1. The *Herpesviridae*

1.1. Nomenclature and classification

The order *Herpesvirales* contains three subfamilies: the *Alloherpesviridae*, the *Malacoherpesviridae* and finally the *Herpesviridae*. The family *Herpesviridae* is the largest and is composed of three subfamilies: the *alpha-*, *beta-* and *gammaherpesvirinae.* This viral family contains at present more than 130 species sharing common features (Ackermann 2004; Davison *et al.* 2005; Davison *et al.* 2009). Indeed, they are spherical viral particles sizing from 150 to 300 nm. They all have a linear double strand DNA (dsDNA, double strand DNA) accompanied by some rare RNA molecules (Bresnahan and Shenk 2000; Bechtel *et al.* 2005; Jochum *et al.* 2012). These nucleic acids are associated with an icosaedral nucleocapsid composed of 162 capsomers, surrounded by a tegument containing proteins with regulatory functions. Lastly, a lipidic viral envelope, made from cellular membranes and containing viral glycoproteins, surrounds this assembly (Figure 1). Besides these morphological properties, several biological properties (Ackermann 2004) are also shared by all the *Herpesviridae*. Assuredly, they all have their own enzymatic machinery dedicated to the synthesis of their nucleic acids, which occurs in the nucleus of the infected cell, as well as the assembly of viral capsids. In addition, all the viruses belonging to this family produce a lytic cycle, often leading to the death of the infected cell, before establishing latency in certain cell types (Roizman and Pellet 2007). The term latency is used to describe the state of infection both at cell-level and at host-level. Indeed, a host is considered latently infected when acute primary infection is resolved (Barton *et al.* 2011).

The classification into three subfamilies was mainly established on the basis of host range, but also on the spectrum of cells capable of supporting viral latency *in vivo* (Roizman 1996), as only some cell types can support latency. Thus, *alphaherpesvirinae* are neurotropic viruses, establishing latency in specific neuronal populations while *beta-* and *gammaherpesvirinae* will establish latency essentially in lymphocytes and thus persist in lymphoid organs (Roizman and Pellet 2007). *Gammaherpesvirinea* are particular in the sense that, contrary to *alpha-* and *betahepresvirinae*, they establish early latency preferably to lytic infection. Moreover, *gammaherpesvirinae* are often associated with lymphoproliferative diseases and/or other non lymphoid cancers (Roizman 1996).

The classification is now completed by more objective molecular criteria and was largely confirmed by phylogenetic analyses (McGeoch *et al.* 1995; McGeoch *et al.* 2000; McGeoch *et al.* 2005; Davison *et al.* 2009). The *gammaherpesvirinae* subfamily classification was recently updated by the *International Committee on Taxonomy of Viruses* (ICTV; http://www.ictvonline.org), and now contains 4 genera (Table 1):

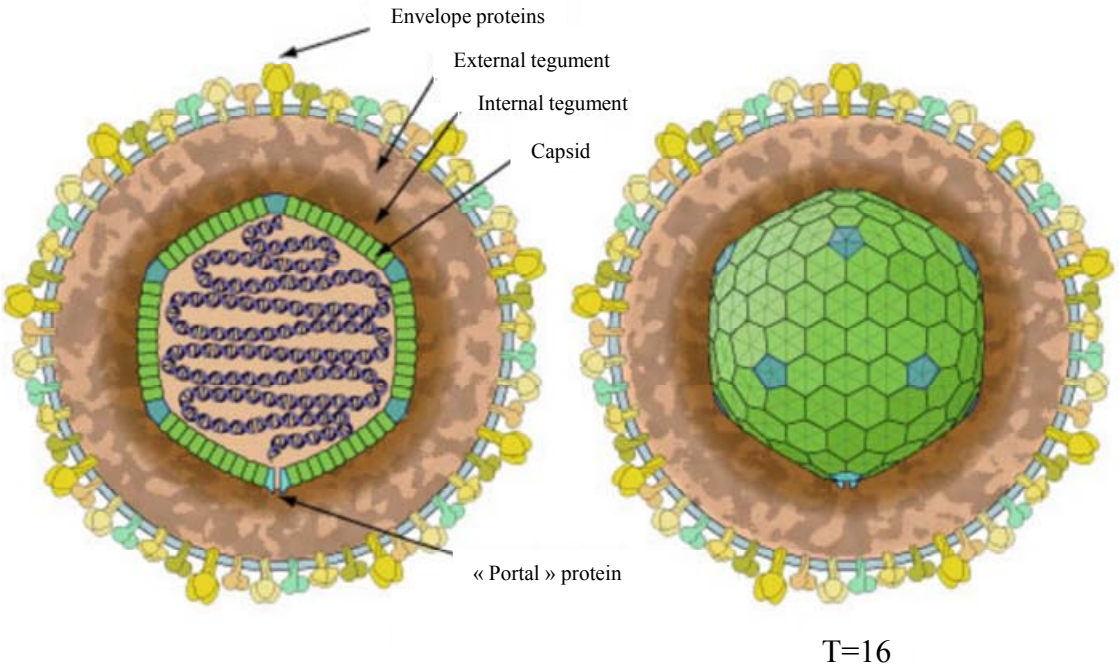


Figure 1. Morphology of herpesviruses. The viral particle is made of a capsid containig the viral genome, an intermediate proteic layer named tegument and a viral envelope with glycoproteins. The capsid is made of 150 hexons (green) and 11 pentons (blue) plus the portal protein to form an isocaedral structure (T=16) with pentons at the tops of the structure. One of the 12 tops is a pore by which DNA is enclosed in the capsid. From http://viralzone.expasy.org/all_by_species/181.html

- the *lymphocryptovirus* with EBV or *human herpesvirus 4* and some viruses infecting primates of the Old World;
- the *rhadinovirus* including KSHV or *human herpesvirus 8*, several viruses infecting primates of the Old and New Worlds but also other mammals;
- the *Percavirus* (standing for **pe**rissodactyl and **ca**rnivore) with the type-species *Equid herpesvirus 2* (EHV-2);
- the *Macavirus* (standing for **Ma**lignant **ca**tarrhal fever) with the type-species *Alcelaphine herpesvirus 1* (AlHV-1).

The newly introduced designations, *Percavirus* and *Macavirus,* take into account the recent characterization and the natural host specificities (artiodactyls versus perissodactyls) of the following viruses: *Bovine herpesvirus 6, Caprine herpesvirus 2, Suid herpesvirus 3, Suid herpesvirus 4* and *Suid herpesvirus 5* (Chmielewicz *et al.* 2001; Chmielewicz *et al.* 2003; Davison *et al.* 2009).

1.2. The viral cycle

1.2.1. The lytic infection

The multiplication cycle of herpesviruses is globally constant among members of the group and represented in figure 2A. The attachment of the viral particle on the cellular surface results from the interaction between one or more viral glycoproteins and cellular receptor(s). Frequently, primary interactions between the virus and the cell are of low specificity and imply the presence of glycosaminoglycans (GAG) at the surface of the cell. It was demonstrated that the ubiquitous presence of GAG at the cellular surface is an important co-factor for the entry of several herpesviruses into the cells, allowing the establishment of the initial contacts between the virus and the target cell (Shukla and Spear 2001). After these low affinity interactions, higher affinity contacts are established, implying one or more cellular receptors and viral envelope glycoproteins. These specific interactions are an important determinant of the sensitivity of the cell to infection and therefore of the viral tropism and host range. As shown for other viral families (Helenius 2007), herpesviruses are also able to use different cellular receptors and several cell surface molecules can be necessary for viral attachment. This has particularly been documented with HSV-1.

When the virus is attached, the viral envelope will fuse with the cellular membrane, leading to the release of proteins from the tegument and from the nucleocapsid into the cytoplasm (Roizman 1996). Two mechanisms of entry are described: on one hand, the entry can be performed by fusion of

Order	Family	Subfamily	Genus	Species
Herpesvirales	*Malacoherpesviridae*			
	Alloherpesviridae			
	Herpesviridae	*Alphaherpesvirinae*		
		Betaherpesvirinae		
		Gammaherpesvirinae	*Lymphocryptovirus*	*Callitrichine herpesvirus 3*
				Cercopithecine herpesvirus 14
				Gorilline herpesvirus 1
				Human herpesvirus 4
				Macacine herpesvirus 4
				Panine herpesvirus 1
				Papiine herpesvirus 1
				Pongine herpesvirus 2
			Macavirus	***Alcelaphine herpesvirus 1***
				Alcelaphine herpesvirus 2
				Bovine herpesvirus 6
				Caprine herpesvirus 2
				Hippotragine herpesvirus 1
				Ovine herpesvirus 2
				Suid herpesvirus 3
				Suid herpesvirus 4
				Suid herpesvirus 5
			Percavirus	***Equid herpesvirus 2***
				Equid herpesvirus 5
				Mustelid herpesvirus
			Rhadinovirus	*Ateline herpesvirus 2*
				Ateline herpesvirus 3
				Bovine herpesvirus 4
				Human herpesvirus 8
				Macacine herpesvirus 5
				Murid herpesvirus 4
				Saimiriine herpesvirus 2

Table 1: Nomenclature and classification of gammaherpesviruses. Murid herpesvirus-4 classification is mentioned by grey areas and type-species are indicated in bold. From http://ictvdb.org/Ictv/index.htm.

the viral envelope with the cellular plasmic membrane and on the other hand, an entry by endocytosis followed by the fusion of the endosomal membrane with the viral envelope is also possible (Roizman 1996). Fusion is mediated by, at least, glycoproteins B, H and L that form the core entry machinery conserved in all the *Herpesviridae*. gB is the fusion protein (Pertel 2002; Sharma-Walia *et al.* 2004; Backovic *et al.* 2007; Vanarsdall *et al.* 2008; Atanasiu *et al.* 2010). The roles of gH and gL are less clear and still controversial.

Once into the cytoplasm, free capsids follow the network of tubulin microtubules to reach a nuclear pore (Granzow *et al.* 1997; Sodeik *et al.* 1997). This phenomenon implies the « dynein/dynactin » proteic motor (Dohner *et al.* 2002). Once transported to the nucleus, the capsid releases nucleic acids at a nuclear pore (Sodeik *et al.* 1997; Peng *et al.* 2010). After its entry into the nucleus, the viral genome is rapidly circularized in the absence of any viral protein synthesis, suggesting a mechanism under dependence of cellular proteins and/or structural viral proteins (Poffenberger and Roizman 1985). The transcription of the herpesviruses' genome then proceeds, following a series of events that are strictly regulated by viral proteins. We can distinguish three transcriptional phases. The first wave of transcription is initiated by the regulatory tegument proteins and allows the transcription of genes called "immediate-early" (IE) or α. The proteins synthesized at this stage essentially act as activators of transcription. Subsequently, the genes "early" (E) or β, including the viral DNA polymerase, are transcribed. The last phase, called late (L) or γ, allows the synthesis of structural proteins including the envelope glycoproteins and the capsid proteins. Replication of viral DNA marks the separation between early and late phases (Honess and Roizman 1974; Honess and Roizman 1975; Jones and Roizman 1979).

