2007

Genetics of Herpes Simplex Virus Type-1 tegument proteins involved in virion morphogenesis and egress

Preston A. Fulmer
Louisiana State University and Agricultural and Mechanical College

GENETICS OF HERPES SIMPLEX VIRUS TYPE-1 TEGUMENT PROTEINS INVOLVED IN VIRION MORPHOGENESIS AND EGRESS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Pathobiological
Sciences

By

Preston Fulmer
B.S. University of Arkansas, 2000
M.S. Louisiana State University, 2003
August 2007

ACKNOWLEDGEMENTS

First and foremost I would like to thank my wife Amanda. Without her patience and understanding, I would not be here. I would also like to thank my parents, Wayne and Paula, for their support and nurturing. Finally I would like to thank the people who have assisted in this research over the past three years in so many different ways: My fellow students Jeff, Chad, Arun, and Anna for their expert advice and opinions. Vladimir for his expert help when I was stuck, and Thaya for her beautiful sequences. Most of all I would like to thank my mentor, Dr. KG Kousoulas for his support and advice throughout this long process. I would not be here without any one of these people.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Herpes simplex virus type-1 (HSV-1) morphogenesis occurs in multiple stages within infected cells. Initially, the virion capsid assembles within the nucleus and buds through the nuclear membrane into the cytoplasm. Within the cytoplasm, additional tegument proteins attach to the capsid and the fully tegumented capsids bud into *trans*-Golgi network (TGN) derived vesicles. Enveloped virions are ultimately secreted to extracellular spaces. The process by which the cytoplasmic capsids bud into TGN-derived vesicles is not well understood. The prevalent model calls for specific interactions among viral tegument proteins and membrane proteins and glycoproteins embedded within TGN membranes. To further investigate the roles of tegument proteins in cytoplasmic virion envelopment, we constructed deletion mutants of UL11, UL20, both UL11 and UL20, and UL16. UL11 is involved in cytoplasmic virion envelopment. The ΔUL11 virus exhibits large amounts of unenveloped capsids in the cytoplasm of infected cells. The phenotype of the double null virus most closely resembled that of the UL20 single null virus (ΔUL20) in all areas: plaque phenotype, growth kinetics, and ultrastructural characteristics. To asses whether UL11 has any affect on UL20/gK localization, confocal experiments to determine the localization of UL11, UL20 and gK were undertaken, revealing that UL11 transport was completely independent of UL20/gK. Taken together these results indicate that UL11 acts at a step in cytoplasmic envelopment downstream of UL20, and UL20 is required for proper UL11 function. However, UL11 is not dependent upon the UL20/gK heterodimer for its transport. To assess the role of UL16 in virion morphogenesis and egress, the YEbac102ΔUL16 virus was constructed using a recently described RED markerless recombination system. ΔUL16 showed a large accumulation of intranuclear capsids not seen in the ΔUL11 virus. This result indicates a two-fold role for UL16 in virion morphogenesis and egress: 1) The nuclear accumulation of capsids seems to suggest that the first and most important role of UL16

is in intranuclear capsid assembly/egress. 2) The cytoplasmic accumulation of capsids suggests that UL16 also plays a role in cytoplasmic envelopment. These results indicate a possible pathway for the juxtaposition of cytoplasmic capsids with TGN-derived vesicles for final cytoplasmic envelopment.

CHAPTER 1
INTRODUCTION

STATEMENT OF PROBLEM AND HYPOTHESIS

Herpes Simplex Viruses are known to be the etiologic agents responsible for many human diseases including mucocutaneous oral and genital lesions, keratoconjunctivitis, and viral encephalitis. The herpesvirus life cycle is characterized by a number of distinct events, including binding of the virus to the cellular membrane and subsequent fusion of the viral and cellular membranes, transport of virions to the nucleus, replication and transcription of viral DNA, assembly of capsids and DNA packaging, and acquisition of a final viral envelope and egress from the cell. Investigating the viral proteins involved these different steps of the herpesvirus life cycle is an area of intense research interest. While the viral molecular determinants involved in virus entry are relatively well understood, the mechanisms involved in viral morphogenesis and egress from infected cells are not well defined.

HSV-1 egress from the nucleus of the infected cell to extra-cellular spaces involves a number of distinct steps, including primary envelopment by budding into the peri-nuclear space, de-envelopment into the cytoplasm, cytoplasmic re-envelopment by budding into vesicles originating from the Trans-Golgi Network (TGN), and translocation of enveloped virions to extra-cellular spaces. Initially, the virion capsid assembles within the nucleus and the virion acquires an envelope by budding into the peri-nuclear spaces (Roizman and Sears, 2001). Subsequently, these enveloped virions fuse with the outer nuclear lamellae leading to the accumulation of un-enveloped capsids into the cytoplasm. Within the cytoplasm, a number of additional tegument proteins attach to the capsid and the fully tegumented capsids bud into

cytoplasmic TGN- derived vesicles. Enveloped virions are ultimately secreted to extra-cellular spaces through the utilization of cellular vesicular trafficking systems (Browne et al., 1996; Granzow et al., 2001; Harley, Dasgupta, and Wilson, 2001; Mettenleiter, 2002; Skepper et al., 2001; Zhu et al., 1995)

The process by which the tegumented cytoplasmic capsids bud into TGN-derived vesicles is not well-understood. The prevalent model calls for specific interactions among viral tegument proteins and membrane proteins and glycoproteins embedded within TGN membranes as key factors that drive cytoplasmic virion envelopment. This model is supported by evidence that specific mutations within tegument proteins and multiple membrane proteins and glycoproteins inhibit cytoplasmic envelopment (Mettenleiter, 2004; Mettenleiter, 2006).

The overall hypothesis of the investigations described in this thesis, has been that there is a sequential and coordinate action of protein-protein interactions among tegument proteins and cytoplasmic portions of viral glycoproteins that bring about the cytoplasmic envelopment of tegumented virions. A priori, this hypothesis predicts that different defects exhibited by individual mutations or deletions of specific tegument proteins and viral glycoproteins that produce virions with partial defects in cytoplasmic virion morphogenesis can be ordered with respect to each other on the basis of their phenotypes and replication properties of mutant viruses that carry one or both sets of mutations affecting one or two different genes.

STATEMENT OF RESEARCH OBJECTIVES

The goal of this research was to investigate the functional relationship between Herpes Simplex Virus Type 1 tegument proteins UL11 and UL16 and membrane proteins UL20 and glycoprotein K (gK) in virion morphogenesis and egress. The specific aims of this research were:

I. To determine potential functional relationships between UL11 and UL20/gK.

1. To delineate UL11 and UL20 functions during the herpesvirus life cycle, UL11-null and UL11/UL20-double null viruses were constructed using a BAC mutagenesis system and extensively characterized by plaque morphology, viral growth (one-step growth curve), and viral ultra-structural characteristics were examined suing electron microscopy.
2. To determine if UL11, a protein that localizes to the TGN during viral infection, can affect the intracellular transport and TGN localization of UL20 or gK. UL20 and gK are co-dependent on each other for transport. Assesment of intracellular transport and TGN localization was achieved through the use of epitope tagged UL11, UL20, or gK were constructed (Foster et al., 2004c). The transport of these proteins and their effect on each other was determined by confocal microscopy.

II. To further investigate the known functinal relationship between the HSV-1 tegument proteins UL11 and UL16.

1. UL11 is known to physically interact with UL16 in transient co-transfection experiments (Loomis, Courtney, and Wills, 2003; Vittone et al., 2005). To further

investigate the UL11 and UL16 functions during the herpesvirus life cycle, UL16-null and UL16/UL11-double null viruses were constructed using a bacterial artificial chromosome (BAC) mutagenesis system. The phenotypes of these mutant viruses were extensively characterized by plaque morphology, viral growth (one-step growth curve), and viral ultra-structural characteristics.

Overall, the results obtained from this research indicate that:

A. UL20 acts at a step previous to that of UL11 during virion morphogenesis and egress, and this function is required for UL11 to function.

B. Although UL11 is localized to the TGN during viral infections, it has no effect on the transport of UL20 or gK.

C. UL16 is not absolutely required for HSV-1 replication in cell culture. However, UL16-null viruses exhibit less growth and cell to cell spread than wild type virus. Also, ΔUL16 exhibits a marked defect in nuclear egress and cytoplasmic envelopment.

The work is presented in individual chapters in a manuscript format having a specific title for the central theme of each chapter.

Chapter 2: The UL20 Protein Functions Precede and are Required for UL11 Functions in Herpes Simplex Virus Type-1 (HSV-1) Cytoplasmic Virion Envelopment

Chapter 3. Herpes Simplex Virus Type-1 (HSV-1) UL16 is Required for Efficient Nuclear Egress and Cytoplasmic Envelopment

LITERATURE REVIEW

History of Herpesviruses

The first description of Herpes Simplex Virus (HSV) can be traced back to the ancient Greeks. Hippocrates used the term "herpes" to describe lesions that appeared to creep or crawl along the skin (Wildy, 1973). Descriptions of lesions resembling HSV were also found on a Sumerian Tablet dated to the 3rd Millennium BC and the Ebers Papyrus, circa 1500 BC (Whitley, 2001). Herodotus is noted as the first to describe an association between the cutaneous lesions and fever caused by HSV, and Galen recognized that recurrent HSV lesions develop at the same anatomical location (Whitley, 2001). During the 18th century, Bateman accurately described the nature of HSV infection as a "restricted group of localized vesicles with a short, self-limiting course" (Bateman, 1814). The first description of the link between HSV and the genital organs did not appear until *De Morbis Venereis* was published by John Astruc, physician for King Louis XIV, in 1736, after studying the afflictions of French prostitutes (Astruc, 1736).

During the late 19th and early 20th century, human volunteers were often used to test the transmission of infectious agents, and Vidal showed that HSV was infectious by passing it from human to another (Vidal, 1873). Gruter, in a switch from human to animal studies, demonstrated that HSV could be transmitted from rabbit to rabbit, and he is widely credited with the isolation of HSV by the virology community (Gruter, 1924). In 1939, Burnett and Williams published an article describing the nature of latency, noting that HSV seems to persist for life and can be reactivated under stressful conditions to produce visible lesions (Burnet and Williams, 1939).

The development of tissue culture technology was critical in the isolation of other members of the human herpesvirus family. Between 1952 and 1956, varicella zoster virus (VZV), the causative agent of chicken pox, and cytomegalovirus (CMV) were isolated (Craig et al., 1957; Rowe et al., 1956; Smith, 1956; Weller and Stoddard, 1952). The eventual cultivation of lymphoblastoid tumor cells and B lymphocytes led to the isolation and study of Epstein-Barr virus (EBV) (Epstein, Achong, and Barr, 1964). In the 1990s, cultivation of T lymphocytes led to the isolation of human herpesviruses 6A, 6B, and 7 (Frenkel et al., 1990; Lopez et al., 1988; Salahuddin et al., 1986). More recently, Representational Differential Analysis (RDA) led to the discovery of human herpesvirus 8 (Chang et al., 1994).

Taxonomy of *Herpesviridae*

Identification of the new and apparently related viruses led to a scientific desire for classification. However, it was not until 1981 that the current herpesvirus classification came into being. All herpesviruses examined to date are capable of establishing a latent infection in their natural hosts in a specific set of cells, which varies from one virus to another. Other biological properties vary, such as the length of the reproductive cycle, and these were used as the basis of classification, before DNA sequences of the viruses were known. Members of the family *Herpesviridae* were initially classified by the Herpesvirus Study Group into three subfamilies: the *Alphaherpesvirinae*, the *Betaherpesvirinae*, and the *Gammaherpesvirinae* (Roizman, Bartha, and Biggs, 1973; Roizman et al., 1992; Van Regenmortel et al., 2000). DNA sequence data has since supported and expanded the platform on which the classification system rests. Table 1.1 shows the classification of the nine known human herpesviruses (bold type) as

well as other commonly studied herpesviruses (Roizman et al., 1992; Van Regenmortel et al., 2000).

Alphaherpesvirinae were classified based on their variable host range, short reproductive cycle, rapid spread in tissue culture, efficient destruction of infected cells, and the ability to establish latent infections primarily in sensory ganglia. The subfamily consists of the genera *Simplexvirus, Varicellovirus, Marek's disease-like virus,* and *Infectious laryngotracheitis-like virus* (Roizman et al., 1992; Van Regenmortel et al., 2000).

Betaherpesvirinae were characterized by a limited host range, long reproductive cycle, and slow infection progression in tissue culture. Cells that are infected often become enlarged (cytomegalia), and the viruses can maintain latency in secretory glands, lymphoreticular cells, kidneys, and other tissues. The subfamily consists of the genera *Cytomegalovirus, Muromegalovirus,* and *Roseolovirus* (Van Regenmortel et al., 2000).

Gammaherpesvirinae were classified by a limited host range and ability to replicate in lymphoblastoid cells, with some viruses also causing lytic infection in some types of epithelial and fibroblastic cells. Viruses are usually specific for either B or T lymphocytes, and latent virus is frequently demonstrated in lymphoid tissue. The *Gammaherpesvirinae* subfamily consists of the genera *Lymphocryptovirus* (EBV), and *Rhadinovirus* (Van Regenmortel et al., 2000).

Table 1.1: Herpesvirus Taxonomy.

