)

from macaques to humans causes encephalitis that is associated with a high case fatality rate in the absence of treatment [3]. In contrast, HSV-1 rarely causes encephalitis and the McHV-1-related viruses Cercopithecine alphaherpesvirus 2 (CeHV-2, also termed simian agent 8) and Papiine alphaherpesvirus 2 (PaHV-2, herpes virus papio 2) are believed to be non-pathogenic in humans. However, the molecular determinants of neurovirulence of these primate simplexviruses are largely unknown [4].

CeHV-2 was identified in African vervet monkeys in 1958 [5] and was subsequently found to also infect baboons [6]. Natural infection of these animals is usually asymptomatic. Although, oral and/or genital lesions can be associated with infection [7]. In contrast, experimental infection of vervets (intraspinally) and rabbits (intradermally) was associated with paralysis, and encephalomyelitis, respectively [6]. The genomic sequence of CeHV-2 has been determined [8] and the virus was found to efficiently replicate in a panel of cell lines of different species, including cell lines of human origin, with only dog-derived MDCK cells being largely resistant to infection [9]. The presumed lack of neurovirulence of CeHV-2 in humans suggested that CeHV-2 and McHV-2 might differentially interact with human neuronal cells, or differentially evade control by human immune responses. Investigating these scenarios requires the establishment of experimental systems that allow genetic modification of CeHV-2 and McHV-1.

Two types of E. coli-based recombinant systems for the production of herpesviruses have been described: Cosmids and bacterial artificial chromosomes (BACs). The rescue of herpesviruses from cosmids has been first described for HSV-1 [10] and subsequently for other herpes viruses. However, the introduction of targeted mutations in cosmids is challenging due to their large size [11,12]. Cloning and rescue of large DNA virus genomes into BACs was first reported for baculovirus [13] and mouse cytomegalovirus [14], and was subsequently also applied to HSV-1 [15–17]. Moreover, the development of recombination-based methods using phage recombinases (recombineering) allows the modification of large viral genomes in E. coli [18–20]. Here, we report cloning of the fragmented CeHV-2 genome into fosmids [21]. This approach has been employed previously for avian herpesviruses and pseudorabies virus [22–25] and has allowed us to rescue infectious viruses and introduce reporter genes into the viral genome via recombineering. Infection by these recombinant viruses could be readily quantified based on reporter gene expression. The viruses exhibited a broad cell tropism, but were unable to efficiently infect macaque cell lines.

2. Materials and Methods

2.1. Plasmids and Oligonucleotides

Plasmids for the insertion of the reporter genes into the viral genome, by en passant mutagenesis, contained positive (kanamycin resistance) and negative (I-SceI site) selection markers that were inserted into the reporter gene. These were flanked by a 50 bp duplication of sequences next to the insertion site, as outlined by [18,19]. pcDNA3-EGFP-en was derived by stepwise insertion of EGFP-N-terminus, I-SceI site/kanamycin resistance marker and EGFP-C-terminus into pcDNA3. pmCherry-EP was derived by inserting a PstI fragment from pEP-mRFP1-in containing I-SceI site/kanamycin resistance marker [19] into pRSET-B-mCherry [26]. pcDNA3-iRFP670-en was generated by replacing N- and C-termini of EGFP in pcDNA3-EGFP-en with sequences of iRFP670 amplified from MXS_iRFP670 [27]. To generate pHW2000GG-seg8-A/PR/8/34-M2A-Gluc-en, a fragment containing I-SceI site/kanamycin resistance marker was amplified by PCR, where one primer provided the Gluc sequence duplication, and inserted into the NruI site of the Gluc reporter gene. In a second step, this modified Gluc gene was inserted into pHW2000GG-seg8-A/PR/8/34-M2A-EGFP to generate a P2A-Gluc fusion.

Oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following oligonucleotides were used for recombineering: ep-SA8-ICP4-Gluc-3: 5′-GGTCGCGGGCGGGGG TCGCGGGCGGGGGTCGCGGGCGGCGGCGCCGGCTAGTCACCACCGGCCCCCTTGATCTT-3′; ep-SA8-ICP4-2A-5: 5′-CGACCCCGGACTGGGACCCGGACGGCGAGCCGGCGGCCGCCGAGGACT GGGGGTCCGGCGGAGCCACAAATTTCTCCCTCCT-3′; ep-SA8-UL10C-R670-5: 5′-ACCACCCCCG

GAGGGTCGAGGAGCCCATCTACGAGACCGTGGGCGAGTGGGGGTCCGGCATGGCGCGTAAGG TCGATCTC-3′; ep-SA8-UL10C-R670-3: 5′-CGTCGCGTTAAGAGGCAGTTTGGTTTTTATTGATCG AGGGGCGCGAGTCAGCGTTGGTGGTGGGCGGCGGT-3′; ep-SA8-UL35-EGFP-5: 5′-CCTACCTG CCGTTTCTGGCCGCCCCCGCCGCCCAGCCCGCGCGCAGCCCCGGAGGGTCCGGCATGGTGAGC AAGGGCGAGGA-3′; ep-SA8-UL35-EGFP-3: 5′-ACAGAGGGGGAGGGGGCTGCGGGGCGGGGCG CGCCCGGGAACCGCGCCTACTTGTACAGCTCGTCCATGC-3′; ep-SA8-ICP4-EGFP-5: 5′-CGACCC CGGACTGGGACCCGGACGGCGAGCCGGCGGCCGCCGAGGACTGGGGGTCCGGCATGGTGAG CAAGGGCGAGGAGCTG-3′; ep-SA8-ICP4-EGFP-3: 5′-GGTCGCGGGCGGGGGTCGCGGGCGG GGGTCGCGGGCGGCGGCGCCGGCTACTTGTACAGCTCGTCCATGCCGAG-3′. The following oligonucleotides were used for control of recombineering products: ep-SA8-UL35C-kon5: 5′-CTG GCGCTGCAGCCGATGTT-3′; ep-SA8-UL35C-kon3: 5′-GTCCCACCCCTCAGGCAACA-3′; ep-SA8-UL10C-kon5: 5′-GGGGAGCCCATCTACGACGA-3′; ep-SA8-UL10C-kon3: 5′-CGGGGGG GAAACTCACACGA-3′; ep-SA8-ICP4-kon5: 5′-CTGCTCGCCGACGCCGAGGA-3′; ep-SA8-ICP4 -kon3: 5′-CGACACACGATCTGCGAGCTT-3′.

2.2. Cell Culture

Vero76, Cos7 (both African green monkey, kidney), cjFF2 (common marmoset *Callithrix jacchus*, fibrobast), A549 (human, lung), LLC-MK2, MaMuK8639 (both rhesus macaque *Macaca mulatta*, kidney, kidney), sMAGI (rhesus macaque *Macaca mulatta*, mammary tumor), TeloRF (rhesus macaque *Macaca mulatta*, fibroblast), and LR-7 cells (mouse) were cultivated in DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin.

The cjFF2 cells were derived using marmoset (*Callithrix jacchus*) foetal material (pregnancy day 70) leftovers from other unrelated, fully approved studies (LAVES license numbers 42502-04-16/2129 and 42502-04-16/2130). Tissue fragments from the dorsal wall and limb buds were digested with a mixture of 1:1 (*v:v*) Accutase (Gibco, Carlsbad, CA, USA) and Collagenase IV (Worthington Biochemical Corporation) (2 mg/mL) at 37 °C for 15 min, disaggregated by pipetting, and cultured in gelatin-coated culture dishes with M15 culture medium (Dulbecco's DMEM containing GlutaMAX (Gibco, Carlsbad, CA, USA) supplemented with 15% FBS (Gibco, Carlsbad, CA, USA), non-essential amino acids (NEAA (Gibco, Carlsbad, CA, USA), and penicillin/streptomycin (Gibco, Carlsbad, CA, USA). The cultures were further split at 1:5–1:6 ratio by disaggregation with Accutase when the cells reached approximately 80% confluence.

2.3. Virus

CeHV-2 was a kind gift by David Brown and Matthew Jones, Public Health England. Sequencing confirmed its identity with strain B264 [8,28]. The virus was propagated on Vero76 cells by infection at MOI 0.01 and harvested once complete cytopathic effect had developed.

2.4. Fosmid Cloning of CeHV-2 DNA

For cloning of CeHV-2 DNA into fosmid vector DNA was prepared from virus particles as follows. CeHV-2 virions from up to 18 mL cell culture supernatant (cells infected for 2–3 days) were concentrated by ultracentrifugation (Thermo WX Ultra 80; Surespin rotor, 28.000 rpm, 70 min). The virion pellet was resuspended in 100 µL PCR lysis buffer (50 mM KCl, 10 mM Tris pH8.3, 1.5 mM $MgCl_2$, 0.001% gelatine, 0.5% triton X-100, 35 µg/mL Proteinase K) and incubated for 1 h at 56 °C. Then the DNA was sheared 5 times through a 27G needle and precipitated after addition of 40 µL 5 M NaCl and 125 µL of isopropanol. After washing twice in 70% ethanol the DNA pellet was dried, dissolved in 10 µL H_2O. Afterwards the DNA was processed for fosmid cloning as recommended by the manufacturer (CopyControl Fosmid Library Production Kit; Lucigen, Middleton, WI, USA). Briefly, the DNA was treated with an End-Repair Enzyme Mix and then separated on a 0.8% low melting point agarose using control fosmid and DNA size marker (GeneRuler High Range DNA Ladder, Thermo Fisher, Waltham, MA, USA) in the neighboring lanes as reference. After staining the reference lanes with

ethidium bromide, a gel slice encompassing about 20–40 kb fragments of the CeHV-2 DNA was cut out, avoiding exposure of this DNA to ethidium bromide or UV light. The gel slice was melted at 70 °C for 15 min and incubated overnight at 45 °C in the presence of GELase. After precipitation of the DNA, as recommended by the manufacturer, the DNA pellet was dissolved in water and 250 ng was used for ligation to 500 ng Eco72I-linearized pCC1FOS vector. The ligation mixture was then packaged into lambda particles using MaxPlax Packaging Extracts, which were used for transduction of E. coli EPI300 cells, according to the protocols supplied by the manufacturer.

2.5. Characterization of Fosmids

Colonies containing fosmids were first screened with PCR primers positioned every 15 kbp of the CeHV-2 genome (NC_006560) [8], to gain information about the part of genome present. The start and end of the fragment was determined by sequencing for selected clones (Figure 1b) and the integrity of the insert was confirmed by restriction digest. A set of five clones with overlapping ends that were able to successfully rescue the virus was finally analyzed by next generation sequencing.

2.6. Rescue of CeHV-2

For virus rescue, Vero 76 cells were seeded in 12 well plates at 10^5 cells per well. Fosmid DNAs were linearized by digestion with HindIII, ethanol precipitated and dissolved in sterile water. A mixture of fosmids covering the whole genome (1 µg for each fosmid) was transfected using Lipofectamine 2000 (Thermo Fisher, Waltham, MA, USA) according to the protocols of the manufacturer. Usually, cytopathic effects (cell rounding, syncytia) were visible after 2–3 days and the virus was harvested once all cells showed signs of infection.

2.7. Replication Kinetics and Plaque Assay

To measure virus replication, Vero76 cells were seeded in 24 well plates at 5×10^4 cells per well. On the next day, the cells were infected in triplicate with a cell-free virus at MOI 1. After 1 h incubation, the cells were washed with PBS and incubated in 1 mL fresh medium until harvest. To measure the cell-free virus, the supernatant was transferred into Eppendorf tubes, centrifuged 5 min at 4000 rpm to remove cellular material and frozen at −80 °C. To determine the titers of cell associated virus, cells were detached with accutase, transferred to Eppendorf tubes, and collected by centrifugation. After resuspension in 1 mL medium, cells were subjected to three freeze-thaw cycles and cleared from cellular material by centrifugation for 5 min at 4000 rpm. The supernatant was frozen at −80 °C.

Virus titers were determined by plaque assay. For this, Vero76 cells were seeded in 24 well plates at 9×10^4 cells per well. On the next day, cells were infected with serial 10-fold dilutions of harvested supernatants for 1 h at 37 °C. After removal of virus inoculum, 0.5 mL overlay medium containing avicel (FMC, Philadelphia, PA, USA) [29] was added and cells incubated for 3–4 days. For harvest, avicel overlay medium was removed and the cells washed in PBS to remove the remains of avicel. Then, the cells were fixed with cold methanol for 10 min at −20 °C and dried after removal of methanol. Fixated cells were stained with crystal violet solution (0.2% crystal violet, 20% ethanol, 3.5% formaldehyde) for 2 min at room temperature and washed twice with water. Plaques were counted and virus titer calculated as plaque forming units (pfu) per mL.

2.8. Recombineering

For recombineering by en passant mutagenesis [19], fosmids were transferred into E. coli GS1783 [18], which expressed phage recombinases after heat induction and I-SceI under arabinose control. Plasmids pcDNA3-EGFP-en, pHW2000GG-seg8-A/PR/8/34-2A-Gluc-en, pHW2000GG-seg8-A/PR/8/34-M2A-mCherry-en, pcDNA3-iRFP670-en were used as template in PCR reaction employing primer pairs ep-SA8-ICP4-EGFP-5/ep-SA8-ICP4-EGFP-3, ep-SA8-ICP4-Gluc-3/ep-SA8-ICP4-2A-5, ep-SA8-UL35-EGFP-5/ep-SA8-UL35-EGFP-3 and ep-SA8-UL10C-R670-5/ep-SA8-UL10C-R670-3, respectively. The resulting PCR products contained the

en passant reporter gene cassette, flanked by 50 bp sequence ends homologous to the targeted gene. In all cases, reporter genes were targeted towards the C-termini of the ICP4, UL35 or UL10 coding regions. PCR products were treated with DpnI to inactivate template plasmids and gel purified.

E. coli GS1783 strains harboring the respective fosmids (SA8c28 and SA8c41 for ICP4; SA8c6 and SA8c18 for UL35; SA8c29 for UL10) were grown in LB-medium in presence of 25 μg/mL chloramphenicol at 30 °C until the culture reached an optical density (600 nm) of 0.5–0.7. Then, the culture flasks were transferred to a water bath shaker and incubated at 42 °C for 15 min, to induce recombinase enzymes. After cooling in an ice water bath for 20 min, the cells were collected by centrifugation and washed three times in cold sterile water. The pellet was finally resuspended in an equal volume of 10% glycerol. For electroporation 100 μL cells were mixed with PCR product and transferred to a 2 mm electroporation cuvette. Electroporation was performed at 2500 V, 25 μF and 200 Ω in a Biorad Gene Pulser. The cells were immediately transferred to 1 mL LB medium, incubated for 2 h at 30 °C and plated on LB agar plates containing 25 μg/mL chloramphenicol and kanamycin. After incubation for 2 days at 30 °C, the colonies were analyzed by colony PCR employing primers positioned outside the respective targeted regions. To remove the kanamycin gene and restore the respective reporter genes, positive colonies were grown over night at 30 °C in LB medium containing 25 μg/mL chloramphenicol and kanamycin. Then 2 mL LB medium containing 25 μg/mL chloramphenicol were inoculated with 100 μL overnight culture incubated for 3 h at 30 °C. To induce I-SceI enzyme in E. coli GS1783 strains 2 mL LB medium containing 25 μg/mL chloramphenicol and 1% L-arabinose were added to the culture and incubated for 1 h at 30 °C. Afterwards the culture was transferred to a water bath shaker at 42 °C for 30 min, followed by incubation at 30 °C at 1–2 h. The cells were then diluted and 100 μL of 10^{-3} und 10^{-4} dilutions were plated on LB agar plates containing 25 μg/mL chloramphenicol and 1% L-arabinose. The colonies harboring the reporter genes without kanamycin marker were identified by colony PCR. PCR amplificates were sequenced to confirm the sequence of the desired clones and fosmids were characterized for integrity by restriction digest.