Once the circularized viral genome is inside the nucleus of the host cell, the tegument proteins that are present will interact with various cellular components to induce IE gene transcription. This first transcriptional step is carried out by the cellular RNA polymerase II. Protein synthesis occurs in the cytoplasm and some of these proteins are then imported into the nucleus to stimulate transcription of E and L genes, but also to inhibit the transcription of IE genes. E genes show a peak of expression 4-9 hours after infection of the cell, while the L gene expression is maximal after the beginning of the synthesis of viral DNA. These genes can be classified into two categories: the expression of partial late genes (or γ1) is increased by the synthesis of viral DNA, whereas the expression of real late genes (or γ2) is entirely dependent on viral DNA synthesis (Roizman 1996). Viral DNA replication is a critical step in the replication cycle of herpesviruses. It is placed under the control of the viral DNA polymerase synthesized during the early phase and starts at one or more origins of replication. The synthesis of viral genomes occurs through the mechanism of "rolling circles" (Jacob *et al.* 1979; Ackermann 2004), generating units consisting of concatemeric structures separated by sequences that

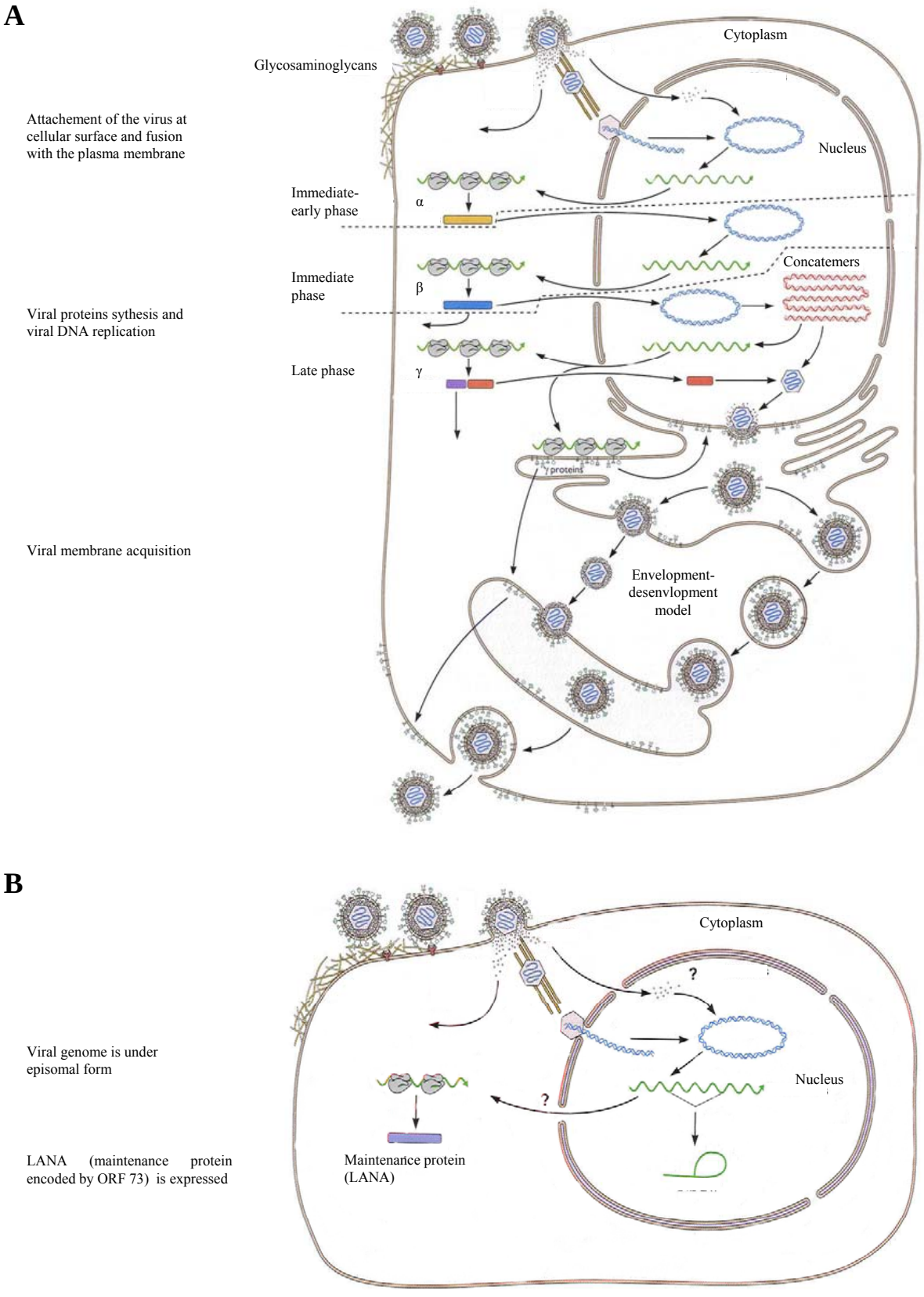


Figure 2. Representation of the general cycle of herpesviruses, comprising the lytic (A) and the latent (B) state. From Flint *et al.*, 2000.

are targets for enzymatic cleavage. Indeed, during the encapsidation of the viral genome, these enzymes recognize and cleave target sequences in order to ensure the encapsidation of one genome by a virus particle (McVoy *et al.* 2000).

Herpesvirus morphology is highly constant, suggesting similar morphogenesis processes (Mettenleiter *et al.* 2009). The proteins of the capsids are synthesized in the cytoplasm and are translocated from the cytoplasm to the nucleus where the capsids are assembled by an autocatalytic process (Homa and Brown 1997). In the structure of the capsids, a portal complex (Chang *et al.* 2007) by which viral DNA is encapsidated is found (Newcomb *et al.* 2006). Mechanisms ruling the egress of the nucleocapsids from the nucleus to the cytoplasm are not well known. However, several mechanisms, presented in figure 3, were proposed: the model of nuclear pore egress, the "luminal" model and finally the "envelopment/deenvelopment" model (Wild *et al.* 2005; Mettenleiter *et al.* 2006). The nuclear pore egress model was proved for Bovine herpesvirus 1 (BoHV-1) (Wild *et al.* 2005). According to this model, the capsids that are present in the nucleus can attain the cytoplasm *via* a previously enlarged nuclear pore. These free cytoplasmic capsids bud in vesicles derived from the Golgi apparatus and the enveloped virions are released at the surface of the cell (Wild *et al.* 2005). The second model proposed, the « luminal » model, suggests the transport of the enveloped virions from the nucleus by a secretion pathway which maintains the integrity of the envelope acquired earlier at the level of the inner nuclear membrane. This model implies *in situ* modifications of the envelope's proteins (Darlington and Moss 1968; Johnson and Spear 1982; Campadelli-Fiume *et al.* 1991). The enveloped viruses are finally released in the extracellular environment (Roizman and Taddeo 2007). However, the most likely model is the envelopment/deenvelopment one, first suggested by Siminoff and Menefee in 1966 as a part of HSV-1 (Herpes simplex 1) morphogenesis and then confirmed by electronic microscopy (Stackpole 1969). According to this model, viral capsids in the nucleus bud at the internal nuclear membrane. Pre-enveloped viruses are then localized between the inner and the outer nuclear membrane. It was demonstrated that nuclear actin filaments are used to mobilize HSV-1 viral capsids (Forest *et al.* 2005). The primary envelope acquired during budding through the inner nuclear membrane is then lost because of the fusion with the external nuclear membrane. This leads to the release of free nude capsids into the cellular cytoplasm (reviewed in: Mettenleiter 2002; Mettenleiter 2004; Mettenleiter *et al.* 2006; Mettenleiter *et al.* 2009). Capsids then transit in the cytoplasm to acquire, on one hand, tegument proteins and, on the other hand, envelope glycoproteins, by budding in Golgi apparatus vesicles. Mature virions are then released at the surface of the cell by exocytosis (Mettenleiter 2006; Mettenleiter *et al.* 2006).

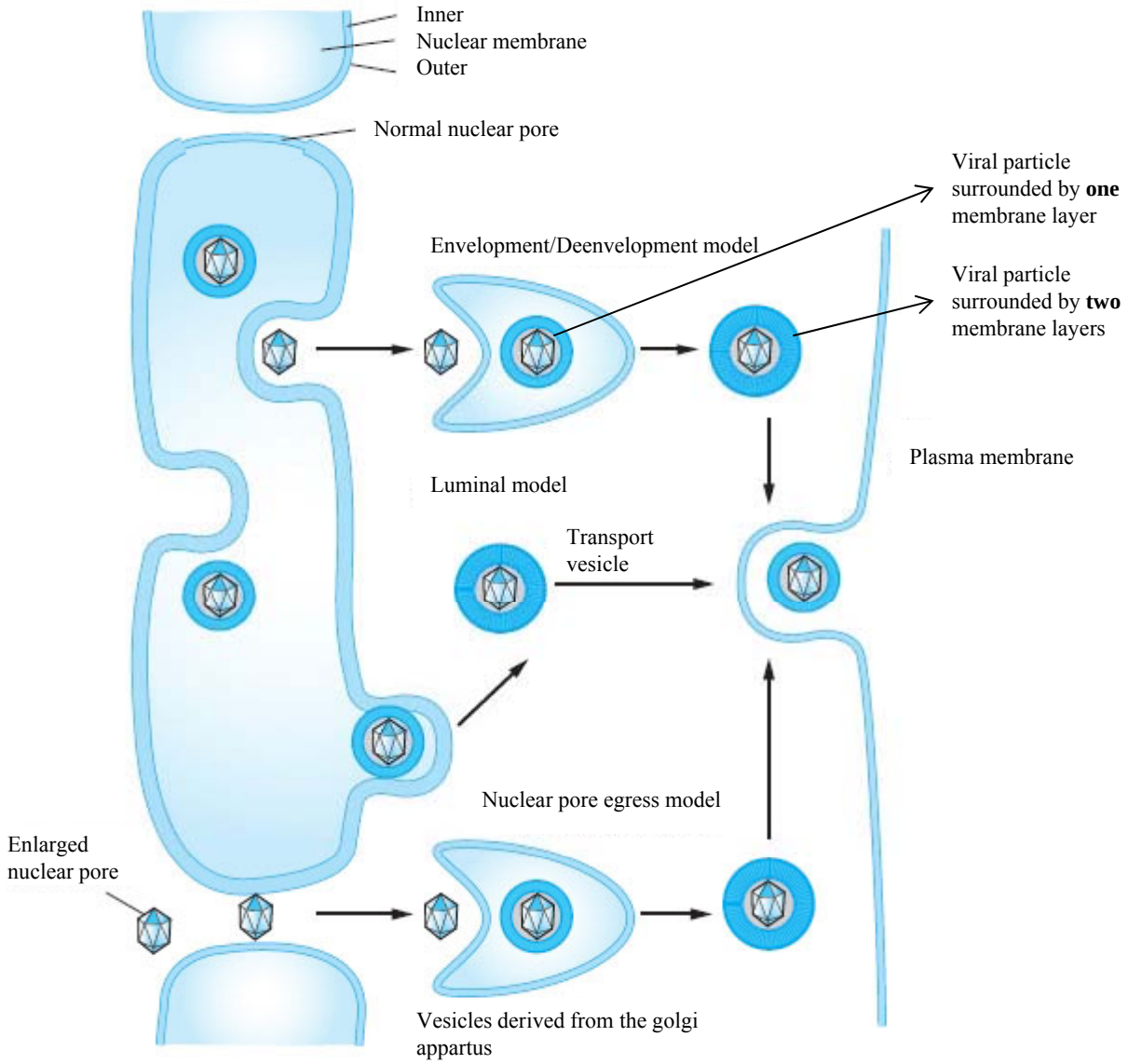


Figure 3: Models for herpesviruses egress from the host cell. 3 pathways are proposed. For the envelopment/deenvelopment model, capsids undergo primary envelopment at the inner nuclear membrane, are deenveloped at the outer nuclear membrane, reenveloped at cytoplamics membrane and then transportated to plama membrane by vesicles. The vesicular memebrane fuses with the plasma membrane to release the virion in the extracellular space. According to the luminal model, capsid is enveloped at the inner membrane and enters a vesicle at the outer nuclear memebrane. The vesicle traffics in the cytaplasm to reach plasma membrane where the virion is released. Finally, in the nuclear pore egress model, capsids exit the nuclei by a nuclear pore, bud into cytoplasmic vesicles to acquire the envelope and are released at the plasma membrane. Adapted from Roizman *et al*., 2007.