Table 1: Members of the family Herpesviridae

Subfamily	Designation	Vernacular Name
Alphaherpesvirinae	***Human herpesvirus 1 (HHV-1)***	**Herpes simplex virus type 1 (HSV-1)**
	Human herpesvirus 2 (HHV-2)	**Herpes simplex virus type 2 (HSV-2)**
	Human herpesvirus 3 (HHV-3)	**Varicella-zoster virus (VZV)**
	Cercopithecine herpesvirus 1 (CeHV-1)	Herpesvirus B, Simian Herpesvirus
	Gallid herpesvirus 1 (GaHV-1)	Infectious laryngotracheitis virus
	Gallid herpesvirus 2 (GaHV-2)	Marek's disease herpesvirus 2
	Suid herpesvirus 1 (SuHV-1)	Pseudorabies virus, Aujesky's disease
	Felid herpesvirus 1 (FeHV-1)	Feline herpesvirus 1, Feline rhinotracheitis herpesvirus
	Ictalurid herpesvirus 1 (IcHV-1)	Channel catfish herpesvirus
Betaherpesvirinae	***Human herpesvirus 5 (HHV-5)***	**Cytomegalovirus (CMV)**
	Cercopithecine herpesvirus 8 (CeHV-8)	Rhesus monkey cytomegalovirus
	Murid herpesvirus 1 (MuHV-1)	Mouse cytomegalovirus
	Murid herpesvirus 2 (MuHV-2)	Rat cytomegalovirus
	Suid herpesvirus 2 (SuHV-1)	Pig cytomegalovirus
	Felid herpesvirus 2 (FeHV-1)	Cat cytomegalovirus
	Human herpesvirus 6A (HHV-6A)	
	Human herpesvirus 6B (HHV-6B)	**Roseolovirus**
	Human herpesvirus 7 (HHV-7)	
Gammaherpesvirinae	***Human herpesvirus 4 (HHV-4)***	**Epstein-Barr virus (EBV)**
	Human herpesvirus 8 (HHV-8)	**Karposi's sarcoma-associated herpesvirus (KSHV)**

Clinical Significance of Herpes Simplex Viruses

Epidemiology

Infections caused by HSV occur worldwide in both developed countries and underdeveloped countries (Black, 1975). There are no known animal carriers for HSV; therefore, humans remain solely responsible for transmitting virus to other humans. Virus transmission from an infected to a susceptible individual occurs during close personal contact.

The frequency of person to person contact appears to be the major mediator of infections (Whitley, 2001). Due to the fact that HSV infection rarely results in fatality and the nature of latency, more than half of the world's population probably has a recurring HSV infection, enabling the transmission of HSV. Initial HSV infection usually occurs in children less than 5 years old and is most often asymptomatic. The mouth area is the most common location of infection (Whitley, 2001). Primary infection leads to the shedding of virus from the mouth and stool for an average of 7 to 10 days (Amir et al., 1997), and neutralizing antibodies appear between 4 and 7 days after the onset of HSV infection and peak at approximately 3 weeks post infection (Buddingh et al., 1953).

The primary factors affecting the rate of HSV infection are location, socioeconomic status, and age. In some studies, by the age of 15, over 95% of children were found to possess antibodies to HSV (Bader et al., 1978; Black et al., 1974). HSV-1 seroprevalence is much more common than its counterpart HSV-2 (Fleming et al., 1997; Nahmias, Lee, and Beckman-Nahmias, 1990). Because HSV-2 is usually acquired through sexual contact, antibodies to HSV-2 are rarely found before the onset of sexual activity. While most genital infections are caused by HSV-2, there is an ever-increasing proportion attributable to HSV-1 (Corey et al., 1983; Kalinyak, Fleagle, and Docherty, 1977). Genital HSV-1 infections are usually both less severe than HSV-2 and less prone to recurrence (Corey et al., 1983; Reeves et al., 1981).

Pathogenesis

Initially, virus must come in contact with a mucosal surface or abraded skin for HSV infection to be initiated. After primary infection, viral replication at the infected location, usually oral or genital mucosal tissue, results in the infection of sensory nerve endings; and virus

is then transported to the dorsal root ganglia (Baringer and Swoveland, 1973; Bastian et al., 1972). In HSV-1 infection, the trigeminal ganglia becomes colonized and harbors latent virus; whereas in HSV-2 infection the sacral ganglia is the site of latency (Whitley, 2001). After the establishment of latency, certain stimuli can cause reactivation to occur, and virus becomes evident at mucocutaneous sites as vesicles or ulcers. A more severe primary infection can result in a higher rate of HSV reactivation.

Cellular changes induced by viral infection include enlargement of infected cells and the appearance of condensed chromatin within the nuclei, followed by degradation of the nuclei. Cells lose intact plasma membranes and form multinucleated giant cells. In infected dermal regions, there is an intense inflammatory response, and the intensity decreases substantially with recurrent disease (Whitley, 2001).

Mucocutaneous Infections

Primary HSV-1 infection can be either totally asymptomatic or can result in a symptoms in any combination of fever, sore throat, vesicular or ulcerative lesions. However, asymptomatic infection is generally the rule rather than the exception (Whitley, 2001). The duration of symptomatic disease in symptomatic children is generally 2 to 3 weeks, with a fever of 101°F to 104°F. The onset of a recurrent HSV-1 infection is generally marked pain, burning, tingling, or itching, which generally lasts for less than 6 hours, and is followed by vesicle formation within 24 to 48 hours (Spruance and Crumpacker, 1982; Spruance et al., 1984; Spruance et al., 1977). Usually, 3 to 5 vesicles appear at the border of the lip and last no longer than 48 hours. Pain is most severe at the outset of vesicle formation and resolves in 4 to 5 days. The frequency of recurrences varies greatly among individuals, and the factors that influence reactivation are

poorly defined but may include fever, stress, and exposure to UV light (Segal et al., 1974; Ship, Miller, and Ram, 1977).

In HSV-2, the most severe clinical symptoms are encountered with primary infection, characterized by the appearance of macules and papules followed by vesicles, pustules, and ulcers. The duration of lesions and viral secretion averages about 3 weeks. Men and women experience both similar and dissimilar symptoms (Corey, 1982; Corey et al., 1983). Preexisting immunity to HSV-1 can have a beneficial effect in reducing the severity of HSV-2 primary infections (Allen and Rapp, 1982; Corey et al., 1981; Kaufman et al., 1973). Recurrent HSV-2 is milder than initial infection and is characterized by the appearance of 3 to 5 vesicles (Adams et al., 1976). Symptoms usually last 7 to 10 days, and virus is shed for an average of 2 to 5 days. The biggest problem involving recurrent genital herpes is the frequency of recurrences, which varies by individual. Recurrences usually occur several times per year; and, whether symptomatic or asymptomatic, transmission of the infection to sexual partners can occur with intimate contact (Corey et al., 1983).

Fetal and Neonatal Infections

Neonatal HSV infections occur at a rate of about 1 in 3000 deliveries per year (Nahmias, Keyserling, and Kerrick, 1983; Nahmias, Keyserling, and Lee, 1989), and infection occurs far less frequently than genital infections in the adult population. The type of maternal genital infection at the time of delivery is directly tied to the risk of fetal infection. If the mother has a primary infection the risk of transmission is approximately 30%, while the risk for recurrent infection is 3% or less (Brown et al., 1991). The most common route of infection, 75% to 80%, is intrapartum contact of the fetus with infected maternal secretions (Whitley, 2001). The

clinical symptoms of neonatal HSV infection are a reflection of the site and extent of viral replication, with infection being almost always symptomatic and often lethal. Babies with HSV infection can have disease: localized to the skin, eye, and mouth; encephalitis with or without skin involvement; or disseminated infection involving multiple organs (Nahmias et al., 1970; Whitley et al., 1981). The highest mortality rate occurs in babies with disseminated infection. In addition, frequently occurring HSV-2 cutaneous lesions defined a group at risk for neurological problems (Whitley et al., 1991).

Infection of an Immunocompromised Host

Patients who are immunocompromised due to immunotherapy, malnutrition or acquired immunodeficiency syndrome are at risk for severe HSV infections, and these patients may develop progressive disease involving the respiratory tract, esophagus, or the gastrointestinal tract (Korsager et al., 1975; Montgomerie et al., 1969). Recurrent HSV infection can occur in theses patients at multiple sites and healing occurs over an average of 6 weeks (Whitley et al., 1984). The repeated treatment required for these patients can lead to viral mutants resistant to antiviral therapy.

Central Nervous System (CNS) Infections

Encephalitis caused by HSV the most common cause of sporadic, fatal encephalitis in this country (Olson et al., 1967). Some studies estimate a rate as high as 1250 cases per year in the United States (Whitley, 2001). Encephalitis is caused when then virus spreads past the dorsal root ganglia, in which latency is usually established, to the CNS. The mechanisms responsible for this aberrant event in the virus life cycle are unclear. The manifestations of HSV encephalitis

include primarily focal encephalitis along with fever, altered behavior, and localized neurological findings. There is usually evidence of a localized temporal lobe disease (Whitley et al., 1977; Whitley et al., 1981). In untreated patients, mortality exceeds 70% and only 2.5% of patients return to normal neurological function (Whitley, 2001).

Keratoconjunctivitis

There are 300,000 new cases of HSV eye infection annually, ranking second behind trauma as the cause of corneal blindness (Binder, 1977). Primary herpetic keratoconjunctivitis can occur in either a single eye or both eyes, and healing of the cornea can take as long as one month even with antiviral therapy. Recurrent HSV eye infections occur at a similar rate to HSV-1 mucocutaneous infections and most often involve only a single eye. Repeated attacks can last for weeks or months and progressive disease can result in vision loss (Whitley, 2001).

Prevention and Treatment of HSV Infection

The two methods for control of HSV infections are antiviral therapy and prevention. Antivirals such as acyclovir and valaciclovir are effective in limiting the extent of HSV infection and therefore helpful in limiting spread to uninfected individuals. However, post-exposure antiviral treatment does not prevent lifelong infection of an individual. Prevention of HSV infection is mainly achieved through avoiding contact with infectious secretions. Vaccination would be the ideal method of HSV prevention; however, to date no HSV vaccine has been clinically successful.

Architecture of the Herpes Virion

Virions of herpes viruses can vary in size from 120nm to 300nm (Roizman and Furlong, 1974), and consist of: an electron-dense core, an icosadeltahedral capsid around the core, an amorphous tegument around the capsid, and an outer envelope containing glycoprotein spikes (Roizman and Furlong, 1974). The variability in the size of herpes virions is due mainly to variability in the makeup of the tegument and the state of the envelope. A model of the virion architecture is presented below in Figure 1.1.

The Core

The core of a mature herpes virion contains the viral DNA in the form of a torus that may appear to be suspended by a proteinaceous spindle to the capsid (Falke, Siegert, and Vogell, 1959; Furlong, Swift, and Roizman, 1972; Nazerian, 1974). The toroidal structure is 50 nm high, with an inside diameter of 18nm and an outside diameter of 70 nm. The arrangement of the viral DNA in the torus is not known.

The Capsid

The capsid is approximately 125 nm in diameter in the form of an icosadeltahedron and features 162 capsomeres, characteristic of all herpesviruses. Capsomeres are either pentons or hexons, consisting of 5 or 6 VP5 (major capsid protein) monomers, respectively. In the capsid, the pentons are located on the icosahedral 5-fold vertices, and the hexons make up the faces and edges. Hexons also contain 6 copies of VP26, attached to the upper edge of VP5 and form a continuous ring around each hexon (Zhou et al., 1995). A heterotrimeric complex known as the

triplex connects the capsomeres; the triplex consists of two copies of VP23 and one copy of VP19C and acts as a sort of scaffold for the capsid (Spencer et al., 1998).

The Tegument

The tegument is contained between the capsid and the virion envelope and appears fibrous on negative staining (Morgan et al., 1959; Morgan, Rose, and Mednis, 1968; Wildy and Watson, 1962). The tegument can be distributed asymmetrically and its thickness can vary depending on the location of the virion particle within the infected cell. There is less tegument that is more symmetrically arranged in peri-nuclear virions than in virions in cytoplasmic vesicles that contain more tegument distributed more asymmetrically (Falke, Siegert, and Vogell, 1959). Tegument proteins are important in various aspects of the virus life cycle and are believed to have key functions in the early events of infection and virion egress. There is ordered tegument density around the pentons, suggesting symmetry where the capsid and tegument interact (Zhou et al., 1999). This density may be due to the VP1-3 protein, an extremely large 336 kDa protein, thought to be involved in nucleocapsid attachment to the nuclear pore facilitating DNA release into the nucleoplasm (Batterson, Furlong, and Roizman, 1983; Knipe et al., 1981; Ojala et al., 2000). However, VP1-3 null mutants also accumulate newly assembled, DNA-filled capsids in the cytoplasm of infected cells, indicating that VP1-3 is involved in various stages of the virus life cycle (Desai, 2000).

The Envelope

The outer covering of the herpesvirus, the envelope, has a typical trilaminar appearance (Epstein, 1962) and appears to be made up of altered cellular membranes (Armstrong, Pereira,

and Andrewes, 1961; Falke, Siegert, and Vogell, 1959; Morgan, Rose, and Mednis, 1968). The herpesvirus envelope contains numerous glycoprotein extrusions, while the amounts of each glycoprotein vary. HSV specifies at least 11 different glycoproteins, and the copy number of each glycoprotein can well exceed 1,000 per virion. Envelope glycoproteins gB, gD, gH, and gL have been shown to be required for virion entry into susceptible cells.