2.9. Infection and Luciferase Assay

For single cycle infection assays, the cells were seeded in 96 well plates at 10^4 cells/well. On the next day, medium was removed and cells infected with SA-ICP4-2A-Gluc virus in a volume of 100 μL with MOI1, 0.1 or 0.01 for 1 h. Subsequently, the cells were washed four times with PBS followed by addition of 150 μL fresh medium. For luciferase measurement 25 μL supernatant was used. All samples were processed as 8-fold replicates.

For multi cycle experiments, cells were seeded in 6-well plates at 2.5×10^5 cells/well. On the next day cells were infected with 100 pfu of SA-ICP4-2A-Gluc virus in a volume of 1 mL for 1 h. Infectious supernatant was then replaced by 2 mL fresh medium. For inhibition of viral replication (Glentham Life Sciences Ltd, Corsham, UK), infection and subsequent cultivation was carried out in the presence of acyclovir (8 and 32 μg/mL). At time intervals of 6–18 h 25 μL, the aliquots were collected and stored at −20 °C. After five days of collecting, luciferase activity was determined for all samples. For multi cycle experiments triplicates of each cell line were measured. Luciferase activities were measured in a Plate Chameleon V (Hidex, Turku, Finland) instrument. As substrate, coelenterazine (PJK, Kleinblittersdorf, Germany) was diluted in D-PBS (with Ca and Mg) to a final concentration of 1.5 mM. We usually measured the background values around 3.000 cps (aliquots of supernatant taken before infection).

2.10. Next-Generation Sequencing and Assembly

Library preparation of fosmid-DNA was performed using the NEBnext Ultra II FS DNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA) following the manufacturer's protocol for <100 ng DNA input (30–46 ng input amount). The quality was controlled using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the libraries were subsequently sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) with a 600v3 sequencing kit (2 × 300 bp paired-end reads). The sequence data were trimmed and quality controlled using fastp [30], and de novo assembled

in the CLC Genomics Workbench (version 10; QIAGEN, Hilden, Germany). Sequence annotations were transferred from the reference vector constructs using Geneious Prime (Biomatters, Auckland, New Zealand). The CeHV-2 sequence coverage was above 99% for all sequenced fosmids and sequences were identical to the reference genome (Genbank NC_006560).

3. Results

3.1. Cloning and Rescue of CeHV-2

To establish a recombinant system for CeHV-2, we first fragmented the viral genome and generated a set of fosmid clones with overlapping ends of at least 1 kb. For this, viral DNA was isolated from virions, sheared to fragments of 30–40 kb, and size fractionated on a low percentage agarose gel (Figure 1a). The recovered fragments were ligated into fosmid vector pCC1FOS and packaged into phage lambda particles for transduction of E. coli cells.

Individual E. coli colonies were then characterized by colony PCR with primer pairs that were positioned every 15 kbp to map the position of the cloned fragments in the viral genome. The 5′ and 3′ ends of selected clones were then sequenced to determine the exact positions of the inserts in the viral genome (Figure 1b), and further characterized by restriction digest to check for integrity of the inserts.

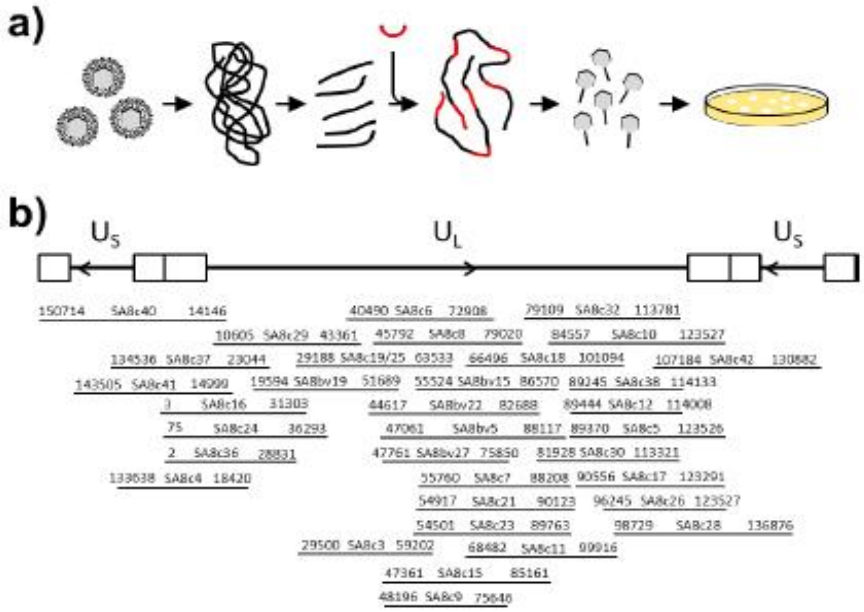

Figure 1. Cloning and characterization of CeHV-2 fosmids. (**a**) Scheme of CeHV-2 genome cloning. Viral DNA was isolated from virus particles, sheared, end-repaired and size fractionated on an agarose gel. Fractionated fragments were ligated into fosmid vector pCC1FOS (red) and packaged into phage lambda particles, which were then used to transduce cells of E. coli strain EPI300. Individual colonies were further characterized by colony PCR, end-sequencing, and restriction digest. (**b**) An overview of all fosmid clones that are characterized by colony PCR and end-sequencing. The genome of CeHV-2 is schematically represented on top, with U_S regions drawn on both sides of the U_L region. The direction of gene order, in the U_L and U_S regions, is indicated by arrows and inverted repeats are drawn as boxes. The positions of fosmid inserts are drawn as lines. The names of the fosmids and nucleotide positions of the insertions are given above the respective lines.

A set of five fosmid clones with ends overlapping between 2.3–6.4 kbp was chosen for the rescue of infectious CeHV-2 (Figure 2a). The fosmid clones were positioned on the genome in such a way that both copies of the inverted repeats were located on separate fosmids. The fosmid DNAs were first linearized at a unique HindIII site present in the multiple cloning site of pCC1FOS and subsequently transfected into Vero76 cells. At 3 days after transfection, clear cytopathic effects were visible, as demonstrated by rounded cells and formation of syncytia (Figure 2b, right panel). While, no changes in the cell monolayer were seen with untransfected cells (Figure 2b, left panel). If one fosmid was excluded during transfection, numerous rounded cells were observed but no formation of syncytia or plaques was detected (Figure 2b, middle panel). The recovered virus could be passaged to fresh uninfected Vero76 cells, and similar results were obtained for a second set of fosmids with overlapping regions shifted relative to the first set (Supplementary Information Figure S1).

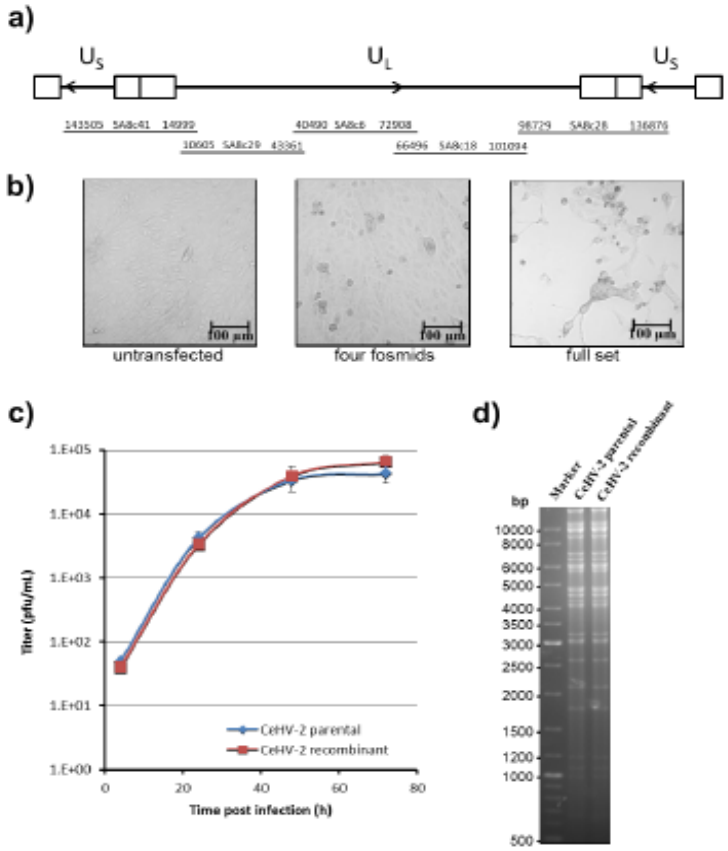

Figure 2. Rescue and characterization of recombinant CeHV-2 virus. (**a**) Schematic depiction of the CeHV-2 genome as in Figure 1b along with the fosmid clones used for rescue. (**b**) Cells were transfected with a linearized set of the fosmids. Brightfield images of untransfected Vero76 cells (left panel) and Vero76 cells transfected with a partial (four fosmids; fosmid SA8c6 missing; middle panel) and a full set (right panel) taken 3 days after transfection are shown below. Scale bars represent 100 µm. (**c**) Replication kinetics of parental and recombinant CeHV-2 viruses on Vero76 cells infected with MOI 1. Culture supernatant was harvested at the indicated time points and virus titer determined by plaque assay. The results of a representative experiment, carried out with triplicate samples, are shown. Similar results were obtained in a separate experiment. Error bars indicate standard deviation (SD). (**d**) Restriction digest with BamHI of genomes of parental and recombinant CeHV-2 viruses isolated from virus particles.

To further characterize the recovered virus, we compared the replication of the recombinant virus with the parental virus. For this, Vero76 cells were infected at MOI 1 with both viruses and culture supernatants were collected. As shown in Figure 2c, both parental and recombinant CeHV-2 viruses exhibited almost identical replication kinetics. Finally, we compared the restriction pattern of viral DNA, prepared from virions of parental and recombinant viruses and found that they were identical (Figure 2d). Thus, we could demonstrate the rescue of a recombinant CeHV-2 virus from fosmids, and we could show that wt and recombinant viruses replicated with comparable efficiency.

3.2. Recombineering of CeHV-2

We next inserted reporter genes into the fosmids, in order to easily detect replication of the recombinant viruses (Figure 3a). For this, we used recombineering and targeted three genes: First, RS1, which encodes the immediate-early protein ICP4. The RS1 gene is located in the inverted repeats of the US region and thus two copies of the gene are present in the viral genome. Since both copies were present on different fosmids, we were able to manipulate both copies independently and in parallel. Second, UL35, a late gene encoding the small capsid protein. Third, UL10, a late gene encoding glycoprotein M. Since UL35 was present in the overlapping region of two fosmids, it was necessary to modify both fosmids. We chose fluorescent proteins EGFP, mCherry and iRFP670 as reporter genes and fused them to the C-termini of ICP4, UL35, and UL10, respectively, with a Gly-Ser-Gly linker separating reporter and viral proteins (Figure 3a). In addition, we fused Gaussia luciferase (Gluc) to the C-terminus of ICP4 and inserted a Gly-Ser-Gly linker and a PTV1-derived 2A stop-go sequence between the two proteins. This should enable secretion of Gluc and allow for the sensitive measurement of infection. All alterations were introduced by en passant mutagenesis and controlled by PCR and sequencing of the modified regions.

To rescue the viruses expressing the modified proteins, the respective linearized engineered fosmids were co-transfected with linearized wt fosmids necessary to cover the SA genome (Figure 3a). Fluorescent plaques demonstrating successful rescue were clearly visible after 3 days for all recombinant viruses bearing fluorescent proteins, and covered large areas of the cell monolayer at 6 days post transfection (Figure 3b). However, plaques generated by CeHV-2 ICP4-EGFP were smaller in size than plaques formed by CeHV-2 UL35-mCherry or CeHV-2 UL10-iRFP670. Apart from the viruses carrying fluorescent reporters, the CeHV-2 ICP4-2A-Gluc virus was also successfully rescued, as judged by development of CPE (not shown) and release of Gluc (see below).

We next characterized the replication of the recombinant reporter viruses and compared it with that of wt parental and wt recombinant viruses. As shown in Figure 3c, virus titers in the supernatant increased up to 2 dpi and then reached a plateau. CeHV-2 UL35-mCherry and CeHV-2 UL10-iRFP670 grew to titers similar to parental or recombinant CeHV-2, while for both viruses with modified ICP4, CeHV-2 ICP4-EGFP and CeHV-2 ICP4-2A-Gluc, the titers were about one log lower (Figure 3c), indicating a moderate replication deficiency. When the titers of cell-associated viruses were compared, they were found to be generally higher and the plateau was reached after 1 dpi (Figure 3d). As in the supernatant, the titers of CeHV-2 ICP4-EGFP and CeHV-2 ICP4-2A-Gluc were about one log lower than for the other viruses. Finally, we asked whether the reduced replication of the viruses encoding ICP4 fused to reporter proteins was due to reduced ICP4 expression. Indeed, ICP4 levels in wt CeHV-2 infected cells were higher than those detected in CeHV-2 ICP4-2A-Gluc and CeHV-2 ICP4-EGFP infected cells [31], suggesting that the reduced replication of the reporter viruses might be due to reduced ICP4 expression.

In summary, we could demonstrate fosmid modification by recombineering and rescue of several viruses carrying reporter genes fused to viral proteins. These viruses grew to high titers, albeit viruses with modification of ICP4 achieved somewhat reduced titers.