1.2.2. The latent infection

The ability to establish a latent state of infection is a fundamental and common characteristic of all herpesviruses (Roizman 1996). This state is described as the maintaining of the virus in the host cell in absence of a productive cycle (Figure 2B). During latency, the viral genome is maintained in the cellular nucleus in a circular form called episome. For the gammaherpesviruses, the viral episome is associated with the cellular genome (Vogel *et al.* 2010). For the rhadinoviruses, the mechanism implies LANA protein or orthologs (Garber *et al.* 2002; Fejer *et al.* 2003). This protein is highly preserved and bifunctional: the N-terminus links to cellular chromatin, whereas the C-terminus interacts with different sequences present in the polyrepetitive DNA (prDNA) units located at the ends of the viral genome (Griffiths *et al.* 2008). This interaction allows the initiation of the viral DNA replication by cellular enzymes and the anchoring of the viral episome to the cellular chromosome (Piolot *et al.* 2001; Ohsaki and Ueda 2012). When the cell is dividing, this anchoring allows the random distribution of episomes between daughter cells, but also avoids their loss in the cellular cytoplasm.

The molecular mechanisms governing the initiation of latency are not well known (Flint *et al.* 2000). However, the establishment of latency always induces a drastic limitation of viral gene transcription associated or not with the production of viral proteins. Thus, a low level of α or β genes can occur but is not sufficient to initiate a productive infection. Alphaherpesviruses only express LATs transcripts (*latency associated transcripts*) (Jones 2003), but beta- and gammaherpesviruses express latency proteins (Lee *et al.* 1999; Ballestas and Kaye 2001; Cardin *et al.* 2009). Maintaining this state on a long term requires the existence of specific and evolved immunoevasion mechanisms allowing the virus to escape the host immune surveillance and persist. The mechanisms implied will be addressed later in this chapter. Lastly, recent studies have demonstrated the existence of miRNA (micro RNA), produced from latency-associated transcripts, for members of all the three subfamilies of herpesviruses (Pfeffer *et al.* 2005). Essentially, their role seems to be in the helping of the maintenance of latency by modulation of cellular immunity and cellular apoptotic pathways, but also by the restraining of the viral lytic cycle (Cai *et al.* 2005; Burnside *et al.* 2006; Lu *et al.* 2008; Umbach *et al.* 2008; Wang *et al.* 2008)

Following an exogenous stimulus, the latency state can be interrupted. Indeed, physiological changes in the cell may provide the needed permissiveness to the cell, allowing it to support a productive infection. The viral genome is then transcribed with more efficiency and is replicated leading to the production of new virions. To date, little is known about mechanisms and stimuli causing efficient viral reactivation of gammaherpesviruses. However, several studies suggest a

role for TLR (*Toll-like* receptor). Indeed, these receptors have a crucial role concerning innate immunity and are able to recognize specific patterns, named PAMP's (pathogen associated molecular patterns), and to provoke a rapid immune response toward pathogens that have induced their activation. Several recent studies highlighted the reactivation of herpesviruses following the activation of TLR's. For example, in 2009, Gargano *et al*. demonstrated that the stimulation of the TLR 3, 4, 5 and 9 by their specific ligands increases the MuHV-4 viral load in mice at 42 days post infection (Gargano *et al.* 2009). In the same way, the activation of the KSHV's lytic genes transcription was observed when the TLR 7 and 8 were stimulated (Gregory *et al.* 2009). Although this mechanism is probably not the only one implied, this data suggests a strong link between innate immunity stimulation and the reactivation of the viral lytic cycle of the gammaherpesviruses. Moreover, the final differentiation of B cells infected by MuHV-4 or EBV into plasmocytes seems to be a signal for the efficient reactivation of these two viruses (Laichalk and Thorley-Lawson 2005; Liang *et al.* 2009).

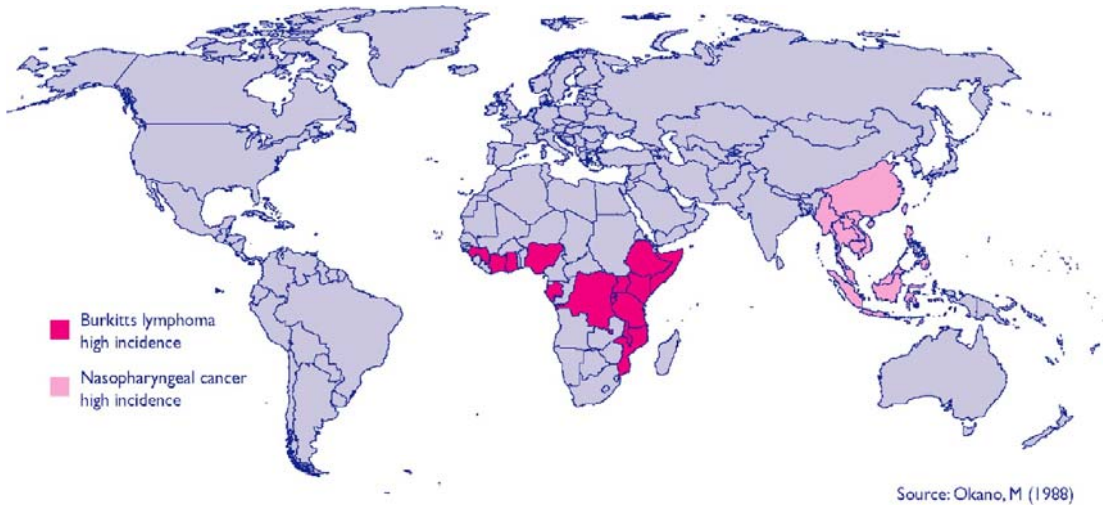
2. The *gammaherpesvirinae*

The *gammaherpesvirinae* are able to infect a wide range of mammals and birds. Among these viruses, some are particularly interesting in terms of animal health, human health or fundamental research. *Alcephaline herpesvirus 1* (AlHV-1), *Ovine herpesvirus 2* (OvHV-2), *Equine herpesvirus 2* (EHV-2), *Bovine herpesvirus 4* (BoHV-4), *Murid herpesvirus 4* (MuHV-4), as well as the human gammaherpesviruses, EBV and KSHV, are certainly the most studied.

AlHV-1 and OvHV-2 are particularly interesting viruses in terms of host specificity. Indeed, even if apparently apathogenic when infecting their natural host, respectively wildebeest and sheep, they are able to infect species as diverse as cattle, swine, but also lagomorphs, rats and hamsters, inducing in these dead-end hosts profound dysregulation of the immune system. (Russell *et al.* 2009). Thus, these two viruses cause a syndrome that is often lethal, known as "MCF" or "malignant catarrhal fever" in sensitive species such as cattle. This clinical entity, with a mortality rate of over 50%, is characterized by high fever and persistent lymphoproliferative lesions reaching all the mucous membranes of the anterior respiratory and digestive tracts, blood vessels and lymphoid organs. Represented by two similar forms of the disease (the African form (AlHV-1) and the European form (OvHV-2)), malignant catarrhal fever has been described clinically in 33 species of domestic and wild ruminants (Metzler and Burri 1990) and occurs on all continents (Mushi and Rurangirwa 1981).

EHV-2 (from the genus *Percavirus*) is also a very well host-adapted virus. With high prevalence, the infection by EHV-2 is distributed worldwide and was isolated from healthy individuals as well as from sick animals, raising the controversial issue of its pathogenicity. While EHV-2 may

A.



B.

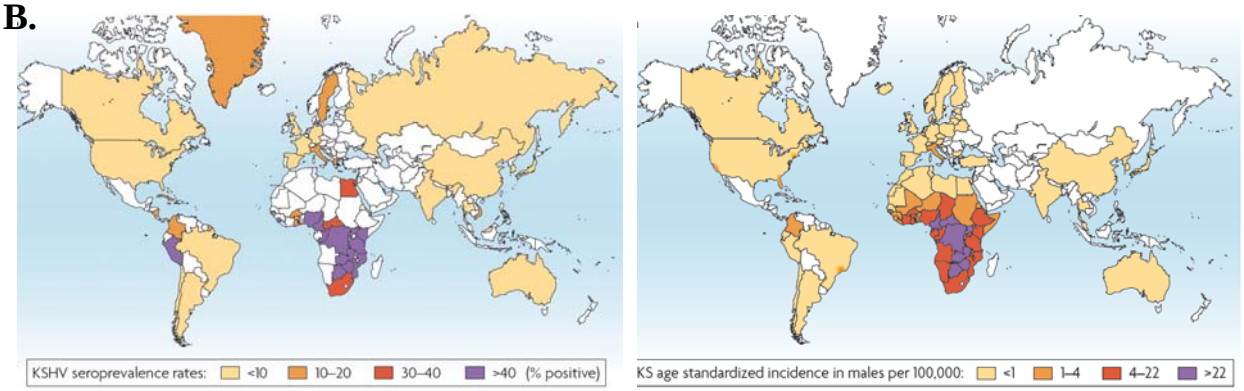


Figure 4: (A) Incidence of Burkitt's lymphoma and nasopharygeal carcinoma associated with EBV infection. From: http://www.cancerresearchuk.org/ (B) Seroprevalence of KSHV and incidence of the associated Kaposi's sarcoma**.** From Mesri *et.al*, 2010.

play a role as reactivator of other equine herpesviruses, this virus has been associated with some upper respiratory diseases, hyperthermia, an overall decline of general health and some forms of keratoconjunctivitis (Dunowska *et al.* 2002; Ruszczyk *et al.* 2004). EHV-2 infection is not strictly limited to domestic horses since the virus was isolated from two other species within the genus Equus, the Przewalski horse and mountain zebra (Borchers *et al.* 1999) while the experimental infection of mice has highlighted the persistence of the virus in the lungs and spleen cells (Rizvi *et al.* 1997; Borchers *et al.* 2002).