Organization of the Viral Genome

The viral DNA of herpesviruses is linear and double stranded, but the DNA becomes circular immediately after release from capsids into the nucleoplasm of the infected cells. The length of the genome of different herpesviruses varies between 120 to 250 kbp, with the size of HSV-1 determined to be 152,261 bp (McGeoch et al., 1988). This variability is different than polymorphism in the genome length of individual viruses, which is due to terminal and internal repeated sequences that can vary in copy number, leading to variations in genome length of more than 10 kbp. The total G+C content of herpesviruses varies from 31% to 75%, and this percentage can vary across the genome (Roizman and Pellett, 2001). HSV-1 and HSV-2 contain approximately 68% and 69% G+C content, respectively (Becker, Dym, and Sarov, 1968; Kieff, Bachenheimer, and Roizman, 1971). The sequence arrangement of herpesvirus genomes varies on the presence and location of reiterated sequences that allow rearrangement to occur. In Herpes Simplex Virus genomes, the sequences from both termini are repeated in an inverted orientation and juxtaposed internally. As a result, the genome is divided into two regions, consisting of the unique long (U_L) and unique short (U_S) regions flanked by inverted repeats (Figure 1.2). Both components are able to invert relative to the other to form four isomers;

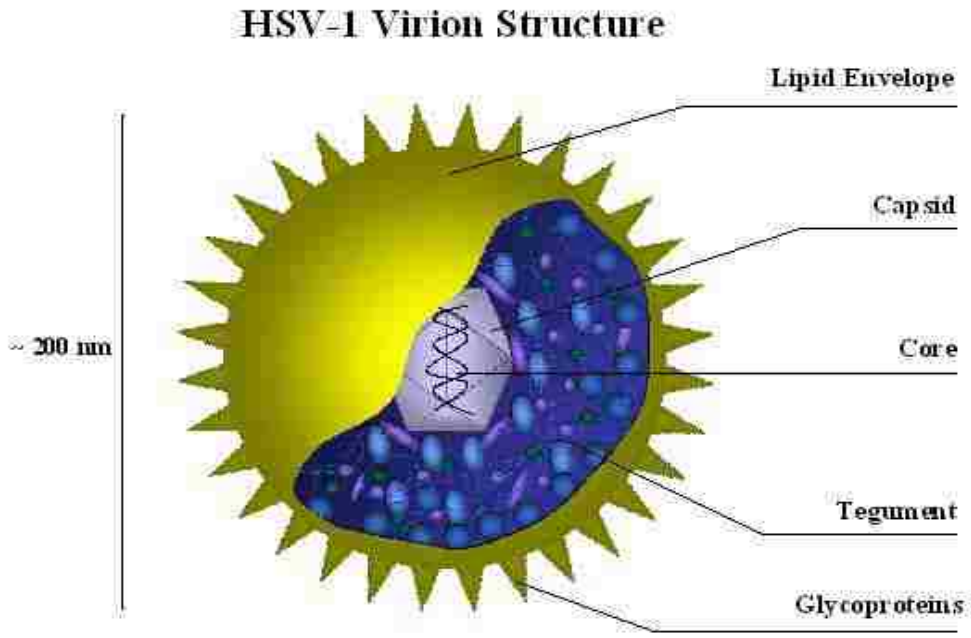


Figure 1.1: Herpesvirus virion structure. Virions of herpes viruses can vary in size from 120nm to 300nm (Roizman and Furlong, 1974). A virion consists of: an electron-dense core containing the viral genome, an icosadeltahedral capsid around the core, an amorphous tegument around the capsid, and an envelope derived from cellular membranes containing glycoprotein spikes (Roizman and Furlong, 1974). Figure from (Melancon, 2003).

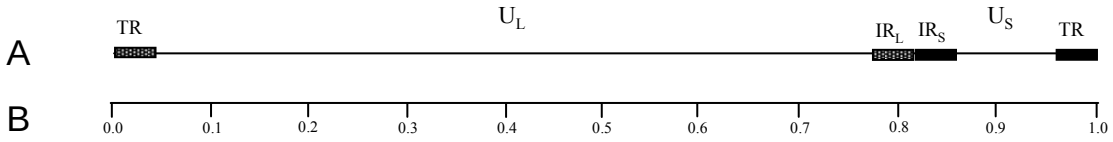


Figure 1.2. Arrangement of the HSV-1 genome. (A) The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (B) The bottom line shows map units of the HSV-1 genome.

and, as predicted, DNA purified from infected cells contains four equivalent populations, which differ in the relative orientation of the unique long and unique short regions (Roizman and Pellett, 2001).

The majority of herpesvirus genes contain: a promoter region 50 to 200 bp upstream of a TATA box, a transcription initiation site 20 to 25 bp downstream of the TATA box, a 5' untranslated leader sequence of 30 to 300 bp, a single major open reading frame (ORF) with a translation initiation codon meeting the host requirement for efficient initiation, 10 to 30 bp of 3' untranslated sequence, and a polyadenylation signal with standard flanking sequences (Roizman and Pellett, 2001). Some exceptions include genes without a TATA box or genes with a second in-frame initiator methionine (Chou and Roizman, 1986; Markovitz, Filatov, and Roizman, 1999). Most transcriptional gene products are not spliced, although every herpesvirus expresses a few spliced genes. Herpesviruses also produce non-coding RNAs, such as the HSV-1 latency associated transcript (LAT) (Roizman and Pellett, 2001). The different members of the herpesvirus family encode between 70 and 200 genes, estimated using various methods

(Roizman and Pellett, 2001). HSV-1 encodes about 90 gene products, with at least 84 of the transcriptional units encoding proteins (Roizman and Knipe, 2001).

The Herpes Simplex Virus Lifecycle

Virus Attachment and Entry

Herpesvirus entry is a multistep process involving multiple viral glycoproteins acting as ligands for multiple receptors on the surface of the target cell. Entry is the most critical step in the HSV life cycle and greatly determines the tropism and pathology of each member of the herpesvirus family. The wide host range of HSV and narrow host range of EBV can be in part explained by the ability of each virus to utilize a different array of cell surface binding and entry receptors. Entry of HSV occurs is three distinct stages: the first step involves virus binding to the surface of the cell, the second step involves an interaction of gD (HSV-1) with an entry receptor, and the third step involves fusion of the viral envelope with the plasma membrane of the cell, releasing the capsid-tegument complex into the cytoplasm of the infected cell (Figure 1.4).

Binding Receptors

Initial contact of herpesviruses with cells usually occurs through binding receptors, in which engagement of the virus with receptor is reversible and changes in the virion envelope required for fusion and entry do not occur. Herpes Simplex viruses initial attachment to cells involves the interaction of glycoprotein C, and glycoprotein B to a lesser extent, with cell surface

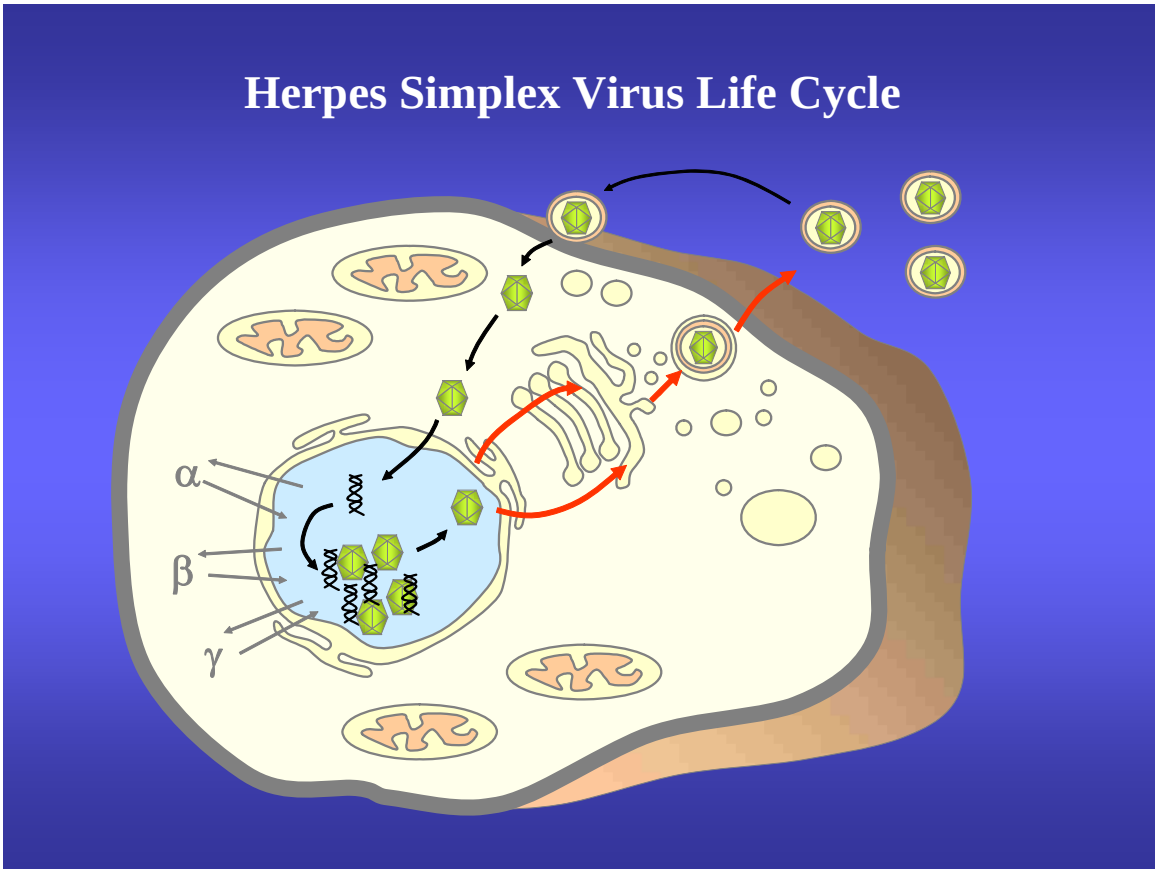


Figure 1.3: The Herpes Simplex Virus Life Cycle. The first stage of the herpes virus life cycle consists of virus entry, capsid transport to the cell nucleus, deposition of viral DNA into the nucleoplasm, coordinate gene expression and viral DNA replication (black arrows). The second stage is virion morphogenesis and egress comprised of primary envelopment at the inner nuclear membrane, de-envelopment at the outer nuclear membrane, final envelopment into cytoplasmic vesicles and transport to extra-cellular spaces (red arrows). Figure from (Melancon, 2003).

glycosaminoglycans, usually heparan sulfate (Shieh et al., 1992; WuDunn and Spear, 1989). Heparan sulfate is synthesized as the glycosaminoglycan component of heparan sulfate proteoglycans, and it is widely distributed on cell surfaces in mammals. The presence of heparan sulfate is not essential for virus entry, but it greatly increases the efficiency of virus entry into cells (Banfield et al., 1995). Similarly, although glycoprotein C confers the greatest efficiency for virus attachment to cell surfaces, increasing the efficiency of virus binding by approximately 10 fold, it is not essential for either virus entry or replication (Heine et al., 1974).

Entry Receptors

Binding of the HSV glycoprotein D to an entry receptor sets in motion an irreversible chain of events leading to the fusion of the virion envelope with the plasma membrane of the target cell. There have been three classes of HSV entry receptors classified to date, indicated below and shown in Figure 1.5.

Tumor Necrosis Factor (TNF) Receptor Family

HVEM / Herpes Virus Entry Mediator A (HveA) is a member of the TNF receptor family and is expressed in a variety of different cell types including lymphocytes, other leukocytes, epithelial cells, and fibroblasts. While HVEM is utilized by herpes simplex viruses as an entry receptor, the natural ligands for HVEM include LIGHT and lymphotoxin-alpha (Mauri et al., 1998). LIGHT can function as a second signal for T-cell activation, and the interactions between LIGHT and HVEM are the focus of active investigations of the regulation of immune responses (Kwon et al., 2003). HVEM serves as an excellent entry mediator for both HSV-1 and HSV-2. Certain studies have shown that HSV-1 entry of activated T-cells is principally mediated by

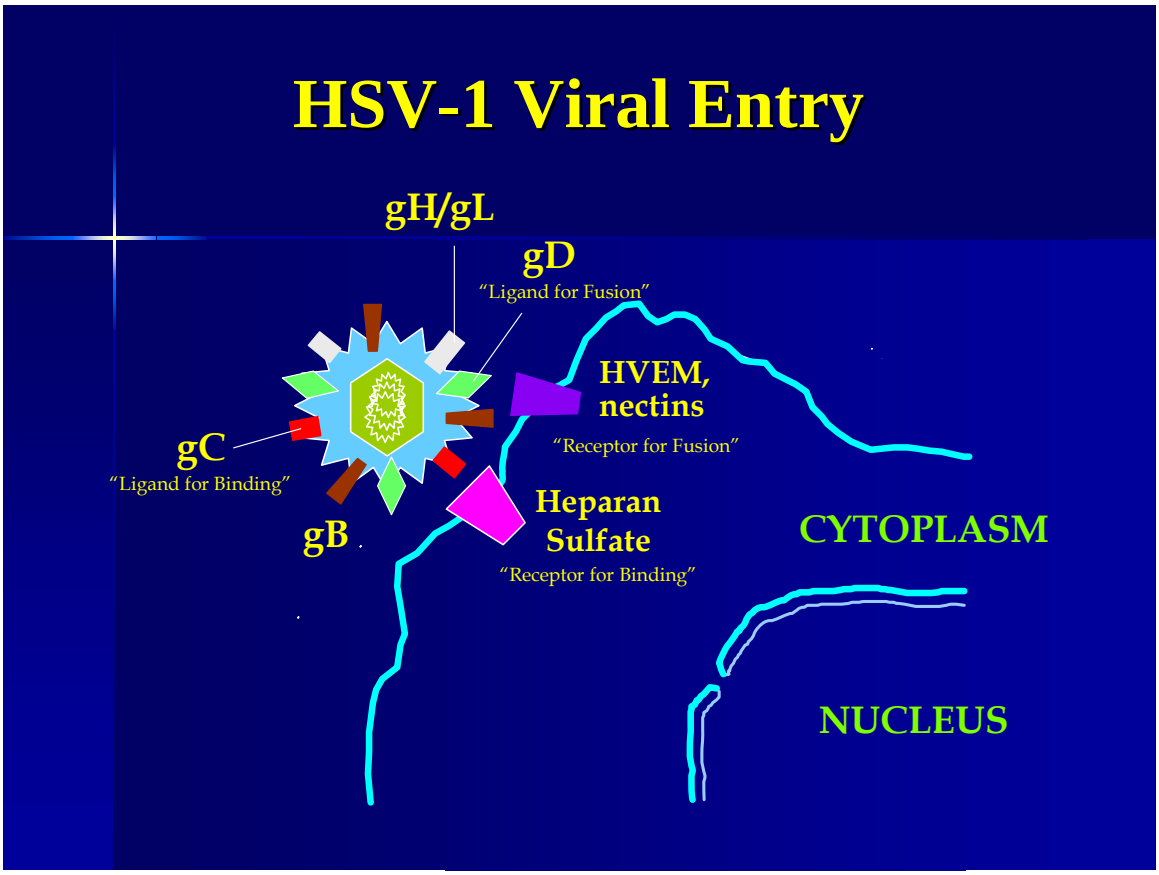


Figure 1.4: Herpes Simplex Virus Entry. The first step of HSV entry is attachment to the plasma membrane of infected cells through interaction of gC (ligand for binding) with cell surface heparan sulfate (receptor for binding). The second step is fusion of the viral envelope with the cell plasma membrane and occurs following attachment of gD (ligand for fusion) to either HVEM or a nectin (receptor for fusion) on cell surfaces. Fusion requires the presence of gD and an entry receptor, as well as gB and the gH/gL heterodimer. Figure from (Melancon, 2003).