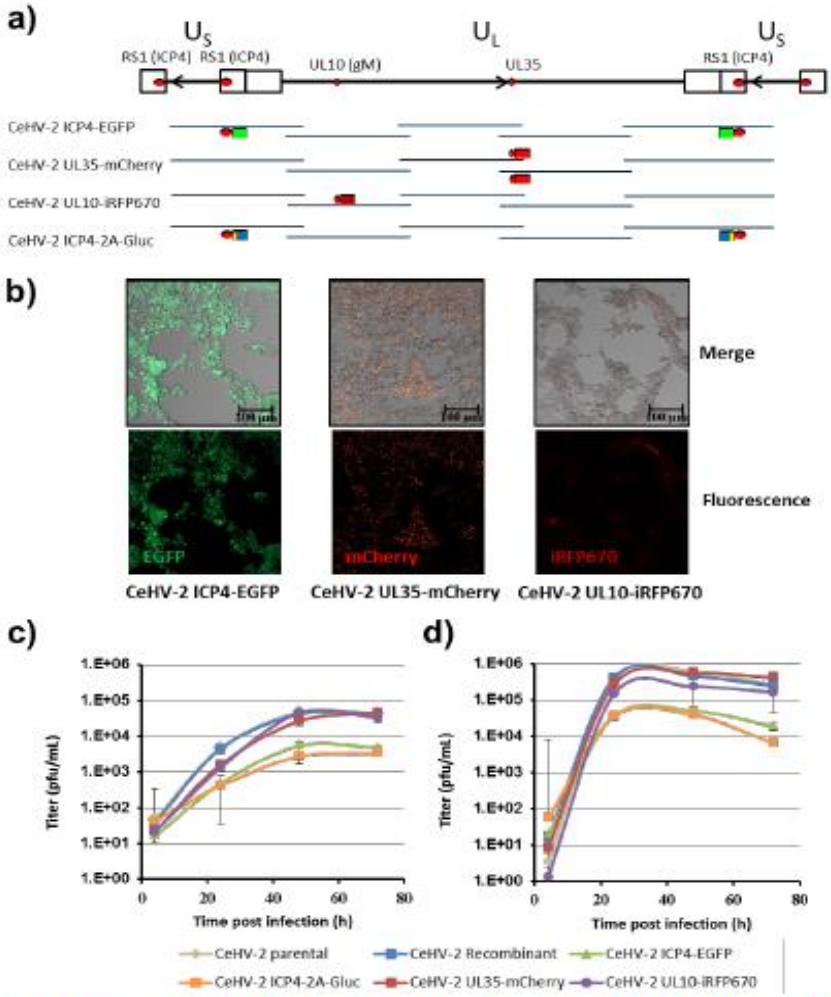

Figure 3. Generation and characterization of CeHV-2 reporter viruses. (**a**) Overview of modifications introduced into CeHV-2 fosmids. The genome of CeHV-2 is drawn, as in Figure 1b. The genes that were modified are indicated as red circles. Reporter genes coding for EGFP (green boxes), mCherry or iRFP670 (bright and dark red boxes) or 2A-Gluc (yellow and blue boxes) were inserted into the respective fosmids. In all cases, the reporter proteins were fused to the C-termini of the viral proteins. The names of the resulting CeHV-2 reporter viruses are indicated on the left side. (**b**) Rescue of CeHV-2 fluorescent reporter viruses. Vero76 cells were transfected with the wt and modified fosmids shown in (a) to generate CeHV-2 ICP4-EGFP (left), CeHV-2 UL35-mCherry (middle) or CeHV-2 UL10-iRFP670 (right). Plaque formation was monitored 6 d after transfection by confocal microscopy. Images show the respective fluorescent signal alone (fluorescence; lower panels) or in an overlay with a brightfield image (merge; upper panels). Scale bars represent 100 µm. (**c**,**d**) Replication kinetics of parental and recombinant CeHV-2 viruses on Vero76 cells infected at MOI 1. Culture supernatant or cells were harvested at the indicated time points. Cells were subjected to three freeze-thaw cycles to release cell-associated virus. Titers were determined by plaque assay for viruses released into the supernatant (**c**) or for cell-associated viruses (**d**). The results of representative experiments, carried out with triplicate samples, are shown in panels c and d, and were confirmed in two separate experiments. Error bars indicate SD.

3.3. Tropism of CeHV-2 for Different Cell Lines

We next employed our reporter viruses to examine the cell line tropism of CeHV-2. For this, we concentrated on CeHV-2 ICP4-2A-Gluc, since Gaussia luciferase (Gluc) allows for highly sensitive detection and, being secreted into the supernatant, continuous sampling. We first analyzed the kinetics of Gluc release from Vero76 cells infected at different MOIs. Upon infection at MOI 1, we observed an increase of Gluc activity in the culture supernatant at 1-2 hpi, which started to level off at 5–6 hpi (Figure 4a). For lower MOIs the rise in Gluc activity started later but for both MOIs a rise of more than 10-fold (MOI 0.01) and 100-fold (MOI 0.1) above background was detected at 24 hpi. Moreover, acyclovir, which impedes viral genome replication, but not immediate early gene expression, reduced Gluc activity close to background, demonstrating that robust reporter activity required viral replication (Figure 4b). Thus, the reporter activity upon CeHV-2 ICP4-2A-Gluc infection is dependent on MOI and viral replication and the reporter virus should be suitable to detect even low levels of infection.

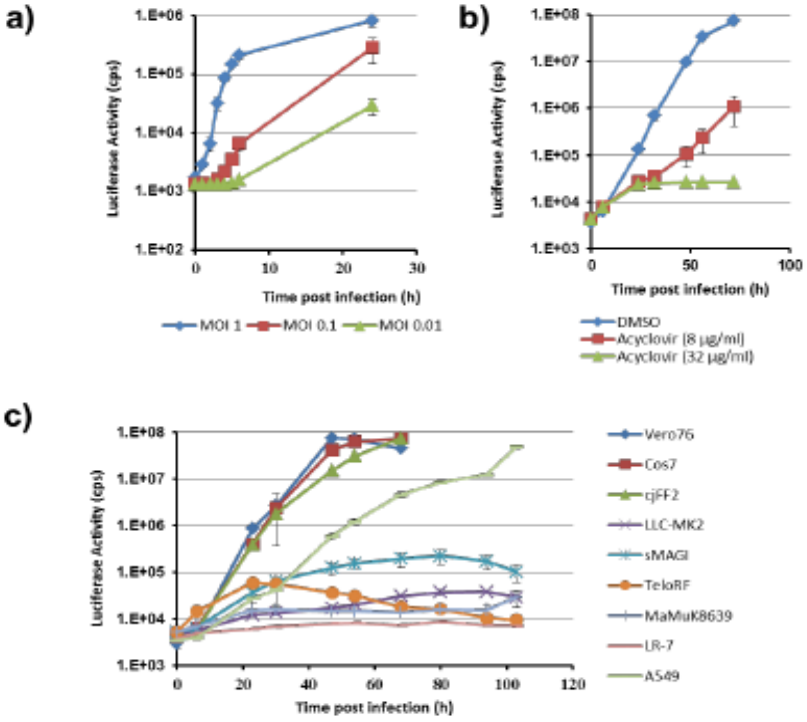

Figure 4. Kinetics of luciferase activity of CeHV-2 ICP4-2A-Gluc in infected cells. (**a**) Vero76 cells were infected at the indicated MOIs with CeHV-2 ICP4-2A-Gluc. Cell culture supernatant was harvested at the indicated time points after infection and luciferase activity determined. The results of a single, representative experiment are shown and were confirmed in a separate experiment. Standard deviation is given for octuplicate samples. (**b**). Multicycle replication kinetics in Vero76 cells infected with low virus dose (100 pfu; MOI 0.0004). Infection and cultivation was done in the medium containing acyclovir or solvent (DMSO). Aliquots of cell culture supernatant were continuously harvested over 3 days followed by measurement of luciferase activity. The results of a single, representative experiment carried out with triplicate samples are shown and were confirmed in a separate experiment. Error bars indicate SD. (**c**) Multicycle replication kinetics on cell lines derived from different species. Cells were infected with low virus dose (100 pfu; MOI 0.0004) and aliquots of cell culture supernatant continuously harvested over 5 days followed by measurement of luciferase activity. The results of a single experiment carried out with triplicate samples are shown and are representative of a total of 2–4 separate experiments. Error bars indicate standard deviation (SD).

We next tested the ability of CeHV-2 to infect different cell lines. For this, cell lines of human, non-human primate (NHP) and mouse origin were infected at MOI 0.0004 and Gluc levels in culture supernatants were measured for five days, a time frame that allows multi-cycle viral replication. As shown in Figure 4c, CeHV-2 replication was most robust in Vero76 and Cos7 cells, both derived from African green monkey, the suspected natural host of CeHV-2. Similar replication was seen in fibroblasts from marmosets (*Callithrix jacchus*). The human lung cell line A549 also supported robust viral replication, while no replication was detected in mouse LR-7 cells (Figure 4c). Finally, all four rhesus macaque-derived cell lines (LLC-MK2, MaMuK8639, both kidney; sMAGI, mammary tumor; TeloRF, fibroblast) were barely susceptible to CeHV-2 replication. Collectively, these results show that CeHV-2 can replicate in diverse cell lines of human and NHP origin but also indicate certain previously undetected limitations in cell type and/or species tropism of CeHV-2.

4. Discussion

Here, we describe a fosmid-based system for the generation of recombinant CeHV-2. This system allowed the rescue of recombinant CeHV-2 that replicated with the same efficiency as uncloned wt virus. Moreover, we successfully equipped CeHV-2 with reporter genes that allowed for convenient quantification of viral replication. Finally, we found that CeHV-2 infected human, African green monkey and marmoset derived cell lines, but failed to infect several rhesus macaque derived cell line. The fosmid-based system described should be suitable for the rescue of diverse herpesviruses, and CeHV-2 plasmids should be helpful in future studies of the biological properties of this virus.

The fosmid approach chosen here combines advantages of cosmids and BACs. BACs are now state-of-the art for modification of herpesviral genomes by recombineering. However, it has been difficult to seamlessly alter diploid genes present in inverted repeat regions of many herpesviral genomes [32]. In alphaherpesviruses these regions are of special interest, as they contain important genes for lytic and latent regulation. In cosmid-based systems, diploid genes could be modified separately [10,11]. Moreover, cosmids offer a high level of biosafety, since only parts of the genome are handled at a time. However, cosmids, are present at medium to high copy-numbers in bacterial cells and have proven difficult to modify using conventional or recombination-based protocols [11,12,33]. In contrast, fosmids are only present at low copies, since they contain a F-factor-derived low copy replication origin for plasmid maintenance and a separate inducible origin for DNA production [21], and the present study confirms that they are amenable to modification by recombineering [22–25]. Thus, the approach employed here for generation and modification of CeHV-2 should also be suitable for other alphaherpesviruses.

The separation of diploid regions of the genome on separate fosmids allowed the parallel and seamless alteration of a diploid gene (RS1/ICP4), thus minimizing the number of consecutively required steps. In this way, we could recover reporter viruses expressing ICP4-EGFP fusion proteins or co-expressing ICP4-2A and Gluc. In addition, reporter genes could be introduced into other regions of the CeHV-2 genome and replication competent viruses were obtained. If viral open reading frames, that are located in the overlap between two fosmids, are to be modified, as UL35 in our case, both copies of the gene need to be modified. The availability of several reporter constructs may allow the rescue of multi-colored viruses, and we recently rescued a CeHV-2 ICP4-EGFP UL35-mCherry virus [34] However, the generation of viruses encoding three reporter genes failed so far and the underlying reasons remain to be investigated. In summary, we applied recombineering to generate CeHV-2 reporter viruses, indicating that our system is suitable to genetically modifying large recombinant DNA viruses, such as herpesviruses.

The Gluc-expressing reporter virus allowed us to analyze cell line tropism of CeHV-2 and inhibition experiments with acyclovir confirmed that the reporter activity, observed in these experiments, reflected viral replication. The virus replicated efficiently in Vero76 and Cos7 cell lines from African green monkey (*Cercocebus* spp.). This is in agreement with previous reports on replication of CeHV-2 in Vero cells [8,9]. In addition, we observed robust replication in marmoset fibroblasts, which has not

been reported so far. Replication in human A549, Huh7.5, HeLa and HEK293T (not shown) was also detected, in agreement with reports on CeHV-2 replication in human foreskin fibroblasts [9], KB (human epithelial carcinoma) or fetal diploid lung cells [35]. In contrast, no appreciable replication was detected in cell lines derived from rhesus macaques (LLC-MK2, sMAGI, TeloRF, MaMuK8639). This is in contrast to a previous report [35] showing productive CeHV-2 infection of the rhesus macaque cell line LLC-MK2. We can, at present, not rule out that these differences are due to use of reporter virus in the present and wt virus in the previous study. However, our preliminary data showed that infection of LLC-MK2 cells with wt virus was very inefficient (not shown). Thus, we cannot dismiss the possibility that CeHV-2 may generally be able to infect rhesus macaque cells and that the cell lines tested here might lack host factors required for viral replication or that it might express antiviral factors that suppress CeHV-2 infection.

In summary, we report fosmid-based cloning of a herpesvirus genome as an alternative approach to generating recombinant viruses, and we demonstrate that CeHV-2 reporter viruses generated by recombineering allow the investigation of CeHV-2 infection.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/11/11/1026/s1, Figure S1: Rescue of recombinant CeHV-2 virus.

Author Contributions: Conceptualization, M.W. (Michael Winkler) and S.P. (Stefan Pöhlmann); methodology, M.W. (Marie Wende); investigation, E.C., S.G., A.R.S., A.M., J.G., and A.D.; resources, S.P. (Stoyan Petkov); formal analysis, E.C., S.G., J.G., and M.W. (Michael Winkler); writing—original draft preparation, M.W. (Michael Winkler) and S.P. (Stefan Pöhlmann); writing—review and editing, M.W. (Michael Winkler), S.P. (Stefan Pöhlmann), S.P. (Stoyan Petkov), and J.G.; supervision, M.W. (Michael Winkler), S.P. (Stefan Pöhlmann), and T.S.

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5.2. Second publication

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A recombinant system and viruses encoding reporter genes for *Papiine alphaherpesvirus 2*

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Individual contribution:

In the following paper, I contributed experiments for Figure 1 A and B: ”Cloning and characterization of PaHV-2”, for Figure 2 A, B, C, and D: “Rescue of PaHV-2 from plasmids”, for Figure 3 A, B, C, D, and E: “Rescue of PaHV-2 bearing reporter genes”, for Figure 4A: “Multicycle replication kinetics in cells of human (A549, 293T, HeLa), AGM (Vero76, Cos7), rhesus macaque (TeloRF, MaMuK8639) or murine (LR-7) origin”, and for Figure 5 A and B: “Inhibition of virus infection”.

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A Recombinant System and Reporter Viruses for Papiine Alphaherpesvirus 2

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Abstract: Primate simplex viruses, including Herpes simplex viruses 1 and 2, form a group of closely related herpesviruses, which establish latent infections in neurons of their respective host species. While neuropathogenic infections in their natural hosts are rare, zoonotic transmission of Macacine alphaherpesvirus 1 (McHV1) from macaques to humans is associated with severe disease. Human infections with baboon-derived Papiine alphaherpesvirus 2 (PaHV2) have not been reported, although PaHV2 and McHV1 share several biological properties, including neuropathogenicity in mice. The reasons for potential differences in PaHV2 and McHV1 pathogenicity are presently not understood, and answering these questions will require mutagenic analysis. Here, we report the development of a recombinant system, which allows rescue of recombinant PaHV2. In addition, we used recombineering to generate viruses carrying reporter genes (Gaussia luciferase or enhanced green fluorescent protein), which replicate with similar efficiency as wild-type PaHV2. We demonstrate that these viruses can be used to analyze susceptibility of cells to infection and inhibition of infection by neutralizing antibodies and antiviral compounds. In summary, we created a recombinant system for PaHV2, which in the future will be invaluable for molecular analyses of neuropathogenicity of PaHV2.