BoHV-4 was isolated from diverse samples from healthy cattle presenting various pathologies such as endometritis, abortion, respiratory or digestive troubles and mammary skin lesions (Donofrio *et al.* 2005). It was also demonstrated that African buffaloes can be infected, reflecting the possibility of inter-species transmission (Dewals *et al.* 2005). However, little studies allowed the experimental reproduction of the clinical disease and the role of BoHV-4 as a pathogenic agent remains, for now, uncertain (Thiry 1989). Although cattle have always been considered as the natural host of BoHV-4, the virus probably originates from African buffalo (Dewals *et al.* 2005). However, the virus is able to infect a large diversity of species such as guinea pigs and rabbits. The latter of these species is actually considered as the best model in experimental *in vivo* studies of BoHV-4

The two important gammaherpesviruses in human health are EBV and KSHV. EBV belongs to the *lymphocryptovirus* genus and was indentified about fifty years ago in cells isolated from Burkitt's lymphoma (BL) that are B cell derived tumors (Epstein *et al.* 1964). From an epidemiological point of view, it is estimated that about 90% of the adult population is infected (Henle *et al.* 1969; Andersson 2000) (Figure 4A). Primary infection can be asymptomatic, but can also lead, to a clinical entity named infectious mononucleosis (IM). The clinical signs and the gravity of IM can be highly inconstant (Thorley-Lawson and Gross 2004). The acute phase is characterized by cervical lymphadenopathy, fever and pharyngitis followed by general sickness and acute fatigue that can last several months (Callan *et al.* 1996). The host immune system is then able to control the infection, essentially by $CD4^+$ and $CD8^+$ T cells. Therefore, a homeostasis state between the host and the virus is set up and the virus will persist in the host organism in a latent state throughout its life. During this period, T cell surveillance is necessary for the control of the infection as evidenced by the development of lymphoproliferative disorders in patients receiving immunosuppressive therapies (Rickinson and Kieff 2001). Moreover, in these patients, but also in those with AIDS (Aquired ImmunoDeficiency Syndrom), neoplasic diseases such as Burkitt's lymphoma, Hodgkin's lymphoma or nasopharyngeal carcinoma can be developed (Rickinson and Kieff 2001).

In vitro, EBV infects B lymphocytes and almost always causes transformation of these cells into proliferative lymphoblasts (LCL, lymphoblastoïd cell lines) (Diehl *et al.* 1968; Pope *et al.* 1968). This effect depends on the expression of viral proteins during latency. The expression profile of the genes responsible of malignant transformation is called the « *growth program* ». The study of LCL cells has allowed the evidencing of a limited set of genes expressed during the infection *in vitro*. Thus, six nuclear proteins are described: *Epstein Barr nuclear antigen 1*, (EBNA-1), EBNA-2, EBNA3A, EBNA3B, EBNA-3, EBNA-LP. Four membrane proteins are also described : *Latent membrane protein 1* (LMP1), LMP2A, LMP2B, BHRF1 and a variable number of non-coding RNA (Rowe *et al.* 2009). Moreover, besides the characterization of the EBV infection in LCL cells, the study of the expression of genes associated with latency in tumoral tissues has shown the complexity of the EBV cycle *in vivo.* Indeed, several transcription programs are used by the EBV to infect cells, but also to maintain a long-term infection (Thorley-Lawson and Gross 2004). These programs are characterized by distinct expression of viral and cellular genes.

KSHV was identified in 1994 in very characteristic tumoral lesions, known as Kaposi's sarcoma (KS) and in HIV (Human Immunodeficiency Virus) seropositive patients (Chang *et al.* 1994). Later, it was demonstrated that KSHV is also associated with the development of other lymphoproliferative malignancies such as Castelman's disease and primary effusion lymphoma (PEL), that are rare B cell lymphomas essentially observed in patients with AIDS (Staskus *et al.* 1997; Ensoli *et al.* 2001; Schulz 2001). Cancerous lesions associated with KSHV are at this time the most frequent tumours in patients infected by HIV (Mesri *et al.* 2010). Seroprevalence can reach 50% (Butler *et al.* 2011) in some Sub-Saharan African regions and 10 to 25% in the Mediterranean area. The rest of the world is at low risk with prevalence ranging from 2 to 5% (Chatlynne and Ablashi 1999) (Figure 4B). Historically, four forms of KS are distinguished: the classical form in the Mediterranean region, the epidemic form or the form associated with AIDS, the endemic form in Africa (Oettle 1962) and finally the iatrogenic form in patients under immunosuppressive treatment following transplant (Siegel *et al.* 1969). These forms are different in terms of clinical etiology, with variations in aggressiveness, injured anatomical sites, mortality and morbidity. However, in all of the four forms, individuals are co-infected with HIV and KSHV (Dourmishev *et al.* 2003). In KS lesions, HHV-8 was detected at the level of vascular endothelial cells and in « *spindle cells* » constituting a histological signature of pathology (Staskus *et al.* 1997; Ensoli *et al.* 2001). Other characteristics of the KS lesions are the large diversity of cell types that can be transformed (Regezi *et al.* 1993; Herndier and Ganem 2001) as well as the early and high level of neovascularization (Hanahan and Folkman 1996). Clinical signs evolve from dermal flat lesions to edematous lesions, finally becoming purplish nodules. Although the virus is proven present in transformed endothelial cells, initial target cells are B lymphocytes (Ambroziak *et al.* 1995) as it is the case for the majority of gammaherpesviruses.

During latency, KSHV expresses a small number of genes: the genes coding for the latency-associated nuclear antigen (LANA), for the proteins v-cyclin and v-FLIP (*viral FLICE inhibitory protein*), for Kaposines A, B, C and finally 18 miRNAs (Cai *et al.* 2005; Samols *et al.* 2005). The majority of these proteins are implied in pathogenesis and malignant transformation associated with the infection by KSHV through diverse mechanisms including inhibition of apoptosis, interferrence with the host immune system, angiogenesis and cell cycle manipulation (Moore and Chang 1998; Moore and Chang 2003).

Lastly, while the oncogenic potential of both human gammaherpesviruses is now clearly established, the impact of this process may be underestimated. Indeed, several human tumors, in which no virus is yet isolated, potentially have a viral origin (Shimizu *et al.* 1994; Srinivas *et al.* 1998). This "hit and run" effect suggests that infection with an oncogenic virus can induce genetic instability and/or epigenetic dysregulation responsible for initiation and maintenance of the transformation of the infected cell (Niller *et al.* ; zur Hausen 1999; Pagano *et al.* 2004). Subsequently, the loss of the viral genome does not affect neoplasic progression following the alteration of cellular functions (Niller *et al.* ; Shen *et al.* 1997).

3. The *Murid herpesvirus 4*

3.1. Host range

The MHV-68 strain of MuHV-4 has been isolated from bank voles (*Myodes glareolus*) caught in Slovakia in 1980, and this, concomitantly to the strains 60 and 72 (Blaskovic *et al.* 1980). During the same study, the strains 76 and 78 were isolated in yellow neck mice (*Apodemus flavicollis*) (Blaskovic *et al.* 1980). Recently, very close viruses were isolated in other species such as field voles (*Microtus agrestis*), field mice (*Apodemus sylvaticus*) (Blasdell *et al.* 2003; Hughes *et al.* 2009) and shrew (*Crocidura russula*) (Chastel *et al.* 1994). To date, no consensus exists with regards to the determination of the natural host(s) of MuHV-4. However, from an epidemiological point of view, several field studies were conducted to determine the species which can be naturally infected. In 2003, a study conducted in England showed that seroprevalence was much higher in populations of wood mice in this country (Blasdell *et al.* 2003). Moreover, these results were confirmed in 2007 (Telfer *et al.* 2007) and studies concluded that the most probable natural host for MuHV-4 could be the wood mouse but that this does not exclude that other species could also be natural hosts for MuHV-4. In addition, in 2009, Hughes *et al*. isolated WMHV (Wood mouse herpesvirus) in a wood mouse (Hughes *et al.* 2009). This newly isolated virus is very close to MuHV-4. Consequently it cannot be excluded that natural populations tested during the two studies mentioned above were infected by

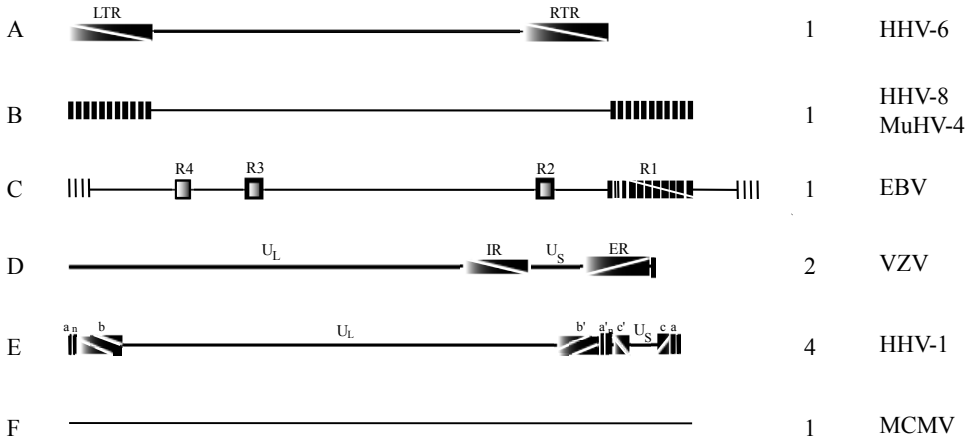


Figure 5: A schematic representations of the six genomic organizations of the viruses of the family *Herpesviridae*. The horizontal lines represent unique or quasi-unique regions. The repeated domains are shown as rectangles and are designated as left and right terminal repeats (LTR and RTR) for group A. In group B, the terminal repeats are reiterated numerous and variable times at both termini. In the case of the group C, the R1 to R4 repeats are internal to the sequence and internal and terminal (IR and ER) repeats are observed for group D. For the group E, the termini is composed of two elements. One terminus contains *n* copies of sequence a next to a larger sequence designated as *b*. The other terminus has one directly repeated *a* sequence next to a sequence designated *c*. The terminal *ab* and *ca* sequences are inserted in an inverted orientation separating the unique sequences into a long (U_L) and short (U_S) domains. Group F genomes do not contain repeat. The components of the genomes in classes D and E invert. In class D, the short component inverts relative to the long. Although (rarely) the long component may also invert, most of the DNA forms two populations differing in the orientation of the short component. In the class E genomes, both the short and long components can invert and viral DNA consists of four equimolar isomers. Adapted from Roizman and Pellet, 2007