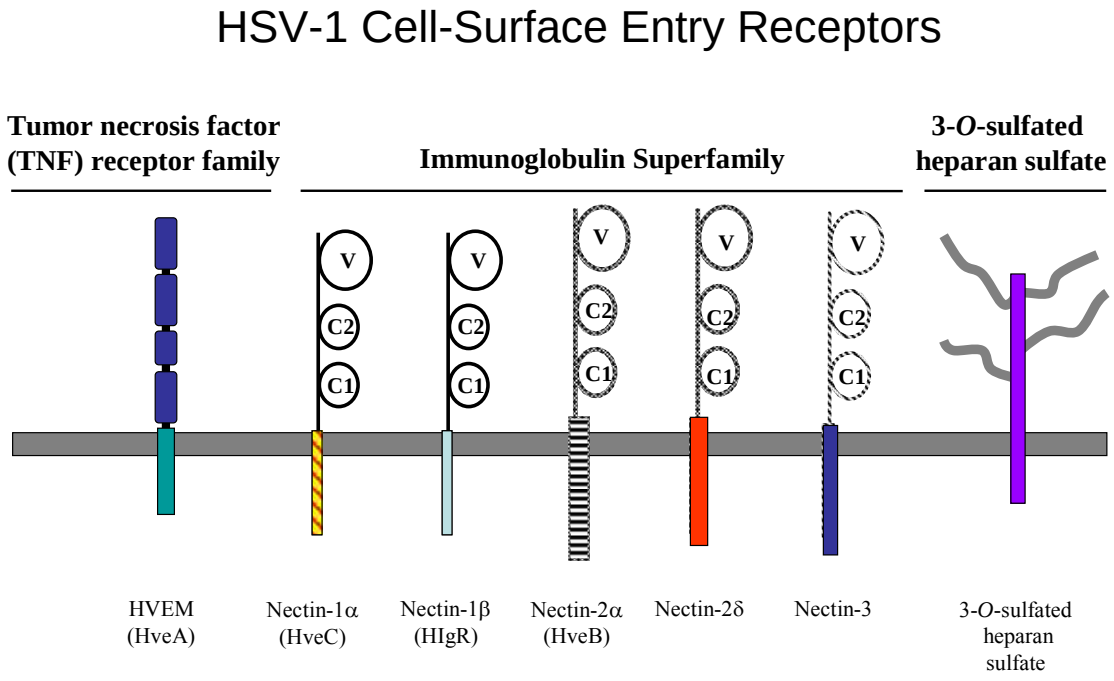


Figure 1.5: Herpes Simplex Virus entry receptors. The three classes of cell surface receptors for HSV entry are: the tumor necrosis factor (TNF) receptor family consisting of HVEM, the immunoglobulin superfamily consisting of the nectins, and 3-*O*-sulfated heparin sulfate. Only viral attachment can occur in the absence of an HSV entry receptor. Figure from (Melancon, 2003).

HVEM, although in variety of other cell types HVEM was shown not to be the primary mediator of virus entry (Montgomery et al., 1996).

Immunoglobulin Superfamily

The second family of herpes simplex virus entry receptors is part of the immunoglobulin superfamily and includes several isoforms present in both human and nonhuman cells encoded

by mRNAs that can vary due to alternative splicing. Members of this family have been shown to act as intercellular adhesion molecules localized at adhesion junctions, in which the carboxyl-terminal domains bind to L-afadin, a PDZ-binding protein that anchors the receptors to the cytoskeleton and adheren junctions. These receptors have been named "nectins" on account of their cellular function (Takahashi et al., 1999). Nectins are highly conserved among mammalian species in respect to structure, function, and the ability to mediate HSV entry (Milne et al., 2001; Shukla et al., 2000). The nectins are expressed in a variety of cell types including epithelial cells, fibroblasts, and neurons (Takai and Nakanishi, 2003). In addition, the distribution of this class of receptors in human tissues reflects the susceptibility of cells to infection and probably accounts for both virus entry and cell-to-cell spread (Roizman and Knipe, 2001).

Nectin-1α (Herpes Virus Entry Mediator C, HveC) and nectin-1β (Herpesvirus Immunoglobulin-like receptor, HIgR) are two mRNA splicing variants containing a common ectodomain and are expressed on epithelial, fibroblastic, neural and hematopoietic cells; in keratinocytes; and in human tissues that are the target of HSV infection including skin, brain, and spinal ganglia (Cocchi et al., 1998; Geraghty et al., 1998). Nectin-1α and nectin-1β have the ability to mediate entry of all HSV-1 strains, HSV-2, Pseudorabies virus (PrV) and bovine herpes virus 1 (BHV-1) (Geraghty et al., 1998).

Nectin-2α (Herpes Virus Entry Mediator B, HveB) and nectin-2δ are also mRNA splice variants, and they mediate the entry of HSV-2, PrV, and certain viable mutant forms of HSV-1 but not wild-type HSV-1 (Lopez et al., 2000; Warner et al., 1998). Nectin-1 and nectin-2 are related to nectin-3 and nectin-4, and the poliovirus receptor (Takai and Nakanishi, 2003).

Nectin-3 has been shown to be expressed on J cells, which lack HVEM, nectin-1, and nectin-2. An HSV-1 mutant, designated HSV-1 (JMP), was able to enter into J cells expressing

nectin-3, but wild-type HSV-1 was not (Cocchi et al., 2004). HSV-1 (JMP) contains mutations in glycoprotein K as well as glycoprotein D. The mutations in both gK and gD were shown to be required for HSV-1 (JMP) entry into J cells (Cocchi et al., 2004).

3-*O*-sulfated Heparan Sulfate (3-OS HS)

Modification of heparan sulfate by D-glucosaminyl 3-*O*-sulfotransferase isoforms-3 and/or -5 creates 3-*O*-sulfated Heparan Sulfate (3-OS HS), generating a gD-binding site that allows heparan sulfate proteoglycans to function as entry receptors for HSV-1. 3-*O*-sulfated heparan sulfates are broadly distributed on human cells and tissue and mediate HSV-1 but not HSV-2 entry (Shukla et al., 1999).

Other herpesvirus receptors

Only alphaherpesviruses (except VZV) encode members of the gD family. Betaherpesvirus and gammaherpesviruses rely on other viral proteins to serve as ligands for cellular receptors. EBV uses gp42, which becomes a member of a gH-gL-gp42 complex, to serve as a ligand for human leukocyte antigen (HLA) class II molecules on B lymphocytes. Human CMV encodes glycoprotein O (gO), which is unrelated to gp42 but also forms a complex with gH-gL (Huber and Compton, 1998). In addition, HHV-6A encodes glycoprotein Q (gQ), which forms a complex with gH-gL (Mori et al., 2003). However, it is unclear whether or not gO or gQ are required for entry in CMV or HHV-6A, respectively.

Virus-to-Cell Fusion

After binding of gD to a HSV-1 virus entry receptor, the last step in virus entry is fusion of the virion envelope with the plasma membrane of the target cell (Morgan, Rose, and Mednis, 1968). The current belief is that gD (Ligas and Johnson, 1988), gB (Sarmiento, Haffey, and Spear, 1979), and the gH/gL heterodimer (Forrester et al., 1992) are required for the virus-to-cell fusion process to occur. Since gD is required for entry receptor binding, it would be logical to hypothesize that receptor binding triggers a conformational change in gD that translates to a change in gB and gH/gL, resulting in activation of the HSV-1 membrane fusion machinery. In some cells types, notably CHO cells that express an entry receptor such as nectin-1 and HeLa cells, endocytosis and acidification of endosomes are required for efficient virus entry. Interestingly, it was found that endocytic uptake of HSV virions from the cell surface is rapid and independent of any known gD receptor. In addition, efficient entry through endocytosis requires cellular PI 3-kinase activity and the viral glycoproteins gB, gD, and gH/gL (Nicola, McEvoy, and Straus, 2003; Nicola and Straus, 2004). While these two entry pathways are spatially distinct, they are still functionally similar in that both require the same set of viral glycoproteins and a gD entry receptor.

Recent studies involving the HSV-1 fusion machinery indicate that these glycoproteins act in a sequential manner, with gD being involved in Phase I, gH/L in Phase II, and gB in Phase III (Gianni, Forghieri, and Campadelli-Fiume, 2006; Subramanian and Geraghty, 2007). Additionally, the crystal structure of HSV-1 gB has been solved, revealing a remarkable homology to vesicular stomatitis virus (VSV) gB. Along with the similarity to VSV gB, two domains that indicate HSV-1 gB is intricately involved in virus cell fusion were identified: an alpha-helical coiled-coil core reminiscent of class I fusion proteins, and two extended beta

hairpins with hydrophobic tips which are indicative of class II fusion proteins (Heldwein et al., 2006). Subsequent mutagenesis studies on HSV-1 gB showed that hydrophobic tips of the loops were vital for proper HSV-1 gB function (Hannah et al., 2007).

Host Protein Shutoff

HSV shuts off the synthesis of cellular protein products in two stages. First, the virus mediates degradation of the host cell mRNA. Second, there is inhibition of further synthesis and processing of host mRNA. The virion host shutoff (*vhs*) function involves structural components of the virion and does not require de novo protein synthesis after infection (Fenwick and Walker, 1978; Nishioka and Silverstein, 1977; Nishioka and Silverstein, 1978a; Nishioka and Silverstein, 1978b). The isolation of *vhs*$^-$ mutants, which failed to shutoff host cell protein synthesis in HSV-1 infected cells, allowed for more detailed study of the viral requirements for host shutoff (Read and Frenkel, 1983). The UL41 ORF was identified as being responsible for the phenotype in the *vhs*$^-$ mutants (Fenwick, Morse, and Roizman, 1979; Kwong, Kruper, and Frenkel, 1988; Oroskar and Read, 1987). The UL41 protein (VHS) is expressed as a γ_1 gene (Frink, Anderson, and Wagner, 1981) and is translated to a 58,000 or 59,500 kDa phosphoprotein (Read, Karr, and Knight, 1993). In addition, VHS was conclusively shown to be capable of functioning in the absence of any other viral proteins (Zelus, Stewart, and Ross, 1996). VHS was shown to function at least in part by inducing endoribonucleolytic cleavage of mRNA *in vitro* (Elgadi and Smiley, 1999), and the 5' end of mRNA is degraded before the 3' end (Karr and Read, 1999). VHS appears to form a complex with the transcription factor eIF-4H, and the interaction is required for RNase activity possibly through targeting to polyribosomes. The VHS and eIF-4H complex appears to decap cellular mRNA from the 5' end (Roizman and Knipe, 2001).

In the context of a *vhs*$^-$ mutant, host protein synthesis is not shut off early in infection and α and β gene expression is extended in comparison to a wild-type virus. These effects are due to the fact that VHS accelerates the degradation of both cellular and viral mRNAs. As a result, during the context of a HSV-1 infection, cellular mRNA levels diminish and as viral mRNA levels accumulate it is preferentially translated. In this way, VHS may facilitate the transition from α to β to γ gene expression through shortening the life of the viral mRNAs (Kwong and Frenkel, 1987; Oroskar and Read, 1987). As a γ_1 gene product, VHS accumulates late in infection, but does not degrade viral mRNA as would be expected. An interaction between VP16 and VHS at late times blocks the degradation of RNA by VHS (Lam et al., 1996). As a result, the accumulation of VHS into tegument complexes accomplishes two crucial functions: it brings VHS into the infected cell to shutoff host protein synthesis, and it regulates the activity of VHS late in infection when mostly viral mRNA is present.

Virion Transport to the Nucleus

After fusion of the virion envelope with the plasma membrane of the infected cell, the capsid with its associated tegument complex is deposited into the cytoplasm. Some tegument proteins disassociate from the complex and remain in the cytoplasm, while others, such as VP16 (αTIF) are transported to the nucleus to effect their functions. The remaining capsid-tegument complex is then transported along the cell's microtubule network to a nuclear pore. Studies have shown that dynein, the microtubule dependent motor, is bound to capsids after entry (Sodeik, Ebersold, and Helenius, 1997), and these results prompted the theory that incoming capsids bind to microtubules and utilize the cell's dynein motor to transport them to nuclear pores. Other experiments have shown that microtubules in proximity to the plasma membrane become

disrupted after entry, suggesting that the presence of the capsid-tegument complex may destroy normal cellular microtubule interactions (Ward et al., 1998). After intracytoplasmic transport to the nucleus, capsids accumulate at the nuclear envelope and become associated with nuclear pore complexes. Experiments have shown that the VP1/2 gene is required for DNA release at the nuclear pore (Batterson, Furlong, and Roizman, 1983; Knipe, Ruyechan, and Roizman, 1979), and antibodies to nuclear pore transport components block capsid binding (Ojala et al., 2000). It is believed that binding to the nuclear pore complex produces a structural change in the HSV capsid, resulting in viral DNA release into the nucleus and an empty capsid docked at the nuclear pore.

Coordinate Gene Expression

During the course of an HSV infection, there are more than 80 gene products expressed from the viral genome in a highly regulated cascade fashion (Figure 1.6) in a number of coordinately expressed groups (Honess and Roizman, 1974). Transcription of viral DNA takes place in the nucleus, and the host RNA polymerase II is responsible for the transcription of all viral genes during infection (Alwine, Steinhart, and Hill, 1974; Costanzo et al., 1977). There are several viral genes that play an important role in the regulation of gene expression at different times post-infection.