Keywords: herpesvirus; Papiine alphaherpesvirus 2; fosmid; transformation-associated recombination; recombineering; reporter virus; Gaussia luciferase; cell susceptibility; antiviral; neutralization

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1. Introduction

Simplex viruses are a subgroup of herpesviruses, which have coevolved with their respective host species [1,2]. After acute infection, they establish lifelong latency in neuronal ganglia of their natural host, from which reactivation and shedding can occur [1,3]. Neuropathogenicity in their respective host species is rare. However, upon cross-species transmission, severe infections of the CNS leading to encephalitis and ultimately death can occur. Thus, zoonotic transmission of Macacine alphaherpesvirus 1 (McHV1; formerly known as monkey B virus) from rhesus macaques to humans causes encephalitis with about 70% case-fatality rate [3].

Papiine alphaherpesvirus 2 (PaHV2) is closely related to McHV1 but has not been reported to cause disease in humans [3–5]. Herpes simplex viruses (HSV) 1 and 2 are more distant, with HSV2 showing a slightly closer overall relationship to PaHV2 than HSV1 [3,5]. Infection with PaHV2 appears to be common among olive and chacma baboons as documented by serological evidence [6]. In general, PaHV2 biology mirrors that of

McHV1 in several respects, including neuropathogenesis in mice and sensitivity to antivirals [7,8], making it an interesting model virus for the study of zoonotic encephalitis caused by simplex viruses. Interestingly, two groups of PaHV2 strains exist, differing greatly with regard to neuropathogenicity in mice [9]. Interstrain hybrids have been used to map the neuropathogenicity determinant to the UL39 gene, which encodes the large subunit of the viral ribonucleotide reductase [10]. However, further molecular analysis of PaHV2 infection has been difficult due to the lack of a recombinant system.

Recombinant systems for viruses provide an opportunity to efficiently introduce alterations into the viral genome before rescuing the virus. Several methods have been applied to generate recombinant herpesviruses, including simplex viruses. First, virus was rescued from a set of overlapping cosmids, as first demonstrated for HSV1 [11]. More recently, fosmids, cosmids with a low-copy F-factor origin, have been used to recue viruses, including Cercopithecine alphaherpesvirus 2 (CeHV2) [12–14]. Second, bacterial artificial chromosomes have been widely applied to clone and modify herpesviral genomes [15–17]. Finally, transformation-associated recombination (TAR) in yeast has been applied to clone and assemble genomes of herpesviruses [18,19] and other viruses [20].

In this work we have established a recombinant system for PaHV2 employing fosmid cloning and TAR. We demonstrate rescue of wild-type and reporter viruses, which show growth characteristics similar to wild-type. Furthermore, reporter viruses were successfully applied to the study of cell susceptibility, antivirals and neutralization.

2. Materials and Methods

2.1. Plasmids, Oligonucleotides and Microorganisms

Plasmids pCC1FOS (Lucigen, Middleton, WI, USA) and YCplac22 [21] were used for fosmid cloning and TAR. For direct cloning of fragments, the multiple cloning site of pCC1FOS was modified by ligating annealed oligonucleotides mcs-FOS-f/mcs-FOS-r into pCC1FOS cut with EcoRI and SphI, to give pCC1FOS-B. Plasmids pcDNA3-EGFP-en [12], pcDNA3-Gluc-en and pHW2000GG-seg8-A/PR/8/34-M2A-Gluc-en [12] were used for recombineering. Plasmid pcDNA3-Gluc-en was constructed by amplification of the Gluc-cassette from pHW2000GG-seg8-A/PR/8/34-M2A-Gluc-en using primers Gluc-5A/Gluc-3StopN and insertion of the purified, Acc65I/NotI digested fragment into pcDNA3 (Invitrogen, Waltham, MA, USA). Oligonucleotides were ordered from Sigma-Aldrich (St. Louis, MO, USA) (see Table 1).

Table 1. Oligonucleotides used for cloning, TAR and recombineering.

Oligonucleotide Name	Oligonucleotide Sequence (Given in 5′-3′ Direction)
Oligonucleotides used for cloning	
mcs-FOS-f	AATTCCTCGAGGCTAGCTTAATTAAGGATCCCACGTGGGATCCATCGATACGCGTCGTACGGCATG
mcs-FOS-r	CCGTACGACGCGTATCGATGGATCCCACGTGGGATCCTTAATTAAGCTAGCCTCGAGG
Gluc-5A	CCGGTACCATGGGAGTCAAAGTTCTG
Gluc-3StopN	CCGCGGCCGCTTAGTCACCACCGGCCCCCTTG
Oligonucleotides used for TAR	
TAR-HVP2-Mid-F	GTAGTGCGCGTCCGCCACCAGCCCCAGCACCGTGTTGGTCGCCGTCTGGAGATCCTC-TAGAGTCGACCTGCAG
TAR-HVP2-Mid-R	GCGGTCTATGTGCTTCACCTGCACGAACTCGCTCACGGTGGTGCGCTTGGGTTTAAACGTCGTGACTGGGAAAACCCTG
TAR-HVP2-Cent-F	CAGCTCGCCGCAGAGCGACTCGTTAAGAGCCAGGAGGTCGGGGTCGAAGGATCCTC-TAGAGTCGACCTGCAG
TAR-HVP2-Cent-R	GCGACACCACCGCCGATCGACCCGCTGTGGAAACCACACGCACATAGACGTTTAAA-CGTCGTGACTGGGAAAACCCTG
TAR-HVP2-G-F	GGCTCGCGTTCGTGATCATCACCGTCATGGTGCTGCGGCCATGCCGTCCGATCCTCTAGAGTCGACCTGCAG
TAR-HVP2-G-R	CCGTGCTGGCGATCAGCCCCTGGCTCGCCACGCGCAGCAGGATCTCGCAGTTTAAACGTCGTGACTGGGAAAACCCTG
Oligonucleotides used for recombineering	
ep-HVPUL4pA-cmv	TCCCGCGGCCCCGGGCGCTCCAGAGACGCGGCGAGACGAATAAACGCGGTGTTGACATTGATTATTGACT
ep-HVPUL3-gGHpA	CGCCTCGCCGATCCCAAGGATGGATGACACACGAATAAATATTTCAAACGTCCCCAGCATGCCTGCTATT
ep-Pa35-Gluc-5	TCCGCCGCCCTCCTCCCGGTCGCCGTTGCGCCCCGCCCGCCGCCCGCGCCATGGGAGTCAAAGTTCTGTTT
ep-Pa35-2A-3	TCGGGGTGAAGCGTGTCGGGCCGGTGGAACTGCGGGACCGCGGCGGACATCGGGCCCGGGTTTTCTTCCAC

For cloning and recombineering, we used the *Escherichia coli* host strains DH10B [22], EPI300 (Lucigen, Middleton, WI, USA), GS1783 [23] and PMC103 [24]. The yeast strain *Saccharomyces cerevisiae* VL6-48N was used for TAR [25].

2.2. Cells and Virus

Vero76, Cos7 (both African green monkey (AGM), kidney), A549 (human, lung epithelial), 293T (human, kidney), HeLa (human, cervix epithelial), MamuK8639 (rhesus macaque, *Macaca mulatta*, kidney) [12], TeloRF (rhesus macaque, *Macaca mulatta*, fibroblast) [26] and LR7 (mouse fibroblast) [27] cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) and 100 U/mL penicillin and 100 µg/mL of streptomycin. The identity of human cell lines was verified by STR analysis using a published protocol [28]. For non-human cell lines, species identity was confirmed by partial sequencing of mitochondrial genes [29].

Papiine alphaherpesvirus 2 strain X313 was a kind gift by David Brown and Matthew Jones, Public Health England. Sequencing of this parental virus demonstrated that all coding regions were identical with the published sequence (DQ149153) [5] with a few exceptions bearing amino acid changes: UL21 P420A, UL29 T533A and US4 H427D. One additional nucleotide change within the coding region of UL44 did not affect the amino acid sequence. Some nucleotide changes were located outside the coding regions, and two extremely GC-rich regions within noncoding regions could not be resolved. The sequence of our strain has been deposited in Genbank (OM021999).

2.3. Fosmid Cloning

Fosmid cloning of PaHV2 DNA was performed as described previously [9]. Briefly, PaHV2 virions were collected from cell culture supernatant by ultracentrifugation (WX Ultra 80, Thermo Scientific Sorvall, Dreieich, Germany; Surespin rotor, 28,000 rpm, 70 min) and the virion pellet resuspended in 100 µL of PCR lysis buffer (50 mM of KCl, 10 mM of Tris pH 8.3, 1.5 mM of $MgCl_2$, 0.001% gelatine, 0.5% triton X-100, 35 µg/mL of Proteinase K) followed by incubation for 1 h at 56 °C. The viral DNA was sheared 7 times through a 27G needle and precipitated after addition of 40 µL of 5 M NaCl and 125 µL of isopropanol. The DNA pellet was dissolved in 10 µL of H_2O and processed (end repair) for fosmid cloning as recommended by the manufacturer (CopyControl Fosmid Library Production Kit; Lucigen, Middleton, WI, USA). After separation on a 0.8% low melting agarose gel, a gel slice encompassing DNA fragments of about 20–40 kbp was cut and DNA recovered by digesting the agarose with GELase. Ligation into 500 ng of Eco72I-linearized pCC1FOS vector was set up using 250 ng size-fractionated virion DNA and subsequently packaged into lambda particles using MaxPlax Packaging Extracts, according to the protocols supplied by the manufacturer. Finally, *E. coli* EPI300 cells were transduced with lambda particles and resulting colonies were screened by colony PCR to identify the region of the genome present in individual clones. Clones chosen for further analysis were partially sequenced (ends of the insert) and analyzed by restriction digest.

For direct cloning of pCC1FOS-B-HVP2-USUL (H-60), unsheared virus DNA was digested with NheI and SspI, separated on a low melting agarose gel, as described above. The DNA recovered from the gel was ligated into pCC1FOS-B cut with Eco72I and NheI, packaged into lambda particles and processed as described above.

2.4. Transformation-Associated Recombination

Cloning of missing fragments by TAR was essentially performed as described in [30,31]. As the vector backbone, we used YCplac22 [21], which was amplified with primers providing the targeting sequences (hooks) for recombination (see Table 1). Primer pairs TAR-HVP2-Mid-F/R, TAR-HVP2-Cent-F/R and TAR-HVP2-G-F/R were employed to produce target vectors for cloning TAR-HVP2-Mid, TAR-HVP2-Cent and TAR-HVP2-G, respectively.

For transformation, *S. cerevisiae* strain VL6-48N [25,31] was grown overnight (100 mL) to an OD_{600} of 2.0–2.5. Yeast cells were then washed in 1 M of sorbitol, followed by resuspension in 20 mL of SPE solution (1 M of sorbitol, 0.01 M of sodium phosphate, 0.01 M of Na_2EDTA, pH 7.5). For spheroplast generation, 40 µL of 2-mercaptoethanol and 60 µL of Zymolyase stock solution (10 mg/mL zymolyase 20T in 25% (*w*/*v*) glycerol) were

added, and cells were incubated at 30 °C with slow shaking (60 rpm). The optimal level of spheroplasting was determined by measuring OD600 of a 1:10 dilution in 1 M of sorbitol or 2% SDS (optimal ratio 3–5) [31]. Spheroplasts were carefully washed twice in 1 M of sorbitol and finally resuspended in 2.0 mL of STC solution (1 M of sorbitol, 0.01 M of Tris-HCl, 0.01 M of $CaCl_2$, pH 7.5.). For transformation, 100 µL of spheroplast suspension were mixed with 0.5 µg of TAR cloning vector and 2 µg of sheared virus DNA. After 10 min incubation at room temperature, spheroplasts were gently mixed with 800 µL of PEG8000 solution (20% (*w*/*v*) PEG8000, 10 mM of $CaCl_2$, 10 mM of Tris-HCl, pH 7.5) and incubated for 15 min at room temperature. Then, spheroplasts were collected by centrifugation and carefully resuspended in 800 µL of SOS solution (1 M of sorbitol, 6.5 mM of $CaCl_2$, 0.25% yeast extract and 0.5% peptone), followed by incubation at 30 °C for 40 min. Finally, cells were mixed with sorbitol-containing Trp-dropout top agar and plated on sorbitol-Trp-dropout agar plates. Plates were incubated at 30 °C until colonies formed (about 5 days). Screening was performed by PCR on pools of colonies, followed by identification of individual positive clones from positive pools [31].

To produce DNA for transfection while preserving unstable regions, the plasmids were transferred from yeast to *E. coli* strain PMC103 [24], which has been described to provide increased stability of palindromic sequences. Positive clones were cultured at room temperature to further increase genetic stability [32].

2.5. Recombineering

For recombineering by en passant mutagenesis [33], required fosmids were transferred into *E. coli* host strain GS1783 [23], which allows for heat-inducible expression of phage recombinases and arabinose inducible expression of I-SceI. PCR fragments for recombineering were prepared using plasmids pcDNA3-EGFP-en [12], pcDNA3-Gluc-en and pHW2000GG-seg8-A/PR/8/34-M2A-Gluc-en [12] as templates as indicated in Table 2. PCR products were subsequently digested with DpnI to remove template. The final products harbored the amplified en passant cassette flanked by 50 bp sequences homologous to their respective target.

Table 2. Recombineering. Templates, primers and target fosmids used for recombineering.