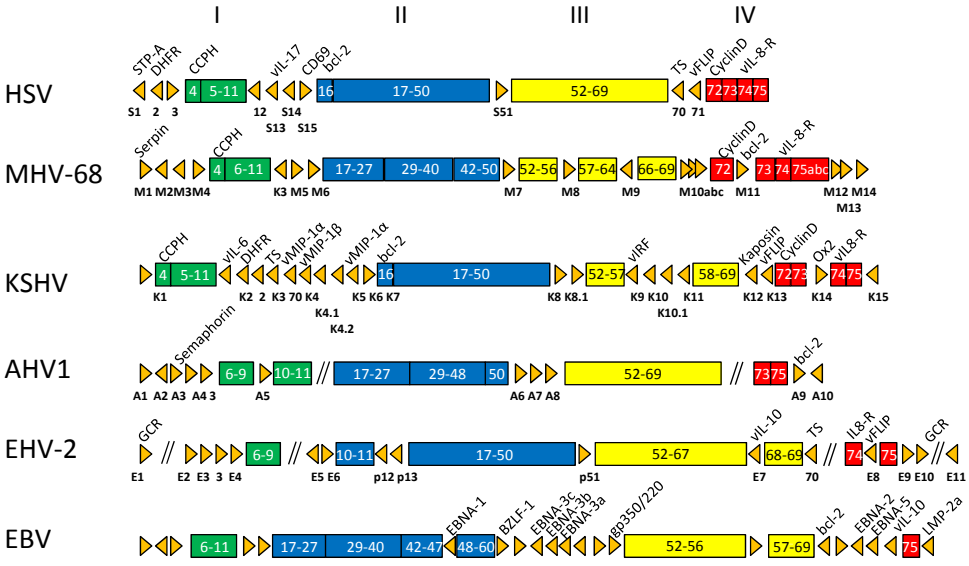


Figure 6: A comparison of the genomic organization of some fully sequenced gammaherpesviruses. As examples, HSV (Herpesvirus saimiri), MHV-68 (*Murid herpesvirus 4*), HHV-8 (*Humam herpesvirus 8* or KSHV), AlHV-1 (*Alcephaline herepsvirus 1*), EHV-2 (*Equid herpesvirus 2*) and EBV (Epstein-Barr virus), are shown. The conserved genome blocks are represented in green, blue, yellow and red and the numbering of the ORF's is relative to the one of HSV. Adapted from Simas and Efstathiou, 1998.

WMHV rather than by MuHV-4. Finally, in 2007, a study designed to identify new herpesviruses in natural rodent populations using PCR (polymerase chain reaction) was conducted by Ehlers *et al.* (Ehlers *et al.* 2007). More than 1,100 samples from rodents caught in the UK, Germany and Thailand were tested, allowing the identification of several new *beta*- and *gammaherpesvirinae*, among which the first gammaherpesvirus naturally infecting house mice (*Mus musculus*), the species from which laboratory mice are derived (Ehlers *et al.* 2007). Although the identification of this new virus opens interesting perspectives concerning the study of a gammaherpesvirus in its natural host, the absence of virus isolation makes it impossible to present any experimental perspective.

3.2. Molecular biology

3.2.1. The viral genome

As already mentioned, MuHV-4 is a gammaherpesvirus belonging to the *rhadinovirus* genus. Rapidly after its isolation, this virus became an essential tool for the study of gammaherpesvirus biology. The genome of MuHV-4 has a B-type structure (Figure 5), and was entirely sequenced in 1997 (Virgin *et al.* 1997). With a length of about 120 kb, the genome of MuHV-4 is composed of a long unique region (118 237 kb) flanked by a variable number of 1.2 kb direct terminal repeats (Efstathiou *et al.* 1990b). The sequence analysis allowed the identification of 80 open reading frames (ORF), 63 of which are homologues of HVS (herpesvirus saimiri), the type species of the genus *rhadinovirus* (Figure 6) (Virgin *et al.* 1997). These 63 ORF's are also present in the genome of KSHV and the majority is present in the genome of EBV. The genome is composed of large blocks of genes conserved among all the gammaherpesviruses between which MuHV-4-specific ORF's are interspersed. These specific ORF's seem to participate in specific biological properties of the virus. This genomic organization, the high positional homology between MuHV-4 and KSHV and the lack of conservation of many proteins involved in the tumoral transformation following EBV infection (Virgin *et al.* 1997) led to the classification of MHV-68 within the *rhadinovirus* genus (Efstathiou *et al.* 1990a). This classification was moreover confirmed later by phylogenetic evidences (Ehlers *et al.* 2008).

The MuHV-4 genome contains 14 unique genes named M1 to M14. The majority of these are only accessory for lytic infection. As an example, the left end of the MHV-68 genome contains the M1 to M4 genes and sequences coding for t-RNAs and micro RNAs (see later), that are absent in the strain 76 isolated simultaneously with the 68 strain. The analysis of the infectivity of the strain 76 in mice has demonstrated that this region is essential for viral pathogenesis as the infection by the strain lacking this region is more rapidly controlled in the lungs and latency is less efficient. Moreover, a

recombinant virus made by the 76 strain complemented with the left end of the 68 strain shows similar infectivity to 68 strain (Macrae *et al.* 2001).

As for all the gammaherpesviruses, numerous cellular homologues are found in the MHV-68 genome. It seems that these DNA sequences were recently acquired at the scale of evolution and are implied in the manipulation of the cellular cycle and the regulation of apoptosis by the virus during the infectious process. For the KSHV, at least 12 ORF's encode proteins with cellular homologues. Concerning MuHV-4, this number is reduced to 4 proteins : a homologue (ORF 4) of a complement regulatory protein whose role could be the inhibition of complement-dependent lysis; a D-cyclin homologue (ORF 72) able to interact with the cyclin dependent kinase 6 (cdk6) and to a lesser extend with cdk4, leading to the alteration of the cell cycle; a receptor for the interleukine 8 (IL8) whose KSHV homologue is known to be implied in tumourigenesis and neoangiogenesis and finally a homologue of the cellular gene *bcl-2* (M11 gene) whose function would be to improve the survival of infected cells, ensuring the maintenance of a pool of latently infected cells (Virgin *et al.* 1997).

The genome also contains eight t-RNA (transfert RNA) type sequences localized at the 5' terminus (Bowden *et al.* 1997; Virgin *et al.* 1997). The role of these RNA is not clearly defined but it is interesting to note that they are abundant in germinal centers when viral latency is established. The expression of the t-RNA is therefore a marker of viral latency (Bowden *et al.* 1997). It is however speculated that those vt-RNA sequences are evolutional remnants serving as promoter for viral microRNA (miRNA) sequences (Zhu *et al.* 2010). Indeed, the expression of viral microRNA during latency was recently demonstrated for members of the three families of herpesviruses (Pfeffer *et al.* 2005). These were proposed to be implied in both lytic and latent infection, possibly acting on viral/host interaction (Sullivan and Ganem 2005; Nair and Zavolan 2006). Pfeffer et al. have predicted 17 miRNAs encoded by MuHV-4, 9 of which were experimentally confirmed (Pfeffer *et al.* 2005). More recently, Zhu *et al*. have systematically analyzed the expression profile of RNAs in lytically and latently MuHV-4 infected cells (Zhu *et al.* 2010). Their results show increased level of miRNAs in latently infected cells in comparison with levels observed in lytically infected cells. Thus these RNAs seems to act positively on viral latency by modulating apoptosis cellular pathways, immunity and repression of viral lytic cycle (Cai *et al.* 2005; Burnside *et al.* 2006; Lu *et al.* 2008; Umbach *et al.* 2008; Wang *et al.* 2008; Lei *et al.* 2010; Forte and Luftig 2011).

3.2.2. Tools

Making great advances in fundamental and applied research about herpesviruses often requires genetic manipulations such as transgene insertion or ORF deletion. While these two types

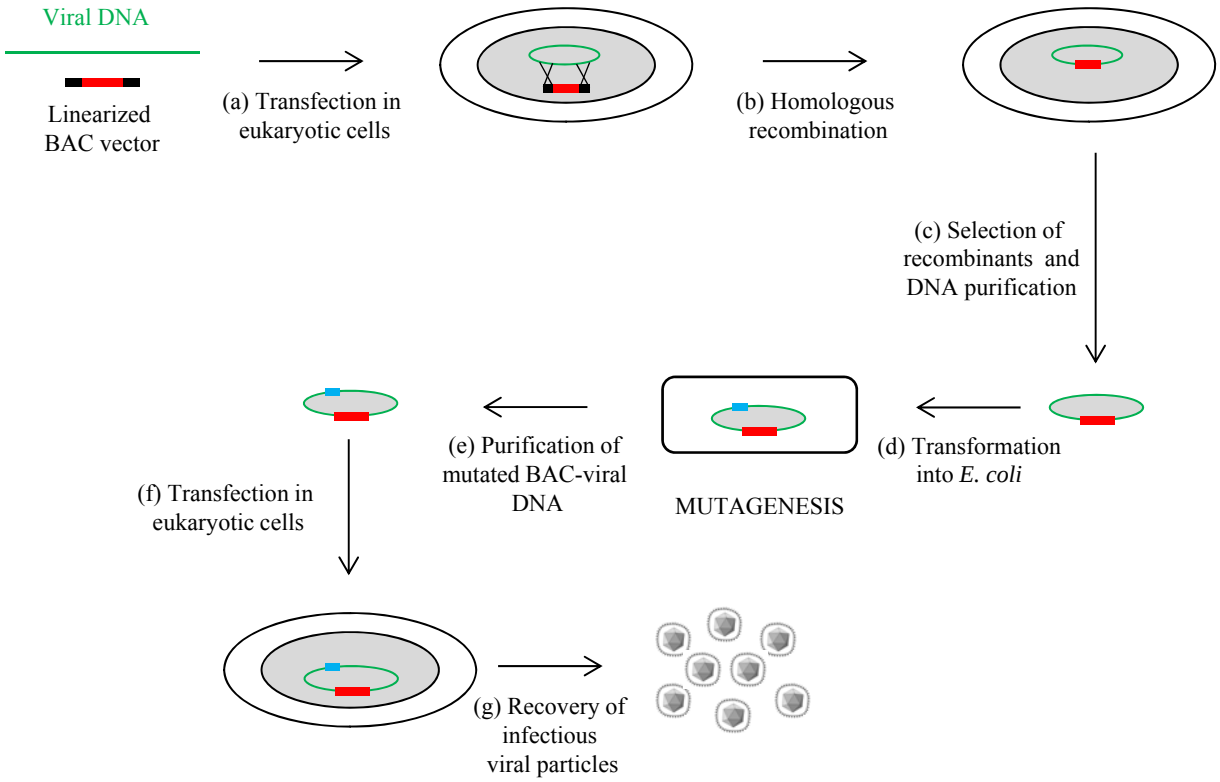


Figure 7: Schematic representation of the generation of a viral BAC-genome. (a) The viral genome and a linearized baterial plasmid containing the BAC cassette surrounded by homologues sequences to viral genome are co-transfected in eukaryotic cells. (b) Homologous recombinaison occurs and viral genomes containig BAC cassette are naturally circularized. (c) Viral genomes containing BAC cassette are selected using a selection marker and viral DNA is extracted from selected plaques. (d) BAC-containing viral genome is used to transform baterial cells. (e) BAC-genome can be mutated by various methods (here the mutation is represented in blue) and isolated from bateria. (f) Viral BAC-DNA may be tranfected in eukaryotic cells and (g) infectious viral particles are recovered.

of modifications can be made by homologous recombination in eukaryotic cells, the approach is extremely long and difficult due to the large genome of herpesviruses and their relatively slow kinetics of replication (Adler *et al.* 2003). Furthermore, with this strategy, genome analysis of the mutant virus is only possible after the experimental procedure, revealing only later accidents such as unwanted genetic recombination, deletions or rearrangements. On the other hand, the selection of target mutants is also difficult. To overcome all these constraints, a new approach for constructing herpesvirus mutants has been developed. The principle of it is based on the cloning of the viral genome as a bacterial artificial chromosome (BAC) in *Escherichia coli* (*E. coli*). The maintenance of the viral genome under the form of a bacterial chromosome in prokaryotic cells allows the researchers to apply well known and controlled mutagenesis methods, using bacterial or phage recombinases, and greatly simplifies genetic modification and selection of recombinants.