HSV encodes a function responsible for transactivation of α genes immediately after infection (Post, Mackem, and Roizman, 1981), and this was termed the α gene transactivating factor (α-TIF). α-TIF was shown to be a component of the tegument capable of inducing the α genes, which contain the "TAATGArATT" promoter response element, which binds Oct-1. VP16 was identified as the viral protein responsible for the α-TIF function (Campbell,

Palfreyman, and Preston, 1984). After entry, VP16 is released from its interaction with VHS and the tegument complex. VP16 then binds to a cellular protein called the host cell factor (HCF) or C1 (Katan et al., 1990; Kristie and Sharp, 1990), and HCF carries VP16 into the nucleus, at which time the VP16-HCF complex binds to Oct-1 that is bound to viral DNA. This event forms the activator complex that is responsible for transactivation of α genes (La Boissiere, Hughes, and O'Hare, 1999). In addition to the Oct-1 binding sites, the promoters for α genes also contain binding sites for other cellular transcriptional activators upstream of a TATA box that may contribute to a basal level of α gene expression in rapidly dividing cells in the absence of VP16 (Roizman and Knipe, 2001). The viral requirement for VP16 may be different in resting cells, such as neuronal cells, where cellular transcription factors are not available. After being deposited into the nucleus of the infected cell, the HSV-1 viral genome localizes to nuclear ND10 structures where the transcription of α genes takes place (Maul, Ishov, and Everett, 1996). At 2 to 4 hours post infection, the viral α genes are expressed at peak levels. There are six viral α genes: ICP0, ICP4, ICP22, ICP27, ICP47, and $U_S1.5$. Five of the six α genes stimulate viral β gene expression in at least some cell types. In particular, ICP4 is required for all post-α gene expression (Clements, Watson, and Wilkie, 1977; Dixon and Schaffer, 1980), and its effect is exerted at the transcriptional level (Godowski and Knipe, 1986). However, the mechanism by which ICP4 exerts its transcriptional control over β gene expression is unclear. ICP4 is also responsible for down regulation of α gene products including itself and ICP0, and the "pre α" gene products ORF P and ORF O. In this case, specific consensus binding sites appear to be responsible for ICP4 mediated transcriptional regulation (Faber and Wilcox, 1986; Gelman and Silverstein, 1987; Kristie and Roizman, 1984; Kristie and Roizman, 1986; Muller, 1987). In addition, ICP4 has different isoforms dependent on different post translational modifications, and

it is possible that the different functions may be somewhat dependent on the specific isoform present at different times during the course of the infection. ICP0 promotes viral infection and gene expression, especially at a low multiplicity of infection (MOI) where its absence leads to a virus yield that is 100 fold less than a wild type virus (Sinclair et al., 1994; Stow and Stow, 1986). ICP0 is a nonspecific transactivator that induces the expression of the HSV α, β, and γ genes. Because ICP0 does not bind DNA directly, it appears to act indirectly in modulation of transcription (Everett, Orr, and Elliott, 1991).

The HSV-1 β genes are produced at peak levels between 4 and 8 hours post infection. Expression of the viral β genes requires the presence of functional ICP4, but is not dependent on viral DNA synthesis. β gene products include proteins involved in viral DNA replication and nucleotide metabolism. These viral proteins promote viral DNA replication, which results in expression of the γ class of genes. The β genes can be divided into two general groups: β_1 genes, which are expressed shortly after the synthesis of α proteins; and β_2 genes, which are expressed with more of a delay after a gene expression (Roizman and Knipe, 2001). β_1 genes are exemplified by the single-stranded DNA binding protein, ICP8, and the large subunit of ribonucleotide reductase, ICP6. β_2 genes are exemplified by the viral thymidine kinase encoded by UL23. Some β_2 genes require ICP27 for expression, and this dependence may correlate with the later expression of these genes (Roizman and Knipe, 2001). The HSV-1 γ (late) genes are produced at peak levels only after viral DNA replication has started, and require ICP4, ICP27 and ICP8 for efficient levels of transcription. The transition from β to γ gene expression is also marked by a change in the nuclear localization of transcription from sites near ND10 domains to

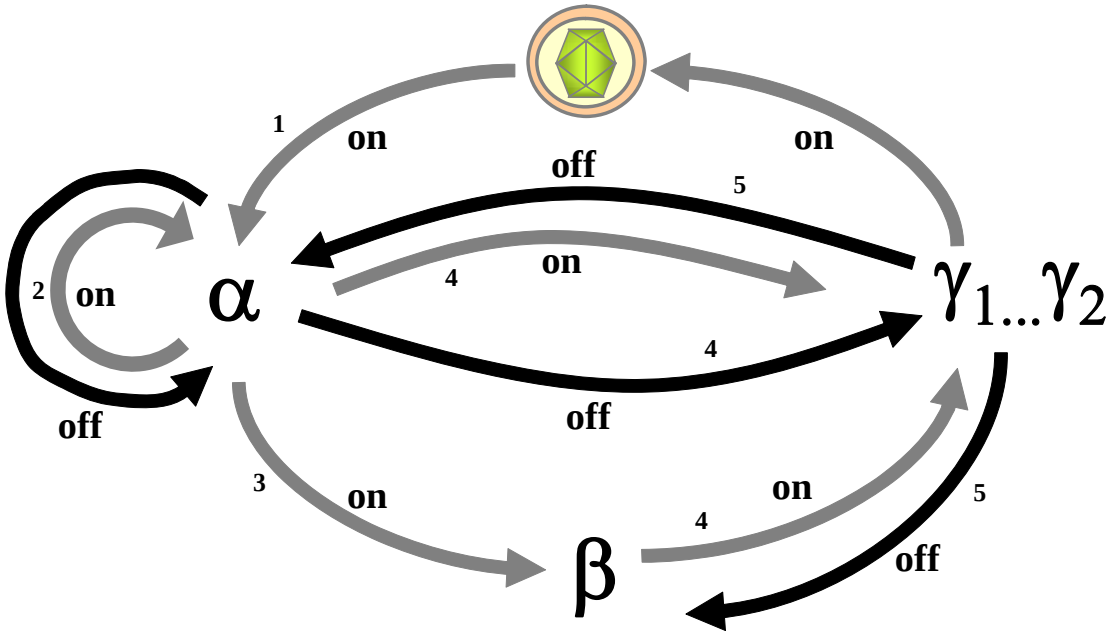


Figure 1.6: Coordinate gene expression in Herpes Simplex Virus. 1) α-TIF, a γ gene present in the tegument, activates initial transcription of the α genes. 2) Autoregulation of gene expression. 3) Activation of β gene expression. 4) Activation of γ gene expression by α and β genes, release of repression of γ genes, and replication of the viral genome. 5) γ genes turn off α and β genes late in infection (Roizman and Knipe, 2001).

replication compartments as evidenced by the localization of ICP4, RNA polymerase II, and ICP22 (Knipe et al., 1987; Leopardi et al., 1997; Rice et al., 1994). γ gene products include structural proteins, glycoproteins, and tegument components required to prepare newly infected cells for an efficient infection. The γ genes have been subdivided into two groups based on timing of expression and their dependence on viral DNA replication: γ_1 (leaky-late) genes, which are expressed relatively early in infection and transcription is increased only a few fold after DNA replication has occurred; and γ_2 genes, which do not accumulate in appreciable amounts until after DNA replication and are not expressed in the presence of inhibitors of viral DNA synthesis (Wagner, 1985). Typical γ_1 genes include the major capsid protein ICP5, gB, gD, and ICP34.5, whereas typical γ_2 genes include gC, UL41 (VHS), UL36, UL38, UL20, and gK.

Viral DNA Replication

After the β genes have been expressed and translated, there are several proteins that are localized to the nucleus where they assemble on the parental viral DNA in punctuate "pre-replicative sites" located near nuclear ND10 structures (Ishov and Maul, 1996; Uprichard and Knipe, 1996). Initially, viral DNA replication initiates on the circular viral DNA, creating a "theta" structure, which as replication progresses transitions to a rolling circle mechanism of replication producing head-to-tail concatemers of viral DNA (Jacob, Morse, and Roizman, 1979). At this point, replication takes place in "replication compartments" that consist of accumulating DNA molecules and replication complexes (Quinlan, Chen, and Knipe, 1984).

There are seven viral proteins absolutely required for viral DNA replication and sufficient to replicate a viral origin transfected into cells. These are the viral DNA polymerase (UL30) (Purifoy, Lewis, and Powell, 1977), its accessory protein (UL42) (Conley et al., 1981), an origin-

binding protein (UL9), the single stranded DNA binding protein (ICP8), and the helicase-primase complex that consists of three proteins: UL5, UL8, and UL52 (Challberg, 1986; Wu et al., 1988). Host cell factors may also be involved in DNA synthesis, and host enzymes that include the DNA polymerase α-primase, DNAligase, and topoisomerase II are almost certainly also required. There are also three origins of replication located on the viral genome: *oriS*, a palindromic sequence of 45 bp that is located in c sequences bounding the S component and present in two copies; and *oriL*, a palindromic sequence of 144 bp that is located between the transcriptional units of ICP8 and the DNA polymerase (UL29 and UL30, respectively) (Deb and Doelberg, 1988; Frenkel et al., 1976; Knopf, 1986; Locker, Frenkel, and Halliburton, 1982; Lockshon and Galloway, 1986; Mocarski and Roizman, 1982; Stow, 1982; Vlazny, Kwong, and Frenkel, 1982; Weller et al., 1985). The reason for the presence of three origins of replication is not clear, although it may reflect the evolutionary history of the virus. Only one origin of replication is needed for replication to occur (Igarashi et al., 1993; Polvino-Bodnar, Orberg, and Schaffer, 1987).

The basic model for the replication of HSV viral DNA proceeds as follows. First, the parental viral DNA is circularized upon being deposited into the nucleus of the infected cell. After α and β gene expression, UL9 binds to specific elements in either *oriL* or *oriS* and begins to unwind the viral DNA. UL9 then recruits the ssDNA binding protein ICP8 to the unwound portion of the viral DNA. At this point, UL9 and ICP8 recruit the remaining five proteins to the replication forks. The helicase–primase and viral DNA polymerase complexes assemble at each replication fork and initiate theta form replication. Through an unknown mechanism, replication switches from theta form to the rolling circle form of replication, and UL9 is not required for rolling circle replication because it is not origin dependent. The rolling circle replication forms

long head-to-tail concatamers of viral DNA, which become cleaved into individual units during packaging of viral DNA into capsids (Roizman and Knipe, 2001).

Capsid Assembly and Packaging

After DNA replication has started, the γ proteins are transcribed, including the HSV-1 capsid proteins. First, empty shells containing an internal scaffold are assembled, with the internal scaffold lost upon viral DNA packaging into the capsid. Then the mature capsid is able to proceed along the viral egress pathway. At least some of the initial stages of capsid assembly occur in the cytoplasm of the infected cell (Nicholson et al., 1994; Rixon et al., 1996). The major capsid protein (VP5), the outer tip of hexons (VP26), and a triplex protein (VP23) are not capable of nuclear localization on their own; however, VP5 can be carried into the nucleus by VP19C, another capsid triplex protein, or by pre-VP22a, a scaffolding protein. VP23 localizes to the nucleus only in the presence of VP19C, while VP26 localizes to the nucleus only when it is expressed with both VP5 and VP19C or pre-VP22a (Nicholson et al., 1994; Rixon et al., 1996). Electron microscopic studies have shown that final envelopment occurs in the nucleus; and three types of capsids, called *A*, *B*, and *C* capsids have been identified by sucrose density gradient ultracentrifugation (Gibson and Roizman, 1972). All three types of capsids are about 120 nm in diameter with an outer shell composed of hexons and pentons made up of VP5. The individual capsomeres are linked by triplex structures consisting of VP19C and VP23, the two minor capsid proteins. Each triplex consists of one molecule of VP19C and two molecules of VP23 (Newcomb et al., 1993). *C*-capsids are mature capsids that contain packaged viral DNA and can proceed to become infectious virions by budding through the nuclear membrane into the peri-nuclear space (Perdue et al., 1976). In contrast, *A* and *B* capsids lack viral DNA, but *B* capsids

are filled with VP22a and VP21, the cleaved scaffolding proteins, and a viral protease VP24 (Gibson and Roizman, 1972; Newcomb et al., 1993). The internal proteins VP22a, VP21, and VP24 are removed upon packaging of viral DNA to form *C* capsids (Davison, Rixon, and Davison, 1992; Gibson and Roizman, 1972). *A* capsids do not contain viral DNA or scaffolding proteins and are believed to be an abortive result of failed attempts at DNA packaging.

In terms of the order of assembly, once in the nucleus, VP5-pre-VP22a complexes come together as a result of self assembly of pre-VP22a. The triplex proteins VP19C and VP23 are then added to form a partial capsid. As hexons and pentons are added, the structure assembles into a round procapsid (Newcomb et al., 1996). At his point, the procapsid undergoes a structural transformation and becomes polyhedral (Church and Wilson, 1997; Thomsen et al., 1995; Trus et al., 1996). It is unknown at this time whether the round or polyhedral *B* capsids are the structure that viral DNA is packaged (Lee, Irmiere, and Gibson, 1988; Perdue et al., 1974; Trus et al., 1996). Encapsidation of viral DNA is a process in which unit length monomers of viral DNA are cleaved from concatamers and packaged into preformed *B* capsids, as originally shown for pseudorabies virus (Ladin, Blankenship, and Ben-Porat, 1980; Ladin et al., 1982). Cleavage of concatamers occurs at specific sites and requires the *pac1* and *pac2* packaging signals (Deiss, Chou, and Frenkel, 1986; Smiley, Duncan, and Howes, 1990; Varmuza and Smiley, 1985). The process of encapsidation of viral DNA into capsids is complex and requires several gene products, including the UL6, UL15, UL25, UL28, UL32, UL33, UL36 and UL37 gene products, but the mechanism of viral DNA packaging is not well defined (Roizman and Knipe, 2001).