Virus Construct	Fosmid	Template	Primer
HVP2-cmvGluc	HVP2a41	pcDNA3-Gluc-en	ep-HVPUL4pA-cmv ep-HVPUL3-gGHpA
HVP2-cmvEGFP	HVP2a41	pcDNA3-EGFP-en	ep-HVPUL4pA-cmv ep-HVPUL3-gGHpA
HVP2-Gluc-2A-UL35	HVP2a3-45	pHW2000GG-seg8-A/PR/8/34-M2A-Gluc-en	ep-Pa35-Gluc-5 ep-Pa35-2A-3

E. coli GS1783 strains harboring the fosmids HVP2a41 or HVP2a3-45 were prepared for electroporation as described before [12]. Briefly, cultures were grown at 30 °C to an optical density (600 nm) of 0.5–0.7 and transferred to a shaking water bath to induce recombinases during a 15 min incubation at 42 °C. Afterwards, cells were cooled in an ice water bath and washed three times in ice-cold sterile water, before the pellet was resuspended in an equal volume of 10% glycerol. PCR fragments were mixed with 100 µL of bacterial suspension and electroporated at 2500 V, 25 µF and 200 W in a Gene Pulser (Bio-Rad, Hercules, CA, USA), followed by addition of 1 mL of LB medium and incubation of 2 h at 30 °C. Recombinant fosmids were selected after plating cells on LB agar plates containing chloramphenicol and kanamycin. Colonies were analyzed by PCR for successful integration of the en passant cassette using primers positioned outside the region used for recombination. For subsequent removal of the kanamycin cassette from positive clones, 100 µL of an overnight culture were inoculated into 2 mL of LB medium containing 25 µg/mL of chloramphenicol and 1% L-arabinose and incubated at 30 °C for 1 h to induce *I-SceI* expression. Then, the culture was transferred to a shaking water bath at 42 °C for 30 min to induce phage

recombinases. After an additional incubation at 30 °C for 1–2 h, dilutions (10^{-3} to 10^{-4}) were plated on LB agar plates containing chloramphenicol and L-arabinose. Colonies negative for kanamycin were identified by colony PCR. The altered region was sequenced to confirm the desired changes.

2.6. Virus Rescue

Rescue of PaHV2 was performed as previously described for CeHV2 [12]. Briefly, Vero76 cells were seeded in 12-well plates at 10^5 cells/well and transfected on the next day with a set of linearized fosmids/plasmids. Transfection was performed with Lipofectamine 2000 (Thermo Fisher, Waltham, MA, USA) according to the protocols of the manufacturer, using 1 µg per plasmid. Cultures were screened for cytopathic effects (rounded cells, syncytia), which usually appeared after 3 days. Supernatant was harvested when all cells showed signs of infection and used to generate virus stocks.

2.7. High-Throughput Sequencing and De Novo Assembly

High-throughput sequencing was performed to validate the constructs and rescued viruses. DNA was isolated from virions collected by ultracentrifugation. Particles were resuspended in lysis buffer (50 mM of KCl, 10 mM of Tris pH 8.3, 1.5 mM of $MgCl_2$, 0.5% Tween 20, 40 µg/mL of proteinase K) and incubated at 56 °C for 1 h. After inactivation of proteinase K at 95 °C for 10 min, DNA was concentrated by ethanol precipitation. Sequencing libraries were prepared using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England Biolabs, Frankfurt, Germany) according to the manufacturer's protocol. In addition, Betaine was used at a final concentration of 1 M during the indexing PCR to mitigate the sample's high GC-content. The libraries were sequenced on an Illumina MiSeq using a 600v3 reagent kit generating 2 × 300 nt paired-end reads. Sequencing reads were trimmed using fastp [34] and de novo assembled using SPAdes [35]. Assemblies were compared to and annotated according to reference construct (DQ149153) using Geneious Prime 2021 (Biomatters, Auckland, New Zealand). The sequences of our parental virus strain and two rescued viruses (set M and set C) have been deposited in Genbank (OM021999, OM021998, OM021997).

2.8. Plaque Assay and Virus Growth Curves

To determine virus titers by plaque assay [12], Vero76 cells were seeded in 24-well plates at 100,000 cells/well. After overnight incubation cells, were infected with 10-fold serial dilutions of harvested virus-containing supernatant. Following 1 h of incubation at 37 °C, inoculum was removed and replaced by 0.5 mL of overlay medium containing 1% Avicel (FMC, Philadelphia, PA, USA). Plates were incubated for 3–4 days until plaques developed. For harvest, overlay medium was aspirated, and cells were washed once with phosphate-buffered saline (PBS) to remove remnants of Avicel. Following fixation with cold methanol for 10 min at −20 °C, plates were dried. To visualize plaques, cells were stained at room temperature with crystal violet solution (0.2% crystal violet, 20% ethanol) for 2 min and washed once with water. Plaques were counted, and virus titer was calculated as plaque-forming units per milliliter (pfu/mL).

Single-step growth curves were performed in 24-well plates as previously described [12]. Briefly, target cells (Vero76) were seeded at 50,000 cells/well, incubated overnight and then infected in triplicates with a multiplicity of infection (MOI) of 1 for 1 h. After infection, inoculum was removed, and cells were washed once with PBS and incubated in 0.5 mL of fresh medium. At fixed time points, supernatants were harvested, centrifuged to remove floating cells and debris and frozen at −80 °C for subsequent titration.

2.9. Microscopy

For microscopy, cells were seeded in plastic multi-well plates as detailed in sections on virus rescue or inhibitor assay. For time series, cells were seeded in lumox 96-well plates (Greiner, Kremsmünster, Austria) and incubated in the presence of cell-permeable nuclear

counterstain Hoechst 33342 (Invitrogen, Karlsruhe, Germany). Microscopic imaging was performed on a Zeiss LSM800 employing 405 nm and 488 nm laser lines, 10× magnification and GaAsP detectors. Brightfield images were taken using the ESID module. Microscopic images were recorded using Zeiss ZEN software. Images were further processed (cropping, adjustment of brightness, scale bar) using Image J/Fiji [36].

2.10. Infection and Luciferase Assay

For infection kinetics, Vero76 cells were seeded in 96-well plates at 10,000 cells/well. On the next day, cells were infected in triplicates with PaHV2 cmvGluc or PaHV2 Gluc-2A-UL35 with MOI 1, 0.1 or 0.01. After 1 h, inoculum was removed, and cells were washed four times with PBS, to remove Gaussia luciferase (Gluc) present in the inoculum. After the final wash, 100 µL of DMEM containing FCS, penicillin and streptomycin was added. Samples of 25 µL of supernatant were collected immediately afterward (0 hpi, hours post infection), and every hour for the next eight hours, followed by 24, 48 and 72 hpi. All samples were frozen at −20 °C.

For multicycle infection, cells were seeded in 6-well plates at 250,000 cells/well. The next day, cells were infected in triplicates with PaHV2-cmvGluc with MOI 0.0004 (100 pfu/well). After 1 h, inoculum was replaced with DMEM containing FCS, penicillin and streptomycin. At certain intervals, samples of 25 µL of supernatant were collected and frozen at −20 °C.

To determine luciferase activities, samples of 25 µL of supernatant were collected in white opaque plates (Nunc/ThermoFisher, Waltham, MA, USA) and measured in a Plate Chameleon V (Hidex, Turku, Finland) instrument by injection of 50 µL assay buffer (DPBS w/o calcium, magnesium, containing 2 µM coelenterazin) and 1 s counting time after a 100-ms delay.

2.11. Inhibitor Assay

For inhibitor assay, we adapted a published protocol [37] to the use of luciferase-based detection of infection. Inhibitors were dissolved in DMSO (acyclovir, ACY) or in water (ganciclovir, GCV; cidofovir, CDV; foscarnet, FOS) in stock solutions of 4 mg/mL. We seeded Vero76 cells in 96-well plates at 5×10^4 cells/well. On the next day, we prepared a dilution of PaHV2 Gluc-2A-UL35 virus to 500 pfu/well (MOI 0.01), as well as a two-fold dilution series of antiviral compounds, both in DMEM without FCS. In parallel, dilution series with diluents (DMSO or water) were performed. Cells were first preincubated for 1 h at 37 °C with the respective dilutions of the antiviral compounds. For infection, medium was aspirated and replaced by diluted virus. After incubation for 1 h at 37 °C, cells were washed at least once with PBS to remove Gluc present in the inoculum, followed by addition of diluted compound in a final volume of 200 µL DMEM without FCS. In addition, controls without virus (negative control) or without virus and compound (positive control) were prepared. All samples were set up in triplicates. After 48 h, 25 µL of cell culture supernatant was removed and assayed for Gluc activity. Sample values were corrected for background by subtracting the mean of negative control and normalized to the positive control.

2.12. Neutralization Assay

For neutralization assay, we adapted existing microneutralization protocols developed for herpesviruses and influenza viruses [38–41]. Blood serum samples were taken from olive baboons (*Papio anubis*) during the annual health monitoring. Sera was tested in a colony surveillance assay (CSA: Panel E Kit) (Intuitive Biosciences, Madison, WI, USA) detecting antibodies against primate simplex viruses among others [42]. We seeded Vero76 cells in 96-well plates at 5×10^4 cells/well. On the next day, we prepared a dilution of PaHV2 Gluc-2A-UL35 virus to 2000 pfu/mL, as well as a 2-fold dilution series of heat-inactivated (30 min at 56 °C) sera, starting at 1:10. Diluted virus (100 pfu in 50 µL) and 50 µL of serum dilution were mixed, giving a 1:20 starting dilution, and incubated for 1 h at 37 °C. For infection, medium was aspirated, and cells were washed with PBS and

subsequently incubated with the virus-serum mixtures. After 24 h, 25 µL of cell culture supernatant was removed and assayed for Gluc activity. Sample values were corrected for background by subtracting the mean of negative control and normalized to the positive control. Values were converted from percent infection to percent neutralization.

2.13. Statistical Analysis

Most data were analyzed and graphed using Excel and its functions to calculate mean and standard deviation (SD) or standard error of the mean (SEM). Statistical significance was calculated by the *t*-test. Data from neutralization or inhibitor assays were first corrected for background (luciferase activity of uninfected untreated cells) and normalized for infection (luciferase activity of infected untreated cells) using Excel. Statistical analysis of normalized data was done using Graphpad Prism using nonlinear regression (curve fit with least squares regression) employing a dose-response calculation for inhibitors assuming a variable slope (Hill equation < 0.0), as detailed in [43].

3. Results

3.1. Cloning of the Papiine Alphaherpesvirus 2 Genome

For cloning of the PaHV2 genome, we initially applied the same strategy as for CeHV2 [12]. DNA isolated from virus particles was fragmented, ligated into fosmid vector pCC1FOS and, after packaging into lambda particles, transduced into *E. coli* (Figure 1a). Individual clones were analyzed by PCR to map the approximate position on the viral genome, followed by sequencing of the insert ends to obtain the exact position of the inserts. Characterization by restriction digest was used as an initial test for integrity of the cloned inserts. In this way, we were able to obtain a number of clones spanning most of the UL and IRL regions (Figure 1b upper panel). However, clones containing US and IRS regions, as well as about 2 kb in the center of the UL region could not be recovered despite screening of more than 2000 clones.

We then attempted targeted restriction enzyme-based cloning of the missing fragments but could only recover one clone (HVP2-H60), covering the US-IRS-IRL-UL junction. Finally, we turned to TAR, to recover the still missing regions as yeast artificial chromosomes in a YCplac22 vector. After transfer of clones from yeast into *E. coli*, plasmids were further characterized, including full sequencing. The initial sequencing results pointed out deletions in three regions of the genome, the center of UL as well as the two IRS regions, affecting the palindromic origin sequences. Therefore, we turned to use *E. coli* strain PMC103 for transfer of DNA from yeast, since this strain shows increased stability of palindromic sequences [24].

In the next step, two sets of five fosmid and two TAR clones each (set M and set C) were chosen for the rescue of infectious PaHV2 (Figure 2a). The plasmids were first linearized using unique restriction enzyme sites in the multiple cloning sites flanking the insert or in the vector backbone and then transfected into Vero76 cells. Cytopathic effects (syncytia formation) could be observed as early as 2 days after transfection (Figure 2b middle and right panels), while no changes were detected in untransfected Vero76 cells (Figure 2b left panel). Infectious virus could be transferred with the supernatant to uninfected cells, and PCR confirmed its identity as PaHV2.

To further characterize the rescued viruses, we compared parental and recombinant viruses in a growth analysis on Vero76 cells infected at MOI 1. As shown in Figure 2c, both recombinant viruses (derived from set C or set M) produced progeny viruses to the same extent and with the same kinetics as the parental virus. Finally, we prepared viral DNA from virus particles and performed restriction digest (Figure 2d). All three genomes showed an identical pattern indicating no major alterations in genome structure. In parallel, DNA was subjected to next generation sequencing. Apart from a few highly GC-rich stretches, which could not be resolved, sequences were almost identical to the parental PaHV2 X313 genome, clearly demonstrating no deletions or frameshift mutations within coding regions. We note a single amino acid change with respect to our parental virus,

which was due to UL13 A424T and was also present in fosmid HVP2a41. Importantly, we did not detect any deletions affecting palindromic origin sequences. Thus, we were able to demonstrate successful rescue of PaHV2 from a plasmid-based recombinant system.

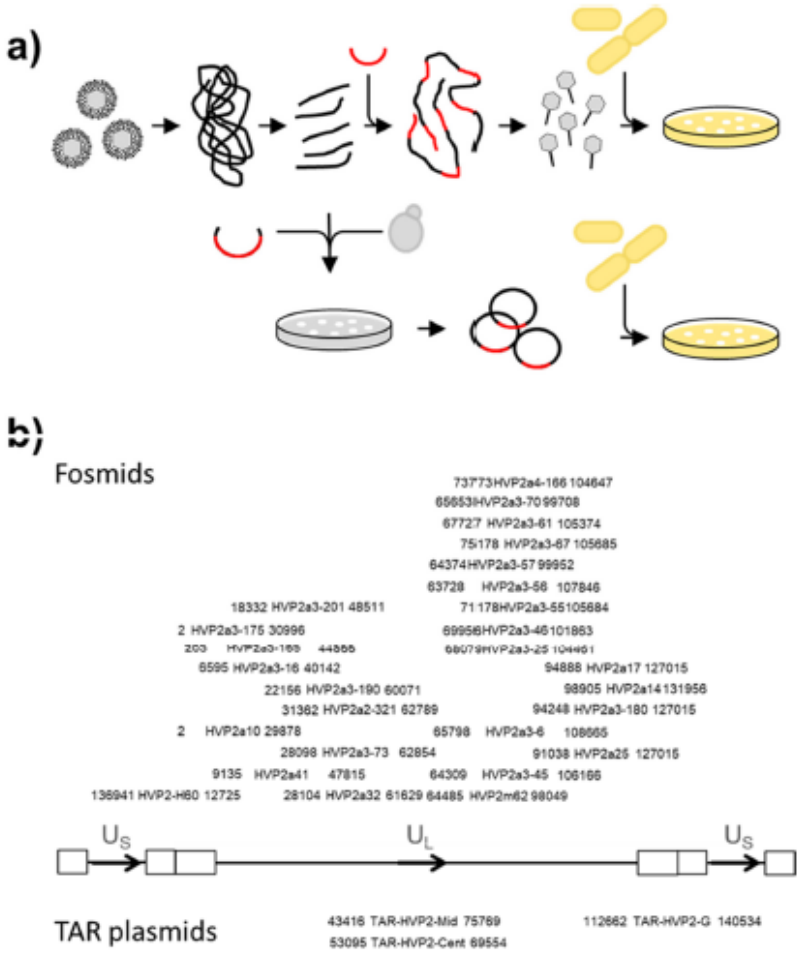

Figure 1. Cloning and characterization of PaHV2 plasmids. (**a**) Scheme of PaHV2 genome cloning (modified from [12]). Viral DNA was isolated from virus particles and sheared. For fosmid cloning (upper panels), viral DNA fragments were end-repaired and size fractionated on an agarose gel. Fractionated fragments were ligated into fosmid vector pCC1FOS (red) and packaged into phage lambda particles to transduce cells of *E. coli* strain EPI300. Individual clones were further characterized by colony PCR, end sequencing and restriction digest. For transformation-associated recombination (TAR) (lower panels), viral DNA fragments were mixed with a yeast shuttle vector harboring targeting sequences (hooks) for recombination and transformed into yeast strain VL6-48N. Positive clones were identified by colony PCR and subsequently transferred to *E. coli* for final characterization by restriction digest and end sequencing. (**b**) Overview of all genomic clones. A concatemeric section of the genome of PaHV2 is schematically represented, with US regions drawn on both sides of the UL region. Direction of gene order in UL and US regions is indicated by arrows and inverted repeats are drawn as boxes. Fosmid clones are shown above the genome representation, whereas TAR clones are shown below. Positions of genomic clones are drawn as lines. Names of the fosmids/plasmids and nucleotide positions of the insertions are given above the respective lines.