To generate a BAC-cloned genome (Figure 7), a BAC cassette composed of genetic sequences needed for DNA replication and segregation in prokaryotic daughter cells as well as selection markers and often *loxP* sites surrounding the cassette is inserted in the viral genome. Infectious viral particles are obtained following transfection of the BAC genome in eucaryotic cells able to support viral lytic infection. The virions therefore contain the BAC cassette which can be removed, if wanted, by the action of *Cre* recombinase on the specifically recognized *loxP* sites (Zhang *et al.* 1998; Wagner *et al.* 2002; Warden *et al.* 2011). Alternatively, the BAC cassette can be excised by the presence of homologous sequences, promoting recombination. This technique of self-excision leads early in the process of virus-reconstitution to viral genomes rid of the BAC cassette thus shorter and therefore preferentially encapsidated (Wagner *et al.* 1999). Excision of the BAC cassette can be an important step, particularly in the context of *in vivo* experiments. Indeed, the presence of these complementary sequences but also the transcription of encoded proteins, mainly the selection markers, may alter viral infectivity in mice by eliciting specific immune response againgt the transgene (Adler *et al.* 2001; El-Gogo *et al.* 2008).

The first BAC cloning of the genome of a herpesvirus was made in 1997 with the cloning of murine cytomegalovirus by Messerle *et al.* (Messerle *et al.* 1997). Today, numerous genomes of herpesviruses have been cloned as BAC (reviewed in Warden *et al.* 2011) : all the human herpesviruses except HHV-7, and also a lot of animal herpesviruses such as BoHV-4 (Gillet *et al.* 2005) or AlHV-1 (Dewals *et al.* 2006a). MuHV-4 was also BAC-cloned in 2000 (Adler *et al.* 2000). Today, a lot of recombinant MuHV-4 viruses are available and the majority has been obtained from the BAC-cloned genome. Subsequently, many studies, both *in vitro* and *in vivo,* were permitted by this molecular tool that has now become indispensable for the specific study of the involvement of specific genes in the pathogenesis of the herpesviruses.

Having this molecular cloning tool has also opened many perspectives in terms of gene therapies and vaccination strategies. In this context, BAC-cloning and mutagenesis methods are constantly evolving with the development of new strategies allowing the rapid construction of vaccinal and therapeutic vectors. For the purpose of gene therapies, viral vectors contain all genes needed for viral replication but lack those needed for virulence. Such HSV-1 (Marconi *et al.* 2009) based vectors have been developed with hopes in treating cancers, (Kuroda *et al.* 2006; Terada *et al.* 2006) and osteoporosis (Xing *et al.* 2004). EBV vectors have also been developed in the context of gene therapies (Magin-Lachmann *et al.* 2003; Hettich *et al.* 2006). In addition, these methods are very useful in fundamental research.

3.3. Pathogenesis of the MuHV-4 infection

MuHV-4 infects and establishes a chronic life-long infection in laboratory mice. The natural way of infection is not known but it is usually considered that the upper respiratory tract should be the most probable entry site. Indeed, a comparative study of intranasal and intravenous infections has proven that the intranasal infection was the most likely to produce clinical signs associated with the infection while representing a more natural way of contamination (Sunil-Chandra *et al.* 1992). Other studies have tested the potential of intraperitoneal, subcutaneous, intracerebral and oral infections. By all these routes, MuHV-4 is able to infect the host, proving the ability of the virus to infect several anatomical sites due to a large tropism for diverse epithelial and fibroblastic cell lines. Regardless of the route of inoculation, B cell infection is a common feature but bypassing the epithelial barrier (as with intraperitoneal infection) leads to more severe disease and seems to make some genes accessory (Jacoby *et al.* 2002). Circulating latently infected cells can theoretically lead to the infection of any other anatomical site explaining the relative equivalence of all routes of infection. The classical experimental infection is intranasal and leads to viral replication in the nasal mucosa during primary infection (Milho *et al.* 2009). The replication then continues in the lungs, causing severe interstitial pneumonia associated with leukocytes in perivascular and peribroncheolar infiltrates. The primo-infection is largely controlled within 10 to 14 days post-infection (Sunil-Chandra *et al.* 1992). Interestingly, no replication is observed in the lungs when the animals are infected without anesthesia (Milho *et al.* 2009). This strongly indicates that replication in the lungs should probably not be considered as a part of natural infection. Simultaneously to the productive infection, latency is established in lymphoid organs, essentially in the spleen and the superficial cranial lymph nodes (SCLN) (Milho *et al.* 2009). Acute infection of these organs is controlled within 14 to 16 days and latency is considered established within 16 to 18 days post-infection. Clinical signs are similar to those observed in the case of an IM following the infection by EBV: lymphadenomegaly, splenomegaly, weight loss, and in the case of mice, dorsal curvature and ruffled fur (Sunil-Chandra *et al.* 1992).

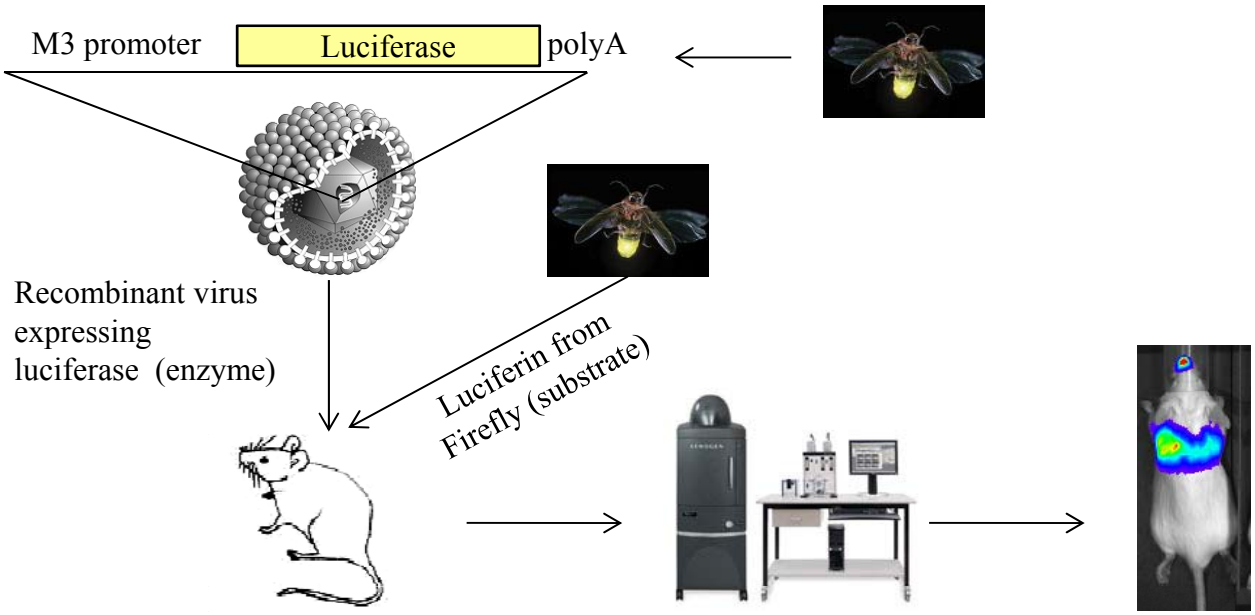


Figure 8: *In vivo* imaging principle (IVIS, *In vivo* imaging system). A recombinant virus expressing luciferase from *Photinus pyralis* under the control of the promoter of a gene associated with the replicative cycle is used to infect animals. Following intaperitoneal injection of luciferase substrate, luciferin, this substrate is oxydized with emission of photons which are detected by a CCD camera. A photagraph and a luminescence image are acquired and superimposed by the software. A color scale is determined to render intensities and quantitative analyzes can be made.

Although many studies using classical virology methods were performed to bring out MuHV-4 pathogenesis, new insights were made by the use of *in vivo* imaging. Indeed, the existence of the MuHV-4 genomic BAC-clone has allowed the construction of a recombinant virus expressing firefly (*Photinus pyralis*) luciferase (Milho *et al.* 2009). This enzyme oxidizes its specific substrate, luciferin, with emission of a photon during the reaction (Figure 8). This mechanism, causing the luminescence of fireflies, is now advantageously used in biology, among others, in order to study infectious processes (Hutchens and Luker 2007). Another important advantage of this method is that the substrate needed for the enzymatic reaction is easily available and can be injected *in vivo* without damaging the animal (Hutchens and Luker 2007) but with an excellent biorepartition and bioavailability in the organism. Also, this method drastically limits the number of animals needed for experiments while minimizing experimental bias caused by the analysis of different groups of animals during kinetics experiments. However, the oxidation reaction needs oxygen and is therefore limited in most anaerobic organs such as the gut. For the MuHV-4 recombinant, the luciferase coding gene was cloned under the dependence of the promoter of the M3 gene (Milho *et al.* 2009), associated with the lytic phase of the viral cycle. Is has to be noted that a similar recombinant was made by another group (Hwang *et al.* 2008). After infection with a luciferase expressing recombinant virus, the infectious process can be monitored continuously using an imaging system composed of a charged coupled device (CCD) camera able to detect photons emitted from the animal. This technique permitted further elucidation of the lytic infection in model animals allowing for example the observation of replication in the nasal mucosa (Milho *et al.* 2009).

3.4. The immune response and the control of the infection

Gammaherpesviruses are the archetype of persistent viruses. The co-evolution of these viruses with their natural host(s) led them to develop near-perfect adaptation between infectivity and long-term persistence. Thus gammaherpesviruses have developed immune evasion strategies from the innate response but also from the adaptative response that allow their persistence in the host and their re-excretion despite the presence of specific antibodies. This ability to evade the immune response is also responsible for the concomitant presence of several related viral strains within the same host organism. Indeed, the host immune response is so limited and circumvented that a close second strain may be the source of infection within an immunized host (Sitki-Green *et al.* 2003; Gorman *et al.* 2006; Muylkens *et al.* 2009). This highlights the difficulty in developing efficient vaccines which induce a sufficient immune response, *in fine* allowing the control of the herpesvirus infection.

Moreover, it is recognized today that the induction of long-term neutralizing response is one of the main antiviral mechanism (Burton *et al.* 2005; Hangartner *et al.* 2006). Therefore, the development

of vaccines against persistent viruses proved to be a major challenge as these viruses have evolved by co-speciation, adapting to their host in order to coexist with the antibody response and resist neutralization (Burton *et al.* 2005). At present, the understanding of the mechanisms of evasion of gammaherpesviruses toward the neutralizing response is very limited. The development of effective vaccine strategies is also complicated by other factors such as low-antigen expression during latency, the manipulation of the elements involved in antigen presentation to the immune system, a tropism that is not confined to a single cell type, the establishment of latency within immune cells in the case of gammaherpesviruses and finally the few available *in vivo* models allowing a detailed study of mechanisms of interaction between the virus and the host immune system. These elements will be discussed in this section.