Herpesvirus Egress

After encapsidation of genomic DNA, mature nucleocapsids acquire an primary envelope by budding through the inner nuclear membrane into the peri-nuclear space (Vlazny, Kwong, and Frenkel, 1982). Two conserved herpesvirus proteins, UL31 and UL34 have recently been shown to be involved in the budding process. UL34 is C-terminally anchored membrane protein present in the inner and outer nuclear membrane (Klupp, Granzow, and Mettenleiter, 2000), while UL31 is a nuclear phosphoprotein also present in the nuclear membrane of infected cells (Fuchs et al., 2002b; Reynolds et al., 2001). The UL31 protein requires UL34 for proper nuclear targeting (Fuchs et al., 2002b), whereas the UL34 protein appears to possess an intrinsic nuclear targeting signal (Klupp, Granzow, and Mettenleiter, 2000). However, the nuclear targeting of UL34 is increased by the presence of UL31 (Fuchs et al., 2002b; Reynolds et al., 2001). Analysis of UL31 and UL34 deletion mutants has indicated that the absence of either protein results in a drastic impairment in primary envelopment with capsids trapped within the nucleus (Chang et al., 1997; Fuchs et al., 2002b; Klupp, Granzow, and Mettenleiter, 2000; Reynolds et al., 2001; Roller et al., 2000). In addition, partial dismantling of the nuclear lamina has been observed after HSV infection and may be required so that intranuclear capsids are able to gain access to the inner nuclear membrane (Scott and O'Hare, 2001). Multiple lines of evidence indicate that UL31 and UL34 play a role in dismantling the nuclear lamina in infected cells. Over expression of the UL31 protein in uninfected cells was sufficient to relocalize lamin A/C from the nuclear rim into nucleoplasmic aggregates, while over expression of UL34 was sufficient to relocalize some lamin A/C into the cytoplasm (Reynolds, Liang, and Baines, 2004). More importantly, both UL31 and UL34 have been shown to directly bind lamin A/C, suggesting that the UL31 and UL34 proteins modify the conformation of the nuclear lamina in infected

cells, possibly by direct interaction with lamin A/C. Given that the nuclear lamina potentially excludes nucleocapsids from envelopment sites at the inner nuclear membrane, the lamina alteration may reflect a role of the UL31/UL34 protein complex in perturbing the lamina to promote nucleocapsid egress from the nucleus (Baines 2004). It is unclear whether additional primary envelope and tegument proteins exist that are required for primary envelopment to take place. The UL11 protein has been proposed to function in primary envelopment and increases the efficiency of envelopment at this step (Baines and Roizman, 1992b; MacLean et al., 1992).

Egress from the Peri-nuclear Space: De-Envelopment

The subsequent steps in HSV-1 egress following primary envelopment have been in dispute for some time. Two models were proposed to explain virion egress. The first model, sometimes called the "lumenal" pathway (CH72 144), suggests that peri-nuclear virions retain their primary envelope and integrity as they leave the cell through the secretory pathway (Campadelli-Fiume et al., 1991; Darlington and Moss, 1968; Johnson and Spear, 1982; Johnson et al., 2001). In this model, virion glycoproteins are modified in transit to the plasma membrane and peri-nuclear virions should contain the entire complement of tegument and envelope proteins present on mature extra-cellular virions. The alternative model proposes that the primary envelope of peri-nuclear virions fuses with the outer nuclear lamellae, resulting in loss of the primary envelope and some tegument components and translocation of the capsid into the cytoplasm of the infected cells (Figure 1.7, step II) (Enquist et al., 1998; Mettenleiter, 2000). In this "de-envelopment re-envelopment" pathway, final tegumentation and envelopment (re-envelopment) occur in cytoplasmic compartments (Figure 1.7, step III). In addition, in this model peri-nuclear and intracytoplasmic/extra-cellular virions should differ in composition.

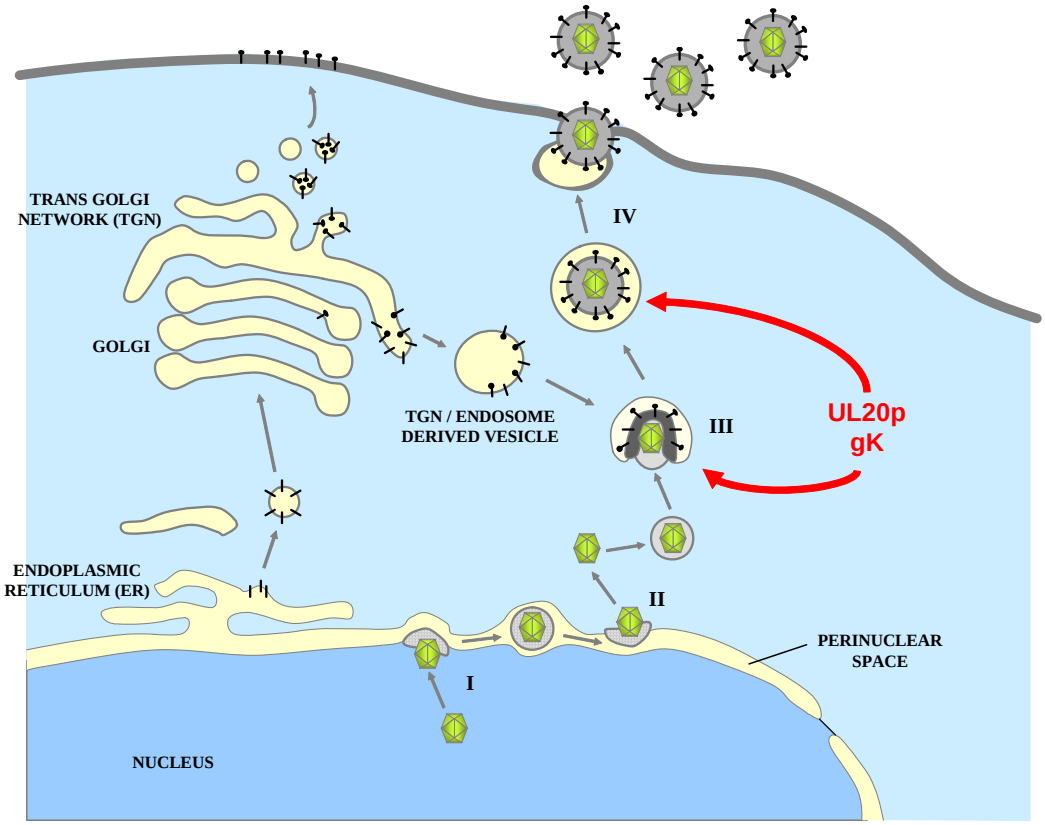


Figure 1.7: Representation of HSV-1 virion morphogenesis and egress. (I) Mature capsids budding through the inner nuclear membrane into the peri-nuclear space; (II) De-envelopment of peri-nuclear virions at the outer nuclear membrane; (III) Re-envelopment of cytoplasmic capsids by budding into cytoplasmic vesicles; (IV) Final egress to the extra-cellular space. The steps at which UL20 and gK are thought to function in virion egress are indicated by red arrows. Figure from (Melancon, 2003).

There is evidence that supports both the "lumenal" and the alternative "de-envelopment re-envelopment" pathways. However, the vast majority of recent evidence supports the "de-envelopment re-envelopment" model of HSV-1 virus egress. The first line of evidence supporting the alternative model is that primary envelope and primary tegument of peri-nuclear virions clearly differ in ultra-structural appearance from the final envelope and final tegument of extra-cellular virions when observed by electron microscopy (Gershon et al., 1994; Granzow et al., 2001). In addition, while UL31 and UL34 localize on nuclear membranes and peri-nuclear virions, the UL31 and UL34 proteins were not detected in extra-cellular virions (Reynolds et al., 2002). In further support, the major tegument proteins UL46 and UL49 are present in intracytoplasmic/extra-cellular virions but absent from peri-nuclear virions (Klupp, Granzow, and Mettenleiter, 2000; Mettenleiter, 2002). Also, the phospholipid composition on the final virion envelope differs substantially from the composition of the nuclear membrane, explained only by a two-step envelopment model of HSV virion egress (van Genderen et al., 1994). Electron microscopic analysis has also demonstrated the fusion of primary envelopes with the outer nuclear membrane with many herpesviruses including HSV-1 (Granzow et al., 2001; Harms et al., 2000). As a result, the two-step "de-envelopment re-envelopment" model of virion egress is congruent with both biochemical and morphological data and constitutes a unified model for the morphogenesis of herpesviruses (Mettenleiter, 2002).

The mechanism of virion de-envelopment from the peri-nuclear space is unclear. Deletion of the major glycoproteins involved in other membrane fusion events does not affect the de-envelopment process as indicated by multiple studies (Cai et al., 1987; Granzow et al., 2001; Jayachandra, Baghian, and Kousoulas, 1997; Steven and Spear, 1997). It is possible that these

glycoproteins function in a redundant manner, and that only deletion of multiple genes encoding glycoproteins would have an observable effect on virion de-envelopment at the outer nuclear membrane.

Tegumentation in the Cytoplasm

While the complexity of the herpesvirus capsid is not much different than that of other icosahedral viral capsids such as the picornavirus capsid, the complexity of the herpesvirus tegument is drastically increased. At least 15 proteins have been identified that are part of the HSV-1 tegument. Even more proteins make up the tegument of other herpesviruses such as VZV or CMV (Gibson, 1996; Spengler et al., 2001). Recent data indicate that tegumentation follows an intricate pattern of protein-protein interactions that contains significant levels of redundancy built in, at least as observed in cell culture (Mettenleiter, 2002). The tegument proteins interact with the capsid on one side and the viral envelope proteins on the other side in order to link the structural components to the final envelope of the HSV-1 virion and to secure the integrity of the virus particle (Figure 1.7) (Mettenleiter, 2002). For a long time the herpesvirus tegument was considered unstructured; however, cryoelectron microscopic analysis has indicated that at least the innermost portion of the tegument located adjacent to the capsid exhibits icosahedral symmetry, resulting from the interaction of a large tegument protein, presumably UL36, with the pentons of the capsid (Zhou et al., 1999). The UL36 gene product has been shown to interact with the major capsid protein VP5, which forms both the pentons and hexons (Machtiger et al., 1980; McNabb and Courtney, 1992; Newcomb et al., 1993). So, it appears that the first layer of tegument around the capsid is structured and composed of UL36. UL37 has been shown to interact with UL36 in co-immunoprecipitation and yeast two-hybrid

experiments, and capsids that accumulate in the absence of UL37 contain UL36 (Klupp et al., 2002). UL36 and UL37 appear to be the only tegument proteins conserved in all herpesvirus subfamilies, and the absence of the HSV-1 UL36 and UL37 proteins abolishes virus maturation (Desai et al., 2001; Desai, 2000; Klupp et al., 2001). Unfortunately, the subsequent steps in capsid tegumentation are still largely undefined. Virion morphogenesis still proceeds in the absence of several other tegument proteins, including UL13, US3 (Purves et al., 1987), UL41, UL46, UL47 (Rafield and Knipe, 1984; Roizman and Knipe, 2001), and UL49 (Mettenleiter, 2002). So far the most dramatic effect has been seen upon deletion of UL48 (α-TIF), which is responsible for transducing α gene promoters and makes up a major part of the virus tegument (Batterson and Roizman, 1983; Heine et al., 1974). The absence of UL48 may interfere with a later step in virion assembly, presumably affecting tegumentation and re-envelopment in the cytoplasm (Mossman et al., 2000). Interestingly, UL48 has been shown to interact with other tegument components, UL49 and UL41 (VHS) (Smibert et al., 1994). Mutant forms of UL41 that do not bind UL48 do not become incorporated into the virion (Read, Karr, and Knight, 1993). Cross-linking studies have indicated that UL48 may interact with gB, gD, and gH (Zhu and Courtney, 1994), although these interactions have not been confirmed through more stringent methods.

Final Envelopment and Egress to Extra-cellular Spaces

Following tegumentation in the cytoplasm, HSV-1 capsids bud into cytoplasmic vesicles derived from the trans-Golgi Network (TGN) or endosomes, acquiring their final envelope (Figure 1.7, step III). Although the mechanism of secondary envelopment is not well defined, the study of mutants that contain deletions of certain glycoproteins has shed light on some

requirements of the final envelopment event. By far the most dramatic effect on secondary envelopment and final egress is caused by the deletion of either UL20 or gK, and ultra-structural study of UL20-null and gK-null infected cells reveals a dramatic accumulation of un-enveloped and aberrantly enveloped capsids in the cytoplasm of infected cells with a marked absence of extra-cellular virus. With regard to gK-null and UL20-null virus titers, there is also a corresponding lack of infectious virus in the supernates of infected cells, with close to wild-type levels of intracellular virus (Foster et al., 2004b; Jayachandra, Baghian, and Kousoulas, 1997). The ultra-structural phenotypes of UL20-null and gK-null viruses are covered in more detail in Chapter III and Chapter IV. Following the final envelopment step, nascent virions must still egress to extra-cellular spaces. The current belief is that cellular transport machinery is utilized in this step, although the mechanisms are unclear. It is apparent that the late stages in viral egress may differ depending on the cell type that is infected. For example, deletion of gE and gI, which have been shown to interact and form a complex, does not impair productive replication of HSV-1 (Enquist et al., 1998). However, in polarized epithelial cells, wild-type HSV-1 virions are sorted predominantly to cell junctions, while gE/gI null virions are non-specifically released into supernatants (Dingwell and Johnson, 1998). Delivery of virus particles to cell junctions would be expected to enhance virus spread and enable viruses to avoid host immune defenses.

Deletion of multiple genes encoding envelope glycoproteins has revealed that certain aspects of final envelopment and egress may feature redundant mechanisms, each of which is sufficient for function, at least in cell culture. For instance, while neither gE/gI or gM is required for productive replication in PrV or HSV-1, simultaneous deletion of gE/gI and gM results in drastic inhibition of plaque formation and replication in PrV (Brack et al., 1999). In addition, simultaneous deletion of UL11 and gM resulted in similar egress defect in PrV (Kopp et al.,

2004). In contrast, simultaneous deletion of gE/gI and gM does not have a major effect in HSV-1 (Browne, Bell, and Minson, 2004); however, a triple mutant lacking gD, gE, and gI had a severe defect in the final envelopment step. The authors proposed that HSV gD and the gE/gI heterodimeric complex act in a redundant fashion to anchor the virion envelope onto tegument-coated capsids (Farnsworth, Goldsmith, and Johnson, 2003). In contrast, it is also possible that deletion of multiple glycoproteins indirectly disrupts the integrity of many different protein-protein interactions required for the final envelopment step and that the resulting phenotype results from the compounded effects of an abnormal glycoprotein profile. More study of the reason for defects in double and triple-null viruses is required before conclusions can be drawn regarding mechanisms of final envelopment.