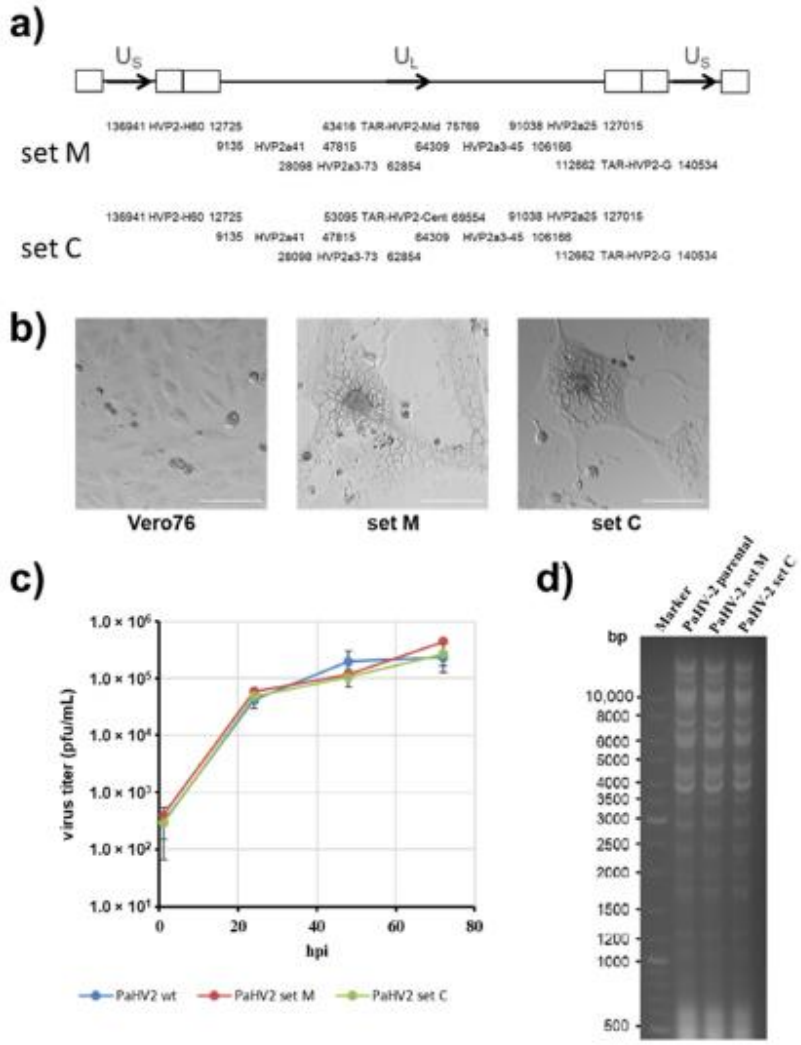

Figure 2. Rescue of PaHV2 from plasmids. (**a**) Schematic depiction of the PaHV2 genome along with two sets of fosmids/TAR plasmids used for rescue. Identical fosmids were used for both sets except for TAR-HVP2-Mid (set M) or TAR-HVP2-Cent (set C). (**b**) Brightfield images of untransfected Vero76 cells (left panel), or Vero76 cells transfected with set M (middle panel) or set C (right panel) taken at 3 days after transfection. Images were taken at 10× magnification. The scale bar represents 100 µm. (**c**) Replication kinetics of parental PaHV2 and recombinant PaHV2, derived from two sets of plasmids, on Vero76 cells infected with MOI 1. The average of three independent experiments, each performed with triplicate samples is shown. Error bars represent standard error of the mean (SEM). (**d**) Restriction digest of viral genomes. Viral DNA isolated from particles of parental or recombinant PaHV2 (set M and set C) was digested with BamHI and subjected to gel electrophoresis. Sizes of marker bands are given on the left.

3.2. PaHV2 Genomes with Reporter Genes

In a next step, we wanted to insert reporter genes into the viral genome. To obtain viruses with immediate expression of the reporter genes (Figure 3a), we chose to insert a reporter gene cassette into the intergenic region between UL3 and UL4, which has

previously been used in the context of herpes simplex virus 1 for gene insertion [16,44]. Our reporter cassettes were driven by the human cytomegalovirus enhancer/promoter and contained either Gaussia luciferase (Gluc) or enhanced green fluorescent protein (EGFP) as reporter, followed by a bovine growth hormone polyadenylation signal. The cassettes were introduced into fosmid HVP2a41 by en passant mutagenesis [23] and controlled by PCR and sequencing of the modified regions. In addition, we fused the gene for Gluc with the 5′-end of the late gene UL35 in the context of fosmid HVP2a3-45. In this construct, both genes are linked with a porcine teschovirus 1 2A "Stop-Go" sequence enabling the processing into separate proteins [45,46].

For rescue of reporter viruses, the wild-type fosmids were replaced by the respective modified fosmids (Figure 3a). After transfection into Vero76 cells, clear cytopathic effects developed within 3–5 days, demonstrating rescue of PaHV2-cmvGluc, PaHV2-cmvEGFP and PaHV2-Gluc-2A-UL35 reporter viruses. Microscopic inspection of plaques formed by PaHV2-cmvEGFP clearly showed expression of EGFP (Figure 3b). The rescued viruses were passaged and subjected to a growth analysis. For this, Vero76 cells were infected with parental PaHV2 and the recombinant reporter viruses at MOI 1. All reporter viruses showed replication similar to parental PaHV2 (Figure 3c). The titers of the reporter viruses were slightly lower than parental PaHV2, but this deviation was not significant except for PaHV2 cmvEGFP at 72 hpi (Student's *t*-test $p = 0.040$).

For further analysis, we focused on the reporter viruses carrying Gluc as a reporter, since this luciferase molecule is secreted into the cell culture supernatant and allows for highly sensitive detection and continuous sampling. We first determined the kinetics of Gluc expression on Vero76 cells infected with different MOI (1, 0.1 or 0.01). For PaHV2-cmvGluc infection at MOI 1 (Figure 3d), a first increase of Gluc activity could be detected as early as 2 hpi, and Gluc activity reached levels close to plateau around 6–8 hpi. When infected with lower MOI, the kinetics of Gluc expression was delayed and dose-dependent. However, in all conditions, maximum Gluc activity could be detected no later than 48 hpi. For PaHV2-Gluc-2A-UL35 (Figure 3e), where Gluc expression is coupled to late gene UL35, the kinetics of Gluc expression was delayed compared to PaHV2-cmvGluc, showing a first rise in Gluc activity between 4–6 hpi when infected at MOI 1. For this virus, Gluc activity was also dose dependent and maximum Gluc activity were detected no later than 48 hpi. Thus, we were able to generate reporter viruses allowing the sensitive detection of PaHV2 infection.

We next employed the reporter viruses for analysis of cell susceptibility of PaHV2. For this, we infected representative human (A549, 293T HeLa), AGM (Vero76, Cos-7), rhesus macaque (TeloRF, MamuK8639) or murine (LR-7) cell lines with PaHV2-cmvGluc at a low dose (MOI 0.0004). Cell culture supernatants were continuously sampled up to 96 hpi, to allow for multicycle replication, and luciferase activity was determined. As shown in Figure 4a, PaHV2-cmvGluc growth is highly robust on Vero76 or Cos7 cells, derived from AGM, and also TeloRF and MamuK8639 cells from rhesus macaques. Growth on human cell lines was more variable, with A549 cells performing best, while replication in HeLa cells was clearly compromised. The murine cell line LR-7 also supported growth of PaHV2-cmvGluc, but at a low level. In parallel, we infected selected cell lines with PaHV2-cmvEGFP at MOI 1 to detect cell susceptibility at the single cell level. At 24 hpi, EGFP expression was clearly detected and slightly increased at 48 and 72 hpi (Figure 4b). Cell lines supporting efficient replication, such as A549, Vero76 and MamuK8639, demonstrated infection of most of the cells and also signs of CPE, such as syncytia formation and chromatin marginalization [47]. EGFP fluorescence was located throughout the cell, but more intense in the cytoplasm. In addition, fluorescence appeared to be less intense in large syncytia, as observed for infected Vero76 and MamuK8639 cell. In contrast, for the murine LR-7 cells, we observed few infected cells, no syncytia formation or chromatin marginalization and apart from rare local foci infection did not spread to other cells.

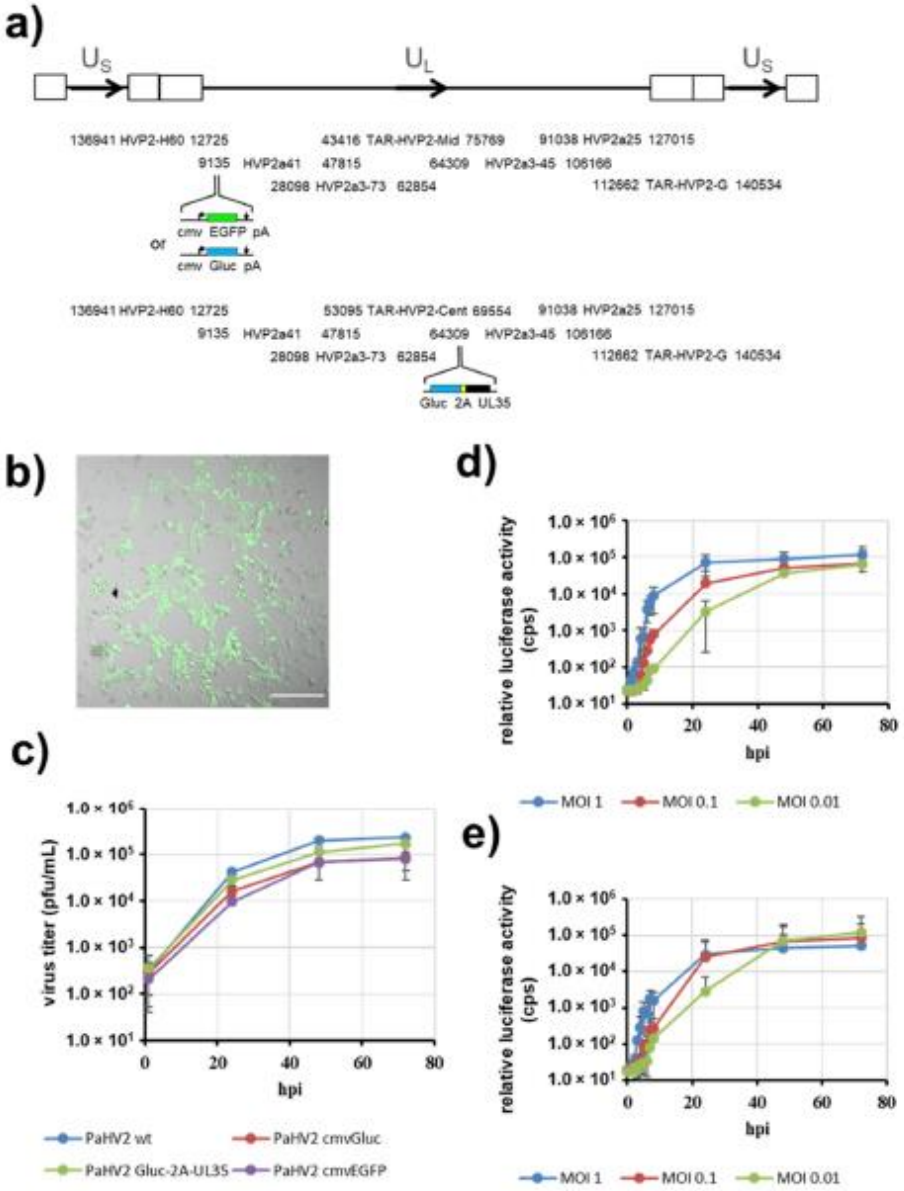

Figure 3. Rescue of PaHV2 bearing reporter genes. (**a**) Schematic depiction of the PaHV2 genome along with the sets of fosmids/TAR plasmids used for rescue. Reporter cassettes introduced by recombineering are indicated. (**b**) Microscopic representation of a plaque formed by PaHV2-cmvEGFP three days after transfection. Brightfield and fluorescent images are merged. The scale bar represents 200 µm. (**c**) Replication kinetics of parental PaHV2 and recombinant PaHV2 bearing reporter cassettes on Vero76 cells infected with MOI 1. The average of two (PaHV2 Gluc-2A-UL35) or three (all other viruses) independent experiments, each performed with triplicate samples is shown. Error bars represent SEM. (**d**,**e**) Kinetics of luciferase activity of PaHV2-cmvGluc (**d**) or PaHV2-Gluc-2A-UL35 (**e**) in Vero76 cells infected at the indicated MOIs. At the indicated time points after infection cell culture supernatant was harvested and luciferase activity determined. The averages of two to four independent experiments, each performed with triplicate samples, are shown. Error bars represent SEM.