In this context, the study of MuHV-4 in the laboratory mouse is very useful. Indeed, we can say that the immune response of laboratory mice has many similarities with that of humans and secondly, a majority of the genes involved in gammaherpesvirus latency was found in MuHV-4. However, it has unique genes but their function appears to be preserved. Thus, the assumption of common strategies for pathogenesis and escape of the immune system can be emitted (Barton *et al.* 2011).

The control of the gammaherpesvirus lytic infection is primarily provided by the cell-mediated immunity and particularly by CD8 + cytotoxic T lymphocytes. Indeed, the end of pulmonary lytic infection coincides with a peak of virus-specific CD8 + T cells (Stevenson and Doherty 1998) and depletion of these cells prior to infection leads to uncontrolled lytic infection and death (Ehtisham *et al.* 1993). However, the gammaherpesviruses have evolved to considerably limit the recognition of their essential epitopes, lytic as well as latent, by the host immune system. In the case of MuHV-4, it was shown that CD8 + T cells, although able to proliferate massively following the viral challenge after previous infection, are unable to control chronic lytic infection (Belz *et al.* 2000). Tests of pre-exposure vaccination designed to increase the efficiency of these CD8 + T cells and to limit the colonization of the infected host have failed (Liu *et al.* 1999; Stevenson *et al.* 1999a). This shows the high capacity of the virus in the escape of the immune system in order to establish an effective state of latency. At a molecular level, there are several genes involved in this process. Among these, the K3 gene product, acts predominantly during the lytic phase to inhibit the presentation of viral antigens in the context of class I major histocompatibility complex (MHC) thus limiting the recognition by CD8 + T cells and limiting the effect of the cytotoxic response (Stevenson *et al.* 2000). This effect is related to the ability of the K3 protein to bind to neoformed molecules of the MHC class I in the endoplasmic reticulum, inducing an ubiquitination complex and its subsequent degradation by the proteasome (Boname and Stevenson 2001). Escaping the CD8 + T cell response is also important during latency.

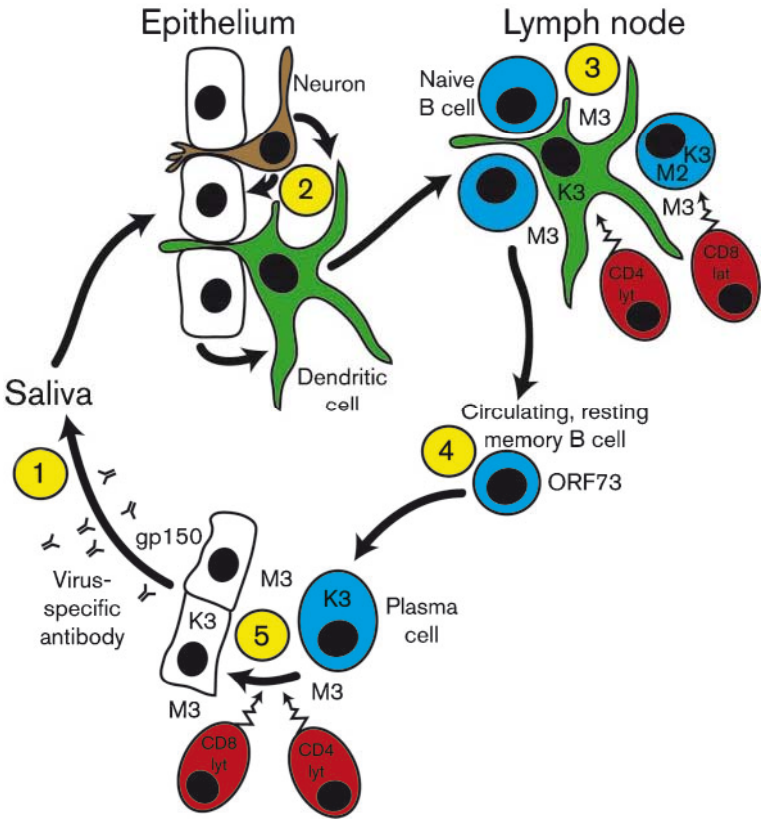


Figure 9: A schematic view of the gammaherpesvirus life cycle for MuHV-4. T cells are shown in red, B cells in blue, epithelial cells in white, neuronal cells are brown and relevant others are in green. Arrows show the movement of virus. Numbers 1–5 indicate definable steps. Key intervention points are latency establishment in naive hosts (steps 2 and 3) and antibody binding to the virions shed by carriers (step 1). Step 1: virions enter a naive host via secretions such as saliva. Since virions come from immune carriers, they are likely to have attached antibody. The virions are not normally neutralized, but neutralization may be possible through boosting fusion-complex-specific antibodies in virus carriers. Nonneutralized MuHV-4 could first infect epithelial cells, IgG Fc receptor-bearing dendritic cells or olfactory neurons. There is no good evidence for direct B cell infection after a non-invasive inoculation. Step 2: local lytic spread could potentially be targeted by antiviral drugs or antibody. Latency establishment seems to occur mainly in draining lymph nodes. Infection may reach these via dendritic cells or via cell-free virions captured by subcapsular sinus macrophages. Step 3: infection next spreads to B cells, which proliferate. Lytically infected myeloid cells secrete evasion proteins such as the M3 chemokine binding protein to protect B cells against CD8+ T-cell attack. K3 protects myeloid cells and perhaps also B cells against CD8+ T-cell recognition. IFN-γ produced by CD4+ T cells may limit M3 production and thereby help latent antigen-specific CD8+ T cells to attack B cells. Step 4: B cells exit germinal centres and differentiate into long-lived, resting memory B cells. Episome maintenance by ORF73 remains below the threshold of antigen presentation. Step 5: virus reactivation probably occurs in submucosal sites, accompanied by B-cell differentiation to a plasma cell phenotype (Sun & Thorley-Lawson, 2007; Wilson et al., 2007). There may be further lytic replication in epithelial cells prior to virion shedding. Lytic antigen-specific CD8+ T cells can potentially inhibit reactivation, but K3 and M3 limit their impact. Consequently, CD4+ T cells seem to protect better against lytic spread. Gp150 promotes virion release and helps to limit neutralization. From Stevenson *et al.*, 2009.

Hence, the M3 protein of MHV-68, that has no counterpart in other gammaherpesviruses, is a chemokine-binding protein (CBP) with a wide spectrum and whose role would be to limit CD8 + T cell migration to sites of latency-antigen expression (Parry *et al.* 2000; van Berkel *et al.* 2000). M3 is expressed during the lytic phase of the infection (Martinez-Guzman *et al.* 2003). In the absence of the M3 protein, amplification of latency is dramatically reduced but can be largely restored after depletion of CD8 + T cells (Bridgeman *et al.* 2001). M3 acts to protect infected B cells from the host's immune system but the way by which this is realized remains unclear as of yet (Stevenson and Efstathiou 2005). We can also mention the LANA protein, present in the MuHV-4 (ORF 73) and in the KSHV, which has a functional homologue in EBV: EBNA-1. This protein, besides its crucial role in maintaining the viral genome as an episome in latently infected cells, significantly limits the expression of viral genes, slows the lytic phase and inhibits epitope presentation during episome maintenance (Bennett *et al.* 2005; Li *et al.* 2008; Wen *et al.* 2009).

CD4 + T cells are also important for the control of infection. Indeed, CD4 + T cells also display a role in the control of the acute phase of infection by MuHV-4. Assuredly, these cells can act as a weapon for the development of a cytotoxic immune response type, but also have a supervisory role by themselves. Therefore, infection of mice deficient in B cells and depleted of CD8 + T cells may be controlled by the action of CD4 + cells. This mechanism appears to depend on the induction of interferon (IFN) production, IFN-γ mainly. Indeed, inhibition of this molecule in these same animals led to a complete loss of control of infection by the organism (Christensen and Doherty 1999). Furthermore, infection of animals that were deficient for the production of IFN-γ led to a chronic lytic infection. Thus, IFN-γ is crucial in the limitation of replication and potentially in that of viral reactivation (Dutia *et al.* 1997; Weck *et al.* 1997).

Finally, a model was suggested to explain the balance between the host's immune system and viral evasion (Figure 9). In this model, it is put forward that tissue damage caused by the lytic phase leads to an acute inflammatory process and therefore to a response during which the CD8 + T cell response is predominant for lytic infection control. During this phase, the host's immune response dominates. Consequently, during the establishment of latency, and during the latency itself, tissue damage is severely limited, as is the inflammation reaction, leading to a dominance of the evasion mechanisms which act by the expression of viral proteins M3 and K3 (Stevenson *et al.* 2002).

4. Epidemiology and transmission of the *gammaherpesvirinae*

Knowledge and understanding of the mechanisms of transmission of herpesviruses in populations are essential to implement large scale antiviral strategies. Indeed, the development of

strategies and/or fully adequate vaccination requires in-depth knowledge not only of the molecular mechanisms governing replication, viral latency, reactivation and re-excretion in a single individual, but also of the mechanisms involved in the dispersion of these viruses at population level, whether human or animal. The association of these diseases with other pathogenesis may also have significant repercussions on prevention of the illness.

In humans, the determination of the mechanisms of transmission at population level is essentially based on sero-epidemiological studies that often involve the interrogation of individuals included in studies. Although this method is probably the oldest source of epidemiological information, many biases may appear in this type of study such as an incomplete questionnaire, the inability to check the accuracy of the answers or a limited or inhomogeneous population sample. At present, molecular biology reinforces these methods allowing for example the assessment of the presence of a pathogen at different anatomical sites. However, access to samples is limited, at least in humans, to body fluids and biopsies.

This section presents a review of current knowledge about the epidemiology and mechanisms of transmission of some gammaherpesviruses of interest in human or veterinary medicine. Indeed, the different gammaherpesviruses share many common properties, whether at a molecular or an evolutionary level, and knowledge of transmission mechanisms involved in the epidemiology of these viruses may therefore be important in the context of the study of another gammaherpesvirus, especially when developing a model of transmission under experimental conditions.

As previously mentioned, two gammaherpesviruses are important in human medicine, namely EBV and KSHV. Associated diseases, but also some epidemiological data have already been mentioned in section 2 of this introduction. This section therefore aims to further explore currently known data concerning the transmission of these two infections.

There are now several studies that tend to demonstrate the presence of EBV in the genital tract and therefore to hypothesize potential sexual transmission. Indeed, by 1986 the presence of EBV in cervical secretions, whether in the form of virions associated with epithelial cells or as free infectious viral particles, was shown (Sixbey *et al.* 1986). Subsequently, many studies have been conducted and have disclosed some interesting elements. Thus, about thirty cases of ulcerative vaginal manifestations associated with EBV have been reported (Halvorsen *et al.* 2006; Leigh and Nyirjesy 2009). Some of these cases were certainly associated with a primary infection by EBV. Indeed, for one, the seroconversion was observed in the month following detection of the ulcerative lesions and secondly, because of the detection by PCR of viral genomes in biopsies of the lesions (Halvorsen *et al.* 2006).