Formation of Light (L) Particles

The presence of egressing capsids in the cytoplasm is not required for tegument assembly and subsequent envelopment to take place. The formation of extra-cellular herpesvirus light (L) particles that lack capsids, consist of only tegument and envelope, and are formed independent of normal virus maturation have been thoroughly studied in HSV-1 (McLauchlan and Rixon, 1992; Rixon, Addison, and McLauchlan, 1992). L particles appear to contain the full complement of tegument proteins as well as an authentic envelope containing all appropriate glycoproteins. Studies of PrV have shown that L particle formation occurs in the absence of UL36 and UL37, which are tegument components that are critical for capsid tegumentation. It is possible that normal tegumentation may be centered on UL36 and UL37 interactions with the capsid and that UL49 may be required for proper interactions with viral glycoproteins in the final budding and

envelopment steps. In the absence of capsids, tegument assembly could proceed anchored on UL49, resulting in the formation of L particles (Mettenleiter, 2002).

HSV-1 Glycoproteins and Their Putative Functions

Glycoprotein B (UL27)

The HSV-1 UL27 gene encodes the 904 aa glycoprotein B (gB). gB is highly conserved across all subfamilies of herpesviruses. HSV-1 gB is homotrimeric (aa) type I membrane glycoprotein composed of a 696-aa ectodomain that is N-glycosylated at multiple sites (Claesson-Welsh and Spear, 1986; Claesson-Welsh and Spear, 1987; Highlander et al., 1991; Laquerre, Person, and Glorioso, 1996; Spear, 1993b; Whitley, 2001), a 69-aa transmembrane domain, and a 109-aa carboxyl-terminal domain. The cytoplasmic domain of gB is the longest among HSV-1 glycoproteins, implying a crucial role for this domain in gB-mediated functions. A variety of evidence indicates that gB plays important roles in membrane fusion phenomena during virus entry and virus-induced cell fusion: HSV-1 mutant viruses lacking gB are not able to enter into cells (Cai et al., 1987) due to a post-attachment defect that can be resolved by polyethylene glycol mediated fusion of viral envelopes with cellular membranes (Cai, Gu, and Person, 1988); Single amino acid substitutions and truncations of the carboxyl terminus of gB cause extensive virus-induced cell fusion (Baghian et al., 1993; Bzik et al., 1984; Cai et al., 1988; Gage, Levine, and Glorioso, 1993); Transient co-expression of gB with gD, gH and gL causes cell-to-cell fusion, which is substantially increased by carboxyl terminal truncations of gB (Foster, Melancon, and Kousoulas, 2001; Haan, Lee, and Longnecker, 2001; Klupp, Nixdorf, and Mettenleiter, 2000; Pertel, 2002). Recent evidence suggests that these glycoproteins act in a

sequential manner, with gD being involved in Phase I, gH/L in Phase II, and gB in Phase III (Gianni, Forghieri, and Campadelli-Fiume, 2006; Subramanian and Geraghty, 2007). The crystal structure of HSV-1 gB has been recently solved, revealing a remarkable homology to vesicular stomatitis virus (VSV) gB. In addition to homology with VSV gB, two domains that indicate HSV-1 gB is intricately involved in virus cell fusion were identified: an alpha-helical coiled-coil core reminiscent of class I fusion proteins, and two extended beta hairpins with hydrophobic tips which are indicative of class II fusion proteins (Heldwein et al., 2006). Subsequent mutagenesis studies on HSV-1 gB showed that hydrophobic tips of the loops were vital for proper HSV-1 gB function (Hannah et al., 2007).

Glycoprotein C (UL44)

The 1,536 bp HSV-1 UL44 gene encodes for the 511 aa long precursor of glycoprotein C (gC) (Frink et al., 1983). gC contains a 25 aa signal sequence at the N-terminus, a long 453 aa extra-cellular domain, a 23 aa transmembrane anchoring domain, and a short 10 aa C-terminal cytoplasmic tail (Homa et al., 1986). The first contact of virions with the surface of susceptible cells is through glycosaminoglycans (GAGs). gC can mediate the initial binding of HSV-1 virus to a cell surface GAG, heparan sulfate (HS). The heparan sulfate binding site of gC has been localized to the N-terminal 120 aa (Tal-Singer et al., 1995). The aminoglycoside neomycin interferes with the binding of virus to HS mediated by gC (Herold et al., 1994). Although gC is dispensable for the infection of cultured cells, its presence can increase the efficiency of virus binding almost 10-fold, at least for HSV-1. The main function of gC binding to GAGs seems to be concentration of the virus on cell surfaces, enabling the more stable interaction of gD with an entry receptor.

Another important function of gC is its ability to inactivate complement to facilitate immune evasion by HSV-1. The gC of many herpesviruses has the ability to bind C3b (Eisenberg et al., 1987; Friedman et al., 1984; Huemer et al., 1993; Huemer et al., 1995). The presence of gC protects the virus and infected cell from antibody (Ab)-independent complement neutralization and cell lysis (Lubinski et al., 1999; Lubinski et al., 1998), antibody dependant complement neutralization (Hook et al., 2006), and is an important virulence factor *in vivo* (Isaacs, Kotwal, and Moss, 1992; Kotwal et al., 1990; Sahu et al., 1998). HSV-1 mutant viruses lacking gC are rapidly inactivated by human complement, resulting in up to a 5000 fold loss of titer (Friedman et al., 1996). While Ab is not required for neutralization of a gC null virus, the presence of Ab enhances neutralization (Friedman et al., 1996; Harris et al., 1990).

An unresolved issue regarding gC function is the relationship of gC to virus-induced syncytia formation. Several spontaneously arising syncytial virus strains were found to be gC-deficient. In particular the MP strain variant MP10311 contains a syncytial mutation in gK and is gC-deficient (Bartoletti et al., 1985). In general, the syncytial phenotype of these strains was found to be more effectively expressed in the absence of gC, especially in Hep-2 cells (Bond, Person, and Warner, 1982; Goodman and Engel, 1991; Manservigi, Spear, and Buchan, 1977; Pertel and Spear, 1996; Tognon et al., 1984). One possible explanation of the enhancement of the syncytial phenotype is that in the absence of gC, because extra-cellular virus does not bind to cell surfaces as rapidly, virus-induced cell fusion acts as the preferential method of cell-to-cell spread of HSV-1.

Glycoprotein D (US6)

The 1182 bp long US6 gene specifies a 394 aa precursor to glycoprotein D (gD). gD consists of a 25 aa signal peptide, a long 315 aa ectodoamin containing three glycosylation sites (McGeoch et al., 1985; Watson et al., 1982), a 22 aa transmembrane domain, and a 32 aa C-terminal cytoplasmic domain (Minson et al., 1986). The ectodomain of gD has 6 cysteine residues at positions 66, 106, 118, 127, 189, 202 forming disulfide bonds (Long et al., 1992). Importantly, gD has been shown to act as the viral ligand for all known HSV-1 entry receptors (Spear, Eisenberg, and Cohen, 2000). As a result, gD is absolutely required for virus entry and virus-induced cell-to-cell fusion to occur. As discussed earlier, HSV-1 gD has the ability to bind HVEM, nectin-1, nectin-2, and other cell surface receptors to mediate virus entry. In addition, cells that express gD are resistant to HSV infection in a dose-dependent manner due to a saturation of the corresponding entry receptors (Campadelli-Fiume et al., 1988; Johnson, Burke, and Gregory, 1990). X-ray structures of HSV-1 gD alone and in complex with HVEM revealed that a portion of gD assumes an Ig-like fold with unconventional disulfide-bonding patterns (Carfi et al., 2001). There is an N-terminal extension from the Ig-like fold that forms a hairpin loop in the complex with HVEM but is disordered in the crystals of gD alone. The contacts in gD for HVEM have been localized to amino acids 7 to 15 and 24 to 32 within the N-terminal hairpin (Spear and Longnecker, 2003). Mutagenic analysis of HSV-1 gD revealed that the first 32 amino acids of the N-terminal extension have a critical role in functional interactions of gD with all the HSV entry-fusion receptors, except for nectin-1, and that the amino acid sequence within this region governs whether nectin-2 can be recognized as an entry and fusion receptor (Yoon et al., 2003; Zago and Spear, 2003).

Glycoproteins E (US8) and I (US7)

The 1652 bp long US8 gene encodes the 550 aa glycoprotein E (gE) (Lee, Para, and Spear, 1982), while the 1172 bp long US7 gene encodes 390 aa glycoprotein I (gI) (Longnecker et al., 1987). HSV, VZV, and PrV express a heterodimer of gE and gI that functions to mediate cell-to-cell spread in epithelial and neuronal tissues (Balan et al., 1994; Card et al., 1992; Cohen and Nguyen, 1997; Dingwell et al., 1994; Dingwell and Johnson, 1998; Jacobs et al., 1993; Johnson and Feenstra, 1987; Johnson et al., 1988; Kimura et al., 1998; Kritas, Pensaert, and Mettenleiter, 1994; Kudelova et al., 1991; Mettenleiter et al., 1987b; Mulder et al., 1994; Neidhardt, Schroder, and Kaerner, 1987; Polcicova et al., 2005; Tirabassi and Enquist, 1998; Tirabassi et al., 1997; Whealy et al., 1993). HSV and PRV gE/gI complexes are required for efficient spread of viruses between certain cultured epithelial cells, neurons, and other polarized cells with extensive cell junctions but are not needed for spread between highly transformed, nonpolarized cells, such as Vero or HeLa cells, which do not form cell junctions (Dingwell et al., 1994; Dingwell, Doering, and Johnson, 1995; Mettenleiter et al., 1987a; Tirabassi et al., 1997; Wisner et al., 2000; Zsak et al., 1992). For example, plaques formed by a gE-negative HSV mutant on monolayers of a keratinocyte cell line included eightfold fewer cells than plaques produced by wild-type HSV-1, yet there was no difference in cell-to-cell spread in monolayers of HeLa cells (Wisner et al., 2000). Moreover, PRV and HSV gE/gI complexes are required for spread within synaptically connected neuronal circuitry in the peripheral and central nervous systems (Babic et al., 1996; Dingwell, Doering, and Johnson, 1995; Mulder et al., 1994; Tirabassi and Enquist, 1998; Tirabassi et al., 1997; Whealy et al., 1993). gE and gI are extensively complexed in virus-infected cells (Johnson and Feenstra, 1987; Johnson et al., 1988), and it is the gE/gI complex that functions in cell-to-cell spread (Dingwell et al., 1994; Dingwell,

Doering, and Johnson, 1995; Johnson and Feenstra, 1987; Johnson et al., 1988; Olson and Grose, 1998; Tirabassi et al., 1997; Zuckermann et al., 1988). In contrast to their effects on cell-to-cell spread, HSV and PRV gE/gI complexes do not appear to be required for entry of cell-free virus, i.e., virus particles applied to the apical surfaces of cells (Dingwell et al., 1994; Mettenleiter et al., 1987a). HSV-1 gE/gI localizes specifically to the TGN during early phases of infection but moves out to cell junctions at intermediate to late times. One mechanism by which gE/gI facilitates cell-to-cell spread of HSV-1 involves the sorting of newly assembled virions to lateral cell surfaces and cell junctions. Mutant HSV-1 lacking gE accumulated more extensively in the cytoplasm, at apical cell surfaces, and in cell culture supernatants than did wild-type HSV-1 particles, which were found predominantly at cell junctions (Alconada et al., 1999; Kimura et al., 1998).

The gE/gI complex is also known as the HSV Fc Receptor (FcR) because of its high affinity for the Fc portion of IgG. Through binding of the Fc portion of IgG, the gE/gI complex contributes to the immune evasion capabilities of HSV-1, reducing the effectiveness of antibody-dependent cellular cytotoxicity (ADCC) targeting the virus and infected cells (Eberle et al., 1995).

Glycoprotein G (US4)

The 716 bp long US4 gene encodes the 238 aa glycoprotein G (gG). During infection, gG is incorporated into nuclear and cytoplasmic membranes in the cell (Frame, Marsden, and McGeoch, 1986; Sullivan and Smith, 1987). The relevant function of gG in the herpesvirus life cycle has been difficult to identify. Mutant HSV-1 lacking US4 showed no discernible phenotypic abnormalities relative to the wild-type HSV-1 virus in non-polarized cells in culture,

and the absence of gG resulted in only marginal attenuation of the virus in certain *in vivo* models (Atkinson, Barr, and Timbury, 1978). However, at the primary site of infection, HSV-1 is required to infect the apical surfaces of mucosal epithelial cells. Recombinant viruses lacking gG were shown to be defective in the gC-dependent infection of the apical surfaces of polarized epithelial cells in culture and also in infection of the apical surfaces of corneal epithelial cells *in vivo* (Tran et al., 2000). It is not known whether the gG function in infection is mediated in a cooperative manner with gC, whereby gG would assist with gC binding, or in an independent manner, whereby gG would be necessary in a second step that renders the gC interaction irreversible.