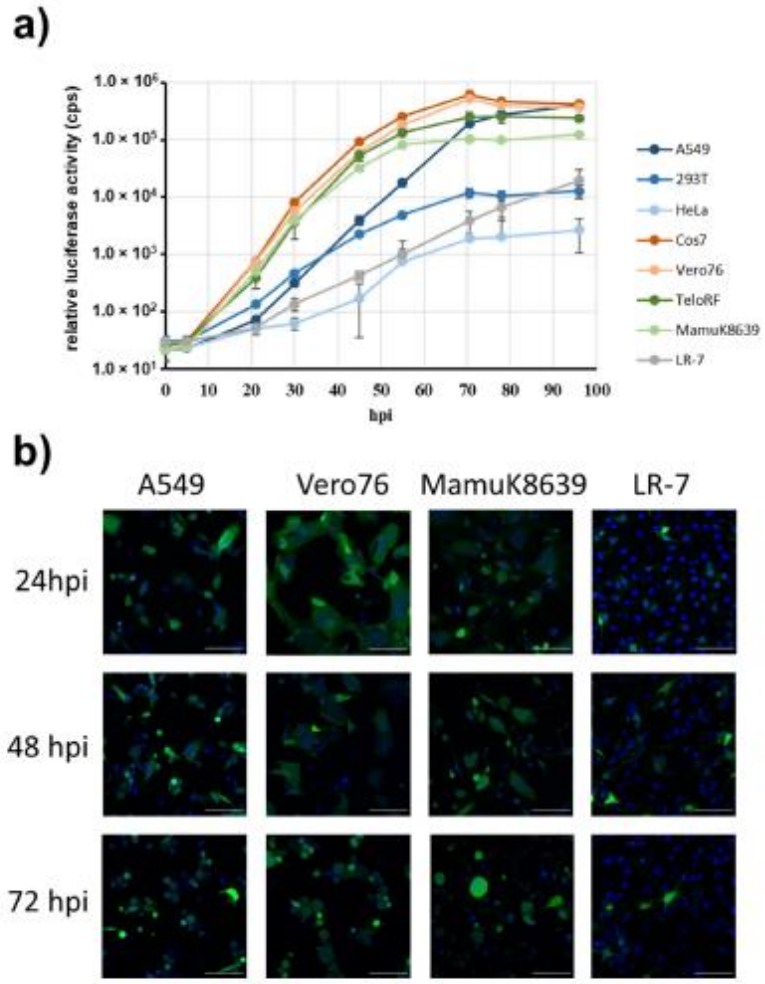

Figure 4. Susceptibility of cell lines. (**a**) Multicycle replication kinetics in cells of human (A549, 293T, HeLa), AGM (Vero76, Cos7), rhesus macaque (TeloRF, MamuK8639) or murine (LR-7) origin. Cells seeded in 6-well plates were infected with PaHV2-cmvGluc at low virus dose (100 pfu; MOI 0.0004) and small aliquots of cell culture supernatant collected over the course of 96 h followed by measurement of luciferase activity. The results of a single, representative experiment are shown, which was confirmed in two separate experiments. Error bars represent SD of triplicate samples. (**b**) Microscopic images of representative human (A549), AGM (Vero76), rhesus macaque (MamuK8639) and murine (LR-7) cell lines infected with PaHV2-cmvEGFP at MOI 1. Cells were incubated with cell permeable nuclear stain Hoechst 33342, and confocal microscopic images were taken at 24, 48 and 72 hpi at 10× magnification. Shown are merged images in which fluorescence from EGFP is colored green, while Hoechst-derived fluorescence is colored blue. The scale bars represent 100 µm.

3.3. Inhibition of Reporter Virus Infection by Antibodies and Antivirals

In a final set of experiments, we wanted to demonstrate the use of our reporter viruses for detecting inhibition of virus replication. First, we measured antibody-mediated neutralization of infection using sera from *Papio anubis*. For this, we set up a microneutralization assay, in which 100 pfu of PaHV2-cmvGluc were mixed with dilutions of sera. We chose two sera from animals tested positive for antibodies against PaHV2 in a commercially available colony surveillance assay and two sera from negative animals. Mixtures of virus and serum

were used to infect Vero76 cells and luciferase levels determined after 24 h. As shown in Figure 5a, the sera from seropositive animals (265, 276) clearly inhibited infection, while sera from seronegative animals (298, 299) were not effective. When luciferase activities were measured after 48 h, the assay changed into a binary readout (infection yes or no), where complete inhibition was detected for 1:20 (serum 276) or 1:40 (serum 265) dilutions, while for sera from seronegative animals, virus replication could be detected for all tested dilutions.

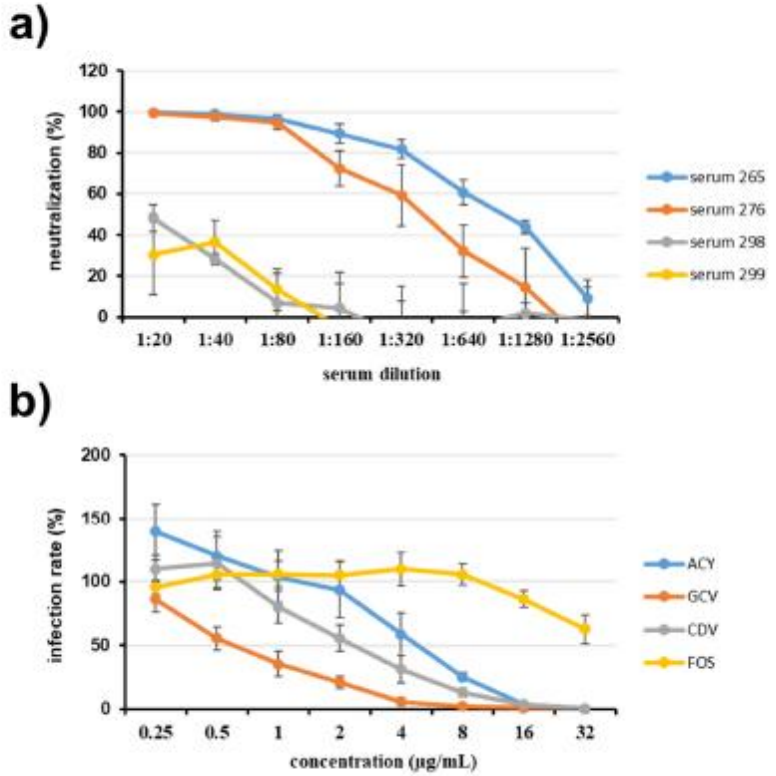

Figure 5. Inhibition of virus infection. (**a**) Neutralization assay. Virus (PaHV2-cmvGluc) and serum dilutions were preincubated for 1 h and then added to Vero76 cells in 96-well plates. After 24 h, activity of Gluc was assessed in the supernatant. Sera were from olive baboons (Papio anubis) previously tested positive (sera 265, 276) or negative (sera 298, 299) for antibodies against PaHV2 in a colony surveillance assay. The average of three independent experiments performed with triplicate samples is shown. Error bars represent SEM. (**b**) Antiviral inhibition assay. Vero76 cells seeded in 96-well plates were preincubated for 1 h with serial dilutions of antivirals acyclovir (ACY), ganciclovir (GCV), cidofovir (CDV) or foscarnet (FOS), followed by infection with PaHV2-Gluc-2A-UL35. After infection culture in presence of antiviral continued until 48 hpi, Gluc activity was measured from the supernatant. The average of three independent experiments, each performed with triplicate samples, is shown. Error bars represent SEM.

Next, we measured the effect of known antivirals effective against herpesviruses. In these experiments we employed PaHV2-Gluc-2A-UL35, since Gluc expression in this virus is coupled to expression of the late gene UL35. Thus, inhibitory effects of the antivirals acyclovir (ACY), ganciclovir (GCV), cidofovir (CDV) or foscarnet (FOS), which target herpesviral replication, should be detected with this virus. We preincubated Vero76 cells with inhibitor dilutions, infected with PaHV2-Gluc-2A-UL35 (MOI 0.01) and then continued incubation in the presence of inhibitor dilutions until luciferase activity was

measured at 48 hpi. As demonstrated in Figure 5b, clear inhibition of infection by ACY, GCV and CDV was observed, while FOS seemed to have only a slight inhibitory effect at the highest concentrations tested. The EC50 values determined for ACY (4.88 µg/mL, 95%CI: 3.14–7.76), GCV (0.67 µg/mL, 95%CI: 0.55–0.82) and CDV (2.48 µg/mL, 95%CI: 1.80–3.48) were slightly lower than determined by plaque assay [8]. In summary, we developed reporter viruses suitable for neutralization and inhibitor assays, which will be useful tools in screening experiments or comparative drug evaluation.

Overall, we were able to establish a recombinant system for PaHV2, which will be useful in comparative research on simplex viruses.

4. Discussion

Non-human primate (NHP) simplex viruses are considered to be similar to herpes simplex virus, but a few notable differences have been uncovered. For a detailed comparison on the molecular level, recombinant systems are required, but presently lacking for most NHP simplex viruses. We have previously succeeded in setting up such a system for CeHV2 [9], and now report a successful rescue of PaHV2.

In the development of the recombinant system for PaHV2, several obstacles had to be overcome. For one, it was not possible to clone parts of US-region using the fosmid methodology. This problem was solved by applying TAR to clone the missing regions. Sequencing of several clones used in initial rescue attempts uncovered deletions in palindromic regions of replication origins, which could be reduced by employing *E. coli* strains with increased palindromic stability [21]. The PaHV2 system, in its current form, still has some limitations. For instance, the TAR-based plasmids in their present form are high-copy plasmids in *E. coli*, making it difficult to apply recombineering. This problem will be solved in the future by conversion to plasmids bearing low copy F factor origins [42]. Recombineering will also help in reducing the extent of fragment overlapping, to make a larger region of the genome accessible to straightforward modification.

For sensitive detection and monitoring of infection, we developed several reporter viruses. For this, we inserted expression cassettes for Gluc and EGFP driven by the HCMV enhancer/promoter into the UL3/4 locus. PaHV2-cmvGluc allowed sensitive detection of infection, where values 100-fold over background were reached after 6 hpi, rendering it a useful tool to quantify infection. PaHV2-cmvEGFP allowed for localization of infected cells, but detection was much less sensitive than with the Gluc-expressing virus, allowing detection after 24 h. Part of this may be explained by the highly sensitive detection of Gluc in an enzymatic assay as compared to the non-enzymatic detection of EGFP [48]. In addition, EGFP has been reported to have limited stability in mammalian cells, while Gluc is highly stable [49,50]. Optimization of promoter or fluorescent protein may allow for a more sensitive detection and will make this an interesting system for single cell analysis, e.g., by flow cytometry. Using PaHV2-cmvGluc, we could demonstrate that the spectrum of cells lines susceptible to infection by PaHV2 appears to be broader than previously observed for CeHV2 [12]. In particular, PaHV2 demonstrated efficient growth in cells derived from rhesus macaques.

In addition, we were able to assess neutralization by sera from olive baboons. Sera from adult or aged animals (15 and 19 years) demonstrated inhibition of infection, while sera from infant animals (<1 year) were not effective, in agreement with in-house chip-based measurements of seropositivity and the published age-dependent seropositivity of baboons [51,52]. Complete neutralization was detected for 1:20 and 1:40 serum dilutions, a range also detected with microneutralization assays for HSV1 and NHP simplex viruses [40,53,54]. Because of the cross-reactivity of anti-PaHV2 antibodies with McHV1 [55,56], the assay could be used as an alternative and sensitive means to detect the presence of neutralizing antibodies against McHV1 in macaques, although this will require an in-depth analysis.

To analyze the effect of antiviral agents, we developed a reporter virus, PaHV2-Gluc-2A-UL35, in which Gluc was co-expressed with the late gene UL35 to allow measurement

of inhibition of virus replication. We could demonstrate inhibition of PaHV2 replication by acyclovir, ganciclovir and cidofovir, but not foscarnet, in line with published data [8]. The IC50 values determined in our assay were somewhat lower compared to published results determined with focus reduction assay. This difference may be explained by the different mode of measurement (late gene expression vs. plaque formation) and the different mode of virus spread (free spread vs. restricted, local spread). Overall, our reporter virus constitutes a highly sensitive assay system, which allows for automatized measurement.

Collectively, we feel that the recombinant system described here will be valuable in the analysis of neuropathogenicity of simplex viruses. Several genes have been implicated in neuropathogenesis, with ICP34.5 believed to be the most important factor for neuroinvasion of HSV1 and HSV2 [1,57]. However, in contrast to simplex viruses from hominid primates [58], simplex viruses from cercopithecid primates (Old World monkeys) lack an ICP34.5 homolog [5,59,60], and at present, it is not clear whether its function was substituted by a different gene. Neuropathogenesis after zoonotic transmission may therefore depend on different virulence genes. Indeed, for neuropathogenesis of PaHV2 in mice, it has been reported that the viral ribonucleotide reductase large subunit (UL39) has a major role, based on analysis of neurovirulent and apathogenic strains [10]. However, a detailed molecular analysis to determine the specific function of UL39 driving neurovirulence has not been performed. The recombinant system for PaHV2 described here will enable such studies and thus make a valuable contribution to the comparative analysis of primate simplex virus neuropathogenesis.

In summary, we developed a recombinant system for PaHV2 and reporter viruses, which will be useful in the analysis of infection, neutralization and antiviral activity. Finally, the system will also prove invaluable in the analysis of determinants of PaHV2 neuropathogenicity.

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6. Discussions

6.1. First publication - A fosmid-based system for the generation of recombinant *Cercopithecine alphaherpesvirus 2* encoding reporter genes

The classic marker transfer method for producing recombinant herpesviruses relies on the transfection of marker expressing plasmids followed by co-infection of wildtype virus. This method is frequently inefficient, labor intensive, and time consuming because of the necessity for virus cloning and purification. Furthermore, insertion of a selection marker is required to discriminate the recombinant virus form the wildtype virus. Therefore, a fosmid-based approach was used for producing recombinant CeHV-2. A mixture of five fosmid clones with ends overlapping between 2.3–6.4 kbp was obtained and allowed to recover infectious CeHV-2 which replicated with the same efficiency as wiltype virus.

The fosmid strategy combines the benefits of bacterial artificial chromososmes (BACs), which stably maintain large fragments of foreign DNA, and cosmids, which separate diploid regions and thus allow parallel engineering. Recombineering of herpesviral genomes using BACs has become the gold standard. However, changing diploid genes, contained in inverted repeat regions of many herpesviral genomes, in a seamless manner has proven difficult (Dai et al., 2012). Unfortunately, these regions are particularly important for *simplexvirus* biology because they contain key genes for lytic and latent control.

In cosmid-based systems diploid genes can be modified individually (Cunningham & Davison, 1993; Gray & Mahalingam, 2005). Furthermore, because only segments of the genome are manipulated at a time, cosmids provide a high level of biosafety. On the other hand, cosmids are present in bacterial cells at medium to high copy numbers and have proven difficult to change using traditional

or recombination-based techniques (Bestman-Smith & Boivin, 2003; Gray & Mahalingam, 2005; Kong et al., 1999). Fosmids, in contrast, are only found in low copies because they have an F-factor-derived low copy replication origin for plasmid maintenance and a separate inducible origin for DNA synthesis (Wild et al., 2002), and the current investigation demonstrates that they are accessible to alteration by recombineering. Recombineering has recently also been applied to fosmids of PRV and MDV (Li et al., 2016; Qi et al., 2020; Zhou et al., 2018). As a result, the fosmid-based strategy used to generate and modify CeHV-2 should be applicable to other herpesviruses as well and should have advantageous as compared to related techniques.