Moreover, a study conducted among women of a rural area of India showed that EBV is detected in genital secretions of about 20% of these women and that it is frequently associated with poor hygiene (for instance, they have no running water), cervical inflammation and genital cancer (Silver *et al.* 2011). Further studies have shown EBV viral particles in male and female genital secretions (Sixbey *et al.* 1986; Israele *et al.* 1991; Naher *et al.* 1992; Thomas *et al.* 2006b). In addition, sero-epidemiological studies conducted on cohorts of university students have correlated EBV seropositivity, history of infectious mononucleosis and the number of sexual intercourses and partners reported (Crawford *et al.* 2002; Woodman *et al.* 2005; Crawford *et al.* 2006). It was also shown that members of a couple often share the same virus isolate (Thomas *et al.* 2006b). However these latest studies do not actually demonstrate sexual transmission as it is not possible to discriminate direct sexual transmission from transmission due to practices associated with sexual intercourse such as kissing. In conclusion, although sexual transmission should be considered as a way of transmission because of the presence of infectious virus in vaginal and urethral secretions, this route does not seem to be the most important, but its incidence remains unclear to date.

KSHV is less prevalent than EBV. Sero-prevalence rates vary greatly depending on the geographical areas considered (see section 2) and are higher among low socio-economic populations, but also among HIV infected individuals (Cannon *et al.* 2001).

A PCR-based detection study of KSHV in body fluids allowed to evidence the presence of the virus essentially in saliva with high titers of viral DNA, but also in the blood, the semen, the skin injured by KS and even in healthy skin (LaDuca *et al.* 1998). The virus was also isolated in the oropharyngeal secretions of homosexuals, but only rarely in samples harvested at the anal and genital regions of the same individuals (Pauk *et al.* 2000). During the studies conducted on biopsies from AIDS patients, prostatic tissues were positive for viral KSHV genomes (Corbellino *et al.* 1996), suggesting that the prostate could be a replication site. KSHV was also shown to be present in the semen and this, often in patients with KS (Diamond *et al.* 1997), sometimes associated with epithelial cells and never associated with spermatozoa (Pellett *et al.* 1999; Ablashi *et al.* 2002). KSHV was also detected in the cervix of some women (Whitby *et al.* 1999). This biological data suggests that sexual transmission should be considered for KSHV. Sero-epidemiological studies were elaborated taking this into account. In 1999, a study conducted on more than 3000 people monitored for more than a year linked the risk of KSHV acquisition to a homosexual or bisexual way of life but not to that of heterosexuals (Smith *et al.* 1999). This was even true in high prevalence areas such as Zimbabwe (Campbell *et al.* 2009). However, in France and Italy, studies tend to show an increased risk of seroconversion of an uninfected partner in a heterosexual relationship with an individual suffering from KS (Brambilla *et al.* 2000; Dupuy *et al.* 2009). Another study has shown that the risk is also

correlated with the number of partners and with infection by HIV (Martin *et al.* 1998). Finally, KSHV was frequently detected in oropharyngeal and cervical secretions of a sample of Zimbabwean women presenting classical KS lesions, but not in the control sample population without KS disease, including women seropositive for KSHV (Lampinen *et al.* 2000). This suggests that sexual or perinatal transmission in high prevalence populations could be linked to the immunodeficient status that allows the shedding at these anatomical sites. This would mean that these transmission routes are probably very limited in immuno-competent individuals (Lampinen *et al.* 2000). High oral and cervical shedding is also correlated with HIV co-infection in Kenya (Taylor *et al.* 2004). In conclusion, despite the highly controversial aspect of the results presented here, it seems that sexual transmission is a major risk of KSHV acquisition among certain populations particularly at risk, but should be very limited in heterosexual and immuno-competent populations (de Sanjose *et al.* 2009).

Mother-to-child transmission, both transplacental or perinatal, seems to be very limited, but could occur in high prevalence areas (Pica and Volpi 2007). However, in women co-infected by HIV, viral reactivation seems to be increased during pregnancy, and this also appears to be the case for perinatal shedding (Lisco *et al.* 2006), but no influence of the infection on the outcome of the pregnancy was observed. Mother-to-child infection exists but seems to be essentially caused by an increase of the mother's perinatal salivary shedding (Dedicoat *et al.* 2004). Additionally, the acquisition of infection during childhood would imply the transmission between young children who shed high levels of viruses in saliva (Plancoulaine *et al.* 2000; Mbulaiteye *et al.* 2006).

The high frequency of association of KSHV and HIV infections, as well as the much more frequent appearance of KSHV-associated diseases such as KS in individuals with AIDS naturally led to evaluate the impact of some factors known to be implied in HIV transmission on the epidemiology of KSHV. Thus, the potency of blood-borne transmission was considered. A study conducted on samples from about 30 infected donnor/healthy patient pairs in West Africa led to the detection of only one case of transmission following the transfusion of a blood sample with high viral load (Gobbini *et al.* 2012). In Uganda, it was shown that the risk of blood-borne transmission from infected donors decreased after 4 days of blood storage (Hladik *et al.* 2006). A similar study but of larger scale was conducted in the U.S.A. revealing no transmission by blood products (Cannon *et al.* 2009). However, another study of lesser extent that used a more sensitive immunofluorescence method showed occasional cases of transmission following blood transfusion in the U.S.A. (Dollard *et al.* 2005). Moreover, the possibility of transmission through needle sharing among populations of injection drug users was also evaluated. A study comparing the seroprevalence of KSHV in a group of intravenous drugs users and a control group has shown that such practices constitute a risk of KSHV acquisition (Sosa *et al.* 2001), regardless of sexual orientation (Atkinson *et al.* 2003). However, another similar

study did not reveal such a correlation and concluded in the dominance of sexual orientation as an important factor regarding the prevalence of KSHV in populations considered at risk (Bernstein *et al.* 2003). Finally it appears that the presence of infectious viral particles in the blood stream greatly depends on the stage of the infection as the massive presence of IgG correlates with a high viral load (Ablashi *et al.* 2002). Globally, this data indicates that the spread by way of blood transmission or needle sharing by drug users can exist but remains of low importance, at least in immune-competent individuals.

AlHV-1 and OvHV-2 are two important gammaherpesviruses in veterinary medicine. As previously mentioned, they are the causative agents of the disease named MCF or malignant catarrhal fever. AlHV-1 is the source of a particularly important epidemiological problem concerning cattle in Africa, but also in the context of zoos hosting many exotic animals. Although the virus and its associated diseases should have been studied for many years, little data actually exists concerning viral transmission. For AlHV-1, it seems that in the natural host, transmission occurs by direct contacts between healthy and infected animals during the first weeks of life. The virus is mostly re-excreted in the nasal and ocular mucosa (Mushi *et al.* 1980; Pretorius *et al.* 2008). An epidemiological study has concluded that the infection is often acquired *in utero* or soon after birth (Pretorius *et al.* 2008). Some serological studies in which geographical elements were taken into account were conducted, mainly in South Africa, over a period of 80 years. The results show that transmission occurs primarily between animals grazing in the same areas, probably *via* viral particles deposited by an infected animal, during parturition as an example, on the grass grazed by a healthy animal. Moreover, it seems that the shedding needed for transmission occurs mainly in young animals that have been recently infected (Barnard *et al.* 1989). However, transmission in the absence of any possibility of direct contact was observed in South Africa, leading the authors to hypothesize transmission through a fly species (Barnard and Van de Pypekamp 1988).

OvHV-2 seems to be predominantly transmitted by nasal secretions from young infected animals (Kim *et al.* 2003). Indeed, a transmission study of the virus was conducted to evaluate the infection ability of samples harvested at the nasal mucosa of sheep, with on one hand recently infected animals and on the other, individuals that had been infected a long time ago (Nishimori *et al.* 2004). The inoculation of naïve animals was realized at the level of nostrils as the virus is thought to infect its host by respiratory route following close contact. Results showed that the efficiency of transmission is a lot higher with nasal swabs from recently infected animals than with those from long-term infected animals, demonstrating shedding in the nasal mucosa of animals undergoing primary infection. Moreover, these results were confirmed during a similar study which also demonstrated that transitory shedding episodes occur in the nasal mucosa of infected sheep (Li *et al.* 2004). All this data indicates

that transmission is primarily executed *via* nasal secretions containing infectious viral particles, often from young and recently infected animals.

BoHV-4 infects cattle and was isolated from various samples harvested from both healthy animals and animals presenting various diseases such as endometritis, abortion, respiratory and digestive problems or even mammary skin lesions (Donofrio *et al.* 2005). This virus is present in European as well as in African, North American or Asian cattle without causing major health damage. Furthermore, experimental infection of cattle causes no clinical signs. The presence of the virus in healthy animals as well as in animals undergoing other pathologies seems to place this virus into the category of secondary pathogens, generating clinical symptoms only in cases of co-infection with other pathogens. These factors result in the low availability of data on the mechanisms of transmission of BoHV-4. However, the virus could be detected in the milk of cows suffering from bacterial mastitis (Kalman et al. 2004), leading to the hypothesis of food-borne transmission to newborn calves. The presence of cell-associated viruses in the milk from infected cows has been confirmed (Donofrio *et al.* 2000). Another study suggests the possibility of *in utero* transmission, as viral DNA was detected in blood samples of calves before colostrum intake. However, during this study, calves were seronegative at birth and showed no symptoms (Egyed *et al.* 2011).

There is less data available concerning the epidemiology and the transmission of MuHV-4. Natural epidemiology has already been evoked (see section 3.1). Regarding transmission, the literature currently available is very limited. However, it was suggested that the virus could be detected in many biological fluids including breast milk and urine (Hricová and Mistríková 2008). This study suggests the possibility of transmission from mother to offspring but also the possibility of transmission through territorial marking behavior. Transplacental transmission was also suggested and Stiglincova et al. published a study in 2011 strengthening this hypothesis (Stiglincova *et al.* 2011). This study also seems to show that the infection, even if it is latent, is the cause of a delay of fetal development as well as of shorter duration of gestation, resulting in fewer births. This study also provided further indications of the presence of virus in breast milk (Stiglincova *et al.* 2011). Globally, available data concerning MuHV-4 transmission are poor and the essential of the epidemiological cycle of the virus in natural population remains unknown.

The diversity of the routes of transmission and the frequent co-existence of multiple routes of dissemination of herpesviruses within their host population makes it relatively difficult to elucidate the preferred mode of transmission of a newly studied herpesvirus. Thus, although assumptions can be made by comparison with other known viruses belonging to the same viral genus, experimental evidence and/or epidemiological studies are needed to determine the existence of a specific mode of

transmission. Indeed, we have evoked that the human gammaherpesviruses seem to be essentially transmitted through re-excretion in saliva, but can also be sexually transmitted and even be associated with genital pathologies. Although current knowledge seems to determine this second mechanism as a secondary route of transmission for gammaherpesviruses, its precise impact remains undetermined so far.