In order to demonstrate parallel engineering and to enable straightforward viral replication detection, reporter genes (EGFP, mCherry, and iRFP670) were successfully fused to three genes: ICP4, UL35 and UL10. These genes were chosen because their successful fusion had been already described (Hogue et al., 2015). The immediate-early protein ICP4, encoded by RS1, is found in the inverted repeats of the US region. The second gene, UL35, codes for the small capsid protein. Finally, UL10 is a late gene that codes for glycoprotein M. The separation of diploid genomic fragments on different fosmids enables for simultaneous and seamless alteration of a diploid gene (RS1/ICP4), reducing the number of steps necessary. Using this approach, reporter viruses that expressed ICP4-EGFP, UL35-mCherry or UL10-iRFP670 fusion proteins were recovered and replicated like the wild type virus. A virus carrying several reporter genes, CeHV-2 ICP4-EGFP UL35-mCherry, was also rescued, demonstrating that several reporter constructs could be combined in one virus. The production of a virus expressing all three reporter genes failed, probably due to the growth defects associated with the reporter cassettes. In

conclusion, the data of the present thesis show that recombineering can be applied to create CeHV-2 reporter viruses. Therefore, it showed that this technology could be utilized to modify large DNA viruses, such as herpesviruses, in one step.

For sensitive quantification of viral replication, Gaussia luciferase (Gluc) was connected to ICP4's C-terminus linked by PTV1-derived 2A stop-go sequence between the two proteins. This should allow for the release of Gluc and thus allow the sensitive and convenient detection of infection. The Gluc-expressing reporter virus was used to investigate CeHV-2 cell line tropism and drug sensitivity. Acyclovir treatment lowered Gluc activity to near-zero levels, suggesting that robust reporter activity was dependent on viral replication. In terms of cell line tropism, the virus replicated efficiently in African green monkey Vero76 and Cos7 (*Cercocebus spp*.) cell lines in agreement with published work (Eberle & Hilliard, 1984; Tyler et al., 2005). CeHV-2 replication was also observed in human A549, Huh7.5, HeLa, and HEK293T cells, which is consistent with published finding that CeHV-2 replicates in human foreskin fibroblasts (Eberle and Hilliard, 1984), KB (human epithelial carcinoma), and fetal diploid lung cells. In addition, efficient replication was observed in marmoset fibroblasts, which has not been reported previously. Cell lines generated from rhesus macaques, on the other hand, showed no evidence of viral replication (LLC-MK2, sMAGI, TeloRF, MaMuK8639). This is in contrast to one older report, which stated that the rhesus macaque cell line LLC-MK2 could be productively infected (Malherbe & Harwin, 1963).

It cannot be ruled out that these differences are related to the use of reporter virus, which demonstrated a mild growth defect, in the current study, while wild type virus was used in the previous investigation. However, preliminary findings suggested that infection of LLC-MK2 cells with wild type virus was inefficient. Finally,

it is possible that CeHV-2 can infect rhesus macaque cells in general, and that the cell lines studied here lack host components essential for viral replication or express a host factor such as TRIM5α, which might block CeHV-2 infection and has been reported to restrict HIV (Zheng et al., 2005) and HSV (Reszka et al., 2010) infection of rhesus macaque cells.

6.2. Second publication – A recombinant system and viruses encoding reporter genes for *Papiine alphaherpesvirus 2*

In this work, the establishment of a recombinant system for PaHV-2 was reported, based on the fosmid strategy applied to CeHV2 (Chukhno et al., 2019). However, here, the fosmid strategy had to be combined with the TAR-based cloning approach. Initially, the same approach as for generating recombinant CeHV2 was applied. However, several challenges had to be addressed in the development of the PaHV-2 recombinant system. In order to get clones spanning the full genome, random fragment (sheared) and direct specific fragment (unsheared) cloning was applied. Despite screening over 2000 clones, clones containing US and IRS regions and the centre of the UL region were not recovered. In parallel, a strategy for cloning the entire, unfragmented genome as a BAC has also been pursued, as this has been widely applied for cloning and modifying of herpesviral genomes. However, all attempts failed and only partial genomes could be recovered from *E. coli*.

TAR-based cloning was applied to fill the gap in the middle of the UL region as well as in the end region of the genome (UL-IR-US). This method was chosen because it is based on a host organism capable of taking up big DNA fragments, yeast, and its application in cloning and assembling genomes of herpesviruses and other viruses has been previously documented (Oldfield et al., 2017; Thi Nhu Thao et al., 2020; Vashee et al., 2017). TAR is a unique method for selective isolation of large fragments (up to almost 300 kb) from complex genomes in the yeast *Saccharomyces cerevisiae* as circular yeast artificial chromosomes (YACs). The missing regions of PaHV-2 could be successfully cloned in yeast using TAR-based cloning. After transfer to *E. coli*, sequencing of clones used in initial rescue attempts revealed deletions in three regions of the genome, which turned out to be the

palindromic regions of viral lytic replication origins (Lockshon & Galloway, 1986; Weller et al., 1985). This problem could be resolved by using *E. coli* strains with increased palindromic stability, such as PMC103 (Doherty et al., 1993; Gietz & Sugino, 1988). Combining fosmid- and TAR-based cloning, recombinant PaHV-2 was successfully rescued and showed the same characteristics as the wildtype virus regarding both growth kinetics and genome structure, as assessed by restriction digest and sequencing. The plasmid system for rescuing PaHV2 has some drawbacks in its current state. For example, TAR-based plasmids in their current form are high-copy plasmids in *E. coli*, which is not optimal for recombineering. Therefore, conversion to plasmids with low copy F factor origins will be needed to overcome this problem in the future (Thapana et al., 2014). Recombineering will also aid in reducing fragment overlaps, allowing for more uncomplicated change of a greater section of the genome.

Several reporter viruses for sensitive detection and monitoring of infection were generated by inserting Gluc (Gaussia luciferase) and EGFP (enhanced green fluorescent protein) expression cassettes, driven by the HCMV enhancer/promoter, into the UL3/4 locus. This locus was chosen, because it has an intergeneric region in which insertion of foreign genes will not compromise growth or virulence (Morimoto et al., 2009; M. Tanaka et al., 2004; S. Tanaka et al., 2004). As a result, the replication of all reporter viruses was indistinguishable from the wild type PaHV-2. PaHV2-cmvGluc allowed for sensitive detection of infection, with reporter activity reaching levels 100-fold over background after 6 hours postinfection, making it a valuable tool for infection quantification. Localization of infected cells was possible with PaHV2-cmvEGFP. However, detection was significantly less sensitive than with the Gluc-expressing virus, permitting detection only after 24 hours. Part of this could

be explained by Gluc's greater sensitivity in an enzymatic assay compared to EGFP's non-enzymatic detection (Tannous et al., 2005). Furthermore, EGFP has been shown to have limited stability in mammalian cells, whereas Gluc is very stable (Verkhusha et al., 2003; Wurdinger et al., 2008). The optimization of the promoter or fluorescent protein may allow for more sensitive detection, to make this a suitable and promising system for single cell analyses using flow cytometry, for example. Using multicycle growth monitoring via Gluc activity, it could be observed that the spectrum of cell lines susceptible to PaHV2 infection is greater than previously reported for CeHV2 (Chukhno et al., 2019). In particular, PaHV2 but not CeHV-2 was found to replicate well in cell lines from rhesus macaques, and these results could be confirmed using wild type PaHV-2 (data not shown).

In order to investigate PaHV-2 sensitivity to antivirals, we created PaHV2-Gluc-2A-UL35, a reporter virus in which Gluc was co-expressed with the late gene UL35. All antiviral compounds tested (acyclovir, ganciclovir, and cidofovir) except foscarnet inhibited PaHV2 replication, which is consistent with previous research (Brush et al., 2014). Acyclovir (ACV) is the “gold standard” for antiherpetic therapy (Schaeffer et al., 1978) and, jointly with ganciclovir (GCV), is also widely used for treatment of herpesvirus infection. However, both antivirals have a number of drawbacks. Importantly, ACV has a low blood-brain barrier penetration with a short plasma half-life, and the bioviability of GCV is also limited (Thust et al., 2000). In agreement with antiviral sensitivity assay with McHV-1, GCV and cidofovir (CDV) also inhibited the virus replication (Maxwell et al., 2020) and were the most efficacious compounds in a mouse model, but none of them will be effective once the McHV-1 has infiltrated the CNS (Brush et al., 2014). In comparison to published data obtained with a focus reduction test, the IC50 values determined with reporter

virus in the present study were slightly lower. This discrepancy could be explained by the difference in measuring methods (late gene expression vs. plaque formation) and virus dissemination modes (cell free spread vs. restricted, cell-associated spread). Finally, antibody neutralization of PaHV-2 was also assessed using the reporter virus. Sera from adult baboons readily neutralized the virus while sera from infant animals did not, suggesting that it could be utilized as an alternative assay to neutralize the presence of McHV-1 in macaques for the antibody cross-reactivity between PaHV-2 and McHV-1.

The recombinant system described here will likely be useful in the study of the genetic determinants of simplexvirus neuropathogenicity. For HSV-1 and HSV-2 neuroinvasion, the RL1 gene, encoding the protein ICP34.5, is generally considered to be the most important factor (Davis et al., 2014; Roizman & Whitley, 2013). However, RL1 is only conserved in simplexviruses from humans or chimpanzees (Luebcke et al., 2006), while an ICP34.5 homolog is lacking in simplexviruses from old world monkeys (Perelygina et al., 2003; Tyler et al., 2005; Tyler & Severini, 2006). It is at present unknown whether its role was replaced by another gene. Studies of PaHV-2 strains differing in neurovirulence revealed that the viral ribonucleotide reductase large subunit (UL39) is a determinant of PaHV-2 neurovirulence in mice (Black et al., 2014). The UL39 protein is important for viral replication in non-dividing cells, such as neurons. In addition, it has been implicated that this gene plays an important role in anti-apoptotic function in infected cells, as well as in the regulation of the interferon response (Black et al., 2014; Dufour et al., 2011; Guo et al., 2015; Lembo & Brune, 2009). So far, deeper molecular investigation has not been undertaken to elucidate the particular function of UL39 in promoting PaHV-2 neurovirulence. Such research will be possible thanks to the

recombinant PaHV2 system developed here, which will contribute to the comparative analysis of primate simplex virus neuropathogenesis.

7. Bibliography

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8. Appendix

List of abbreviations

A549	cell line derived from human lung carcinoma epithelial cell
ADH1	Alcohol dehydrogenase class 1
ARS	Autonomously replicating sequence
ATG	Amino acid which codes for methionine
α-TIF	alpha trans-inducing protein
BAC	Bacterial artificial chromosomes
BSL	Biosafety level
BV	B virus
Cas9	protein which plays a vital role in the immunological defense of certain bacteria against DANN viruses and plasmids
CDC	The Center for Disease Control and Prevention
CeHV-2	Cercopithecine alphehrepesvirus type 2
CEN6	Chromosome VI centromere in yeast
CHEF	Contour-clamped Homogenous Electric Field
cm	chloramphenicol
cmv	citomegalovirus
Cosmid	Plasmid with cos-site
DH10B	Competent bacterial cells of Eschericia coli
DNA	Deoxy-ribonucleic acid
dsDNA	double stranded Deoxyribo Nucleic Acid
E	Early protein
EBV	Epstein barr virus
EGFP	Enhanced Green Fluorescent Protein
EHV	Equine herpesvirus
GaHV-2	Gallid alphaherpesvirus 2
G+C	Guanin + Cytosine
Gluc	Gaussia luciferase
Gly	Glycine
gE	Glycoprotein E
GFP	Green fluorescence protein

GS1783	derivative bacterial cells of DH10B that express lambda Red recombination enzymes in temperature-inducible fashion and the I-SceI restriction enzyme
HCMV	Human cytomegalovirus
HeLa	immortal cell line which is derived from cervical cancer cells
HEK293T	Human Embryonic Kidney 293 cells
HHV-1	Human alphaherpesvirus type 1
HHV-2	Human alphaherpesvirus type 2
HHV-3	Human alphaherpesvirus type 3
HHV-4	human gammaherpesvirus type 4
HHV-5	human betaherpesvirus 5
HHV-6A	human betaherpesvirus 6A
HHV-6B	human betaherpesvirus 6B
HHV-7	human betaherpesvirus 7
HHV-8	human betaherpesvirus 8
HIS3	gene in Saccharomyces cerevisiae, encodes a protein which catalyses the six step in histidine biosynthesis
HTS	high troughput sequencing
Huh7.5	Human hepatoma derived human liver cell line
HSE	Herpes simplex enchepalitis
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HVP2	Herpesvirus papio type 2
HVT	Herpesvirus Turkey
IC50	the half maximal inhibitory concentration
ICP	Infected cell protein
ICTV	International Committee on Taxonomy of Virus
IE	Immediate early protein
IR	Inverted repeat
iRFP670	constitutively fluorescent near ir fluorescent protein which is derived from Rhodopseudomonas palustris CGA009
IRL	Inverted repeat long
Kb	Kilo base
KSHV	Kaposi’s sarcoma-associated herpesvirus

L	Late protein
LLC-MK2	Rhesus Macaca Kidney Epithelial cells
loxP	a site on the bacteriophage P1 consisting of 34 bp
MaMuk8639	Rhesus Macaca mulatta Kidney cells
mCherry	constitutive red fluorescent derived from DsRed of Discosoma sea anemones
McHV-1	Macaccine alphaherpesvirus type 1
McHV-2	Macaccine alphaherpesvirus type 2
McHV-3	Macaccine alphaherpesvirus type 3
MCS	Multiple Cloning Sites
MDV	Marek's disease virus
mRNA	Messanger-RNA
nm	nano meter
NHP	Non-human primates
NIH	National Institute of Health
ND-10	nuclear domain-10
ORF	Open Reading Frame
oriV	origin of replication
pac1	an A rich region flanked by poly C
pac2	CGCGGCG near an A-rich region
PaHV-3	Panine alphaherpesvirus type 3
PaHVP-2	Papiine alphaherpesvirus type 2
parA/B	Partitioning protein A/B
PCR	Polymerase Chain Reaction
PKR	Protein kinase R
PMC103	improved strain of E.coli
PRV	Pseudo rabies virus
RL1	gene codes for neurovirulence factor ICP34.5
Rec-A	Recombination A
repE	replication initiation protein
RNA	Ribonucelid acid
SA-8	Simian Agent 8
Ser	Serine

sMAGI	rhesus macaque epithelial cell line engineered to express human CD4
sopA-B-C	essential protein in plasmid partitioning during cell division
SSRs	Short sequence repeats
TAR	Transformation associated recombination
TeloRF	Telomerase-immortalized rhesus fibroblasts cell line
TK	Thymidine kinase
trfA	a gene encodes the essential replication initiation protein oriV
TRL	Tandem repeat
UL	Unique long
URA3	a marker gene on chromosome V in Saccharomyces cerevisiae
US	Unique short
VHS	Virion host shutoff
VNTRs	Variable number of tandem repeats
VP16	Virion protein 16
VZV	Varicella-zoster virus
WHO	World health organization
YACs	Yeast artificial chromosomes

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