Glycoproteins H (UL22) and L (UL1)

The 2517 bp UL22 gene encodes the 838 aa glycoprotein H (gH). gH is a type I membrane glycoprotein containing an 18 aa signal peptide, a long 785 aa ectodomain, a single 21 aa transmembrane hydrophobic domain close to the C-terminus, and a 14 aa C-terminal cytoplasmic tail. The 675 bp UL1 gene encodes the 224 aa glycoprotein L. gL contains a 25 aa signal peptide; however, unlike other herpesvirus glycoproteins, gL does not contain a transmembrane domain. It appears that gL is not an integral membrane protein; rather, its membrane association and incorporation into virus particles is dependent on its interaction with gH (Dubin and Jiang, 1995). Infected cell membranes contain a heterodimer of gH and gL (gH/gL). When gH is expressed in transfected cells in the absence of gL, the resulting gH polypeptide is neither folded nor processed correctly. The malformed gH remains in the endoplasmic reticulum and undergoes self-aggregation (Foa-Tomasi et al., 1991; Forrester et al., 1991; Roberts et al., 1991). Similarly, cells infected with a gL-null virus do not produce virions

containing gH. When cells are infected with a gH-null virus, gL is neither correctly processed nor incorporated into the plasma membrane or viral envelope (Hutchinson et al., 1992a; Roop, Hutchinson, and Johnson, 1993). Due to the lack of a transmembrane region, the gL polypeptide is secreted into the medium (Dubin and Jiang, 1995). When both UL22 and UL1 are co-transfected into cells, the antigenic conformation of the gH/gL heterodimer is identical to that of virus infected cells, indicating that no other viral proteins are necessary for heterodimer formation. The mutual interaction of both glycoproteins is mediated by the N-terminal region of the first 69 aa of gL after cleavage of the 25 aa signal peptide (Roop, Hutchinson, and Johnson, 1993), while gH interacts through a central region of the its extra-cellular domain. C-terminal cysteine residues are required for proper gH/gL function; however the N-terminal cysteines, which are located within the putative gH/gL binding site are not required for gH/gL interaction or function (Cairns et al., 2005).A properly formed gH/gL heterodimer is required for both virus entry and virus-induced cell-to-cell fusion. Although viruses lacking gH/gL are unable to enter cells, they are able to attach to the cell surface. Therefore, the role of gH/gL in virus entry is in during the virus envelope-plasma membrane fusion event and not required for virion attachment or receptor binding. An interesting result concerning the mechanism of gH/gL functions was obtained when using anti-gL mAbs directed to the C-terminal portion of gL. Certain mAbs inhibited virus-induced cell-to-cell fusion resulting from syncytial mutations but not virus entry, indicating a fundamentally different mechanism may be at work in the two processes (Novotny, Parish, and Spear, 1996).

Glycoprotein J (US5)

The 279 bp US5 gene encodes the 92 aa glycoprotein J (gJ) (Ghiasi et al., 1998). gJ contains a predicted signal peptide as well as a hydrophobic transmembrane domain. The current function of gJ is unknown. Deletion viruses that lack the US5 ORF show no phenotypic deficiencies either *in vitro* or *in vivo* (Balan et al., 1994). More specifically, a gJ-null HSV-1 was capable of multiplying from an inoculation site in mice and entering and replicating in the peripheral and central nervous system (Balan et al., 1994). However, it has been shown that another gene in the unique long (UL) region of the viral genome, UL27.5, has amino acid sequences common to US5. UL27.5 was discovered due to the fact that antibodies generated against the 23 kDa gJ cross reacted with an unknown 43 kDa product (Chang et al., 1998). In contrast to gJ, UL27.5 accumulates in the cytoplasm of infected cells; nevertheless, it may be possible that UL27.5 and gJ could function redundantly and an observable change in phenotype would not be seen unless both US5 and UL27.5 were disrupted.

Glycoprotein K (UL53)

The 1017 bp UL53 gene encodes the 338 aa precursor of glycoprotein K (gK) (Debroy, Pederson, and Person, 1985; Pertel and Spear, 1996). gK contains a cleavable 30 aa signal sequence as well as two asparagine residues at positions 48 and 58 that are glycosylated by N-linked mannose (Hutchinson et al., 1992b; Ramaswamy and Holland, 1992). gK is extremely hydrophobic and was originally proposed to contain four transmembrane domains, with both the N-terminus and C-terminus predicted to lie on the lumenal/extra-cellular side of cellular membranes (Debroy, Pederson, and Person, 1985). In contrast, experiments with *in vitro* translated gK in the presence of microsomal membranes predicted gK to contain three

transmembrane domains, with the C-terminal tail located intracellularly (Debroy, Pederson, and Person, 1985; Mo and Holland, 1997; Ramaswamy and Holland, 1992). However, recent experiments by our laboratory utilizing epitope tags inserted into specific regions of gK confirmed the original prediction for the membrane topology of gK (Foster, Alvarez, and Kousoulas, 2003).

Original attempts to isolate gK-deficient viruses through substitution of UL53 with the *lacZ* gene were unsuccessful and it was determined that gK was essential for HSV replication *in vivo* (MacLean et al., 1991). A subsequent attempt at the isolation of a F-strain gK-null virus was successful, resulting in an insertion-deletion mutation in gK that expressed a fusion protein containing the N-terminal 112 aa of gK. The aberrant virions were significantly less infectious and did not reach the extra-cellular space, with a high number of naked capsids and aberrant virions in the cytoplasm (Hutchinson and Johnson, 1995). Furthermore, cells infected with the F strain gK-null virus caused cell fusion in 143TK- cells. However, a KOS strain containing a more precise deletion of the UL53 gene resulted in a slightly different phenotype (Jayachandra, Baghian, and Kousoulas, 1997). While the egress of virions in the KOS gK-null virus was still severely impaired relative to a wild-type virus, the KOS gK-null did not cause cell fusion in 143TK- cells. A KOS virus that was constructed to mirror the F strain insertion-deletion resulted in syncytia formation in 143TK- cells, so the syncytial phenotype of the "gK-null" viruses was attributed to the expression of the N-terminal 112 aa of gK (Jayachandra, Baghian, and Kousoulas, 1997). Importantly, a precise insertion-deletion gK mutation recently constructed in an F-strain BAC was not syncytial and showed a similar egress defect, confirming previous results (Melancon et al., 2005).

Studies of gK localization in infected cells had previously shown that gK was exclusively localized in the peri-nuclear spaces and contained unprocessed carbohydrates added in the rough endoplasmic reticulum, indicating that gK was not transported to the golgi complex and never reached the cell surface (Hutchinson, Roop-Beauchamp, and Johnson, 1995). Interestingly, while spontaneous mutations leading to syncytia formation can arise in UL20 (Baines et al., 1991; MacLean et al., 1991), UL24 (Jacobson, Martin, and Coen, 1989; Sanders, Wilkie, and Davison, 1982), UL27 (gB) (Bzik et al., 1984; Pellett et al., 1985), and UL53 (gK) (Bond and Person, 1984; Debroy, Pederson, and Person, 1985; Pogue-Geile et al., 1984; Ruyechan et al., 1979), a large majority of syncytial mutations map to the UL53 (gK) gene. The number of syncytial mutation found in gK would seem to be at odds with data indicating that gK never reaches the surface of infected cells; however, data obtained in our laboratory utilizing viruses engineered to contain epitope tags in the putative extra-cellular domains of gK indicates that gK does indeed reach the surface of infected cells, resolving the paradox of how gK could potentially function in virus induced cell-to-cell fusion (Foster, Alvarez, and Kousoulas, 2003). In transiently transfected cells, gK localized in the ER and did not reach the cell surface. It was originally reported for PrV that gK is not correctly processed in a UL20 deletion mutant, and that coexpression of gK and UL20 restored gK processing at least partially (Dietz et al., 2000). In support of these original findings, our laboratory showed that coexpression of gK and UL20 in transfected cells restored the transport of gK to cell surfaces (Foster, Alvarez, and Kousoulas, 2003). Nevertheless, while coexpression of UL20 also restored the transport of syncytial gK genes in transfected cells, syncytia formation did not result in these experiments, indicating that additional viral proteins are needed to activate the membrane fusion potential of gK (Foster, Alvarez, and Kousoulas, 2003).

Glycoprotein M (UL10) and Glycoprotein N (UL49.5)

The 1422 bp UL10 gene encodes the 473 aa glycoprotein M (gM), which contains eight predicted hydrophobic transmembrane regions (Babic et al., 1996; Baines and Roizman, 1993). gM is a component of the virion envelope and is present in the cytoplasmic membranes of infected cells (MacLean, Robertson, and Jamieson, 1993). However, recent evidence suggests a role for gM in nuclear egress (Baines et al., 2007). Viruses deficient in the expression of gM exhibit a 10 to 20 fold reduction in virus titers in Vero or BHK cells and form slightly smaller plaques (MacLean et al., 1991; MacLean, Robertson, and Jamieson, 1993). The 276 bp UL49.5 (UL49A) gene encodes a 91 aa integral membrane protein (Baines and Roizman, 1993). The UL49.5 polypeptide contains a 23 aa cleaved signal peptide and a C-terminal hydrophobic transmembrane domain (Barnett et al., 1992). There are also potential sites for *O*-glycosylation, leading to the naming of UL49.5 as glycoprotein N (gN) in some cases. In PrV, the UL49.5 homolog was found to be *O*-glycosylated and incorporated into virions as a 14kDa envelope protein (Jons et al., 1996). However, the UL49.5 gene product showed no evidence of glycosylation in HSV-1 infected cells, and appeared to be abundantly present in virions, linked to the tegument by disulfide bonds (Adams et al., 1998). While initial attempts at deletion of UL49.5 were unsuccessful and indicated the possibility that UL49.5 was required for virus replication (Barker and Roizman, 1992), a more recent attempt utilizing a more precise deletion resulted in a UL49.5-deficient virus that showed no phenotypic differences in plaque yield or morphology and only a marginal two-fold difference in virus yield (Adams et al., 1998).

gM and UL49.5 (gN) are conserved throughout alpha, beta-, and gammaherpesviruses and have been shown to form a complex in a number of herpesviruses analyzed to date (Jons, Dijkstra, and Mettenleiter, 1998; Koyano et al., 2003; Lake, Molesworth, and Hutt-Fletcher,

1998; Mach et al., 2000; Wu, Zhu, and Letchworth, 1998). The PrV gM has been shown to act as an inhibitor of cell fusion resulting from cotransfection of either the PrV gB, gD and gH/gL or the F protein of bovine respiratory syncytial virus, indicating a general mechanism of fusion inhibition by gM (Klupp, Nixdorf, and Mettenleiter, 2000). While no definitive interaction between has been shown between the HSV-1 gM and UL49.5, an enhancement of function has been demonstrated when both are present, inasmuch as cotransfection of the HSV-1 gM and UL49.5 significantly inhibited cell fusion caused by the HSV-1 gB, gD and gH/gL or the Molony murine leukemia virus envelope protein, while transfection of the HSV-1 gM alone did not inhibit membrane fusion (Koyano et al., 2003). A requirement of both gM and gN for inhibition of cell fusion was also shown for HHV-8 (Koyano et al., 2003). At this point in time, the mechanism by which gM and UL49.5 mediate inhibition of cell-to-cell fusion is unknown.

Characterization of the UL20 Protein

The UL20 ORF

The UL20 open reading frame is situated between the UL19 gene and the recently confirmed UL20.5 gene in the unique long region of the HSV-1 genome. UL20 is expressed as a γ1 (late) gene that does not require viral DNA replication for expression (Ward et al., 1994), and is conserved in the alphaherpesviruses.

Membrane Topology of UL20p

Based on its predicted amino acid sequence, the UL20 protein was suggested to be an integral membrane protein containing two or three hydrophobic transmembrane domains

(McGeoch et al., 1988). However, when the hydrophobic and membrane-spanning domains of UL20p were calculated by using the TMPred and SOSUI computer algorithms (Hirokawa, Boon-Chieng, and Mitaku, 1998; Hofmann and Stoffel, 1993) and used to derive a predicted membrane spanning model of UL20p, four hydrophobic regions were predicted, with no predicted signal peptide. This putative UL20p model features four membrane spanning regions, placing both the 66 amino acid amino terminus and the 14 amino acid carboxyl terminus of UL20p within the cytoplasm (domains I and V). In addition, a third small 10 amino acid domain is predicted to be located intracellularly (domain III), while the two other domains are predicted to be located extracellularly (domains II and IV). Domain II is predicted to contain 7 amino acids, while domain IV is predicted to contain 32 amino acids. As the largest extra-cellular domain, domain IV holds the best chance for interactions with the extra-cellular portions of other membrane proteins. This predicted model of the UL20p membrane topology has been partially confirmed in our laboratory with FLAG epitope tags located either on the N-terminus of UL20p, shown to be intracellular, or inside of domain IV, shown to be present on the extra-cellular/lumenal side of cellular membranes (Foster et al., 2004c).

Interdependence With gK for Transport

Initial reports on the cellular localization of the UL20 protein indicated that it was not expressed at the cell surface but was present in virions purified from extra-cellular fluid and from the cytoplasm. The UL20 protein was found to be present in the nuclear membranes, in the Golgi apparatus, and dispersed in the cytoplasm, but was not detected in the plasma membranes of infected cells (Ward et al., 1994). In contrast, recent experiments performed by our laboratory show that independently expressed gK or UL20p failed to be transported from the ER in Vero

cells. Similarly, infection of Vero cells with either a gK-null or UL20-null virus resulted in ER entrapment of UL20p or gK, respectively. In contrast, transiently coexpressed gK and UL20p predominantly localized to the TGN, and TGN-localized gK and UL20p were shown to originate from internalized gK and UL20p expressed at cell surfaces. In all experiments, UL20p and gK were seen to colocalize, consistent with the hypothesis that gK and UL20p directly interact and that this interaction is important for their TGN localization and function in the virus life cycle (Foster et al., 2004c).

Function of UL20p in the HSV-1 Lifecycle

The original description of the role of UL20p in the virus life cycle was predicated on the observation that an F-strain UL20-null virus, R7225, had a peri-nuclear block in virus egress, resulting in a large accumulation of virions between the inner and outer nuclear lamellae as well as an abnormal level of un-enveloped capsids in the cytoplasm. In addition, the R7225 virus formed small syncytia on 143TK- cells (Baines et al., 1991). However, at the time it was unknown that the UL20.5 gene was located adjacent to UL20, and the R7225 virus contains an aberrantly fused gene consisting of the 5' half of the UL20.5 ORF and the 3' half of the UL20 gene. As such, it was unknown whether or not the description of the UL20-null phenotype could be attributed to the aberrant gene. To resolve the true phenotype of an HSV-1 virus lacking the UL20 gene, our laboratory constructed an insertion-deletion mutation into the HSV-1 KOS-strain UL20 gene that consisted of removal of the 5' region of the UL20 ORF, while maintaining the integrity of both the UL20.5 gene and the promoter for the major capsid protein, UL19. In accordance with the R7225 virus, the KOS strain UL20-null virus exhibited a defect in virion egress; however, the block in virion egress consisted solely of an accumulation of un-enveloped

capsids in the cytoplasm, and no accumulation of peri-nuclear virions was observed (Foster et al., 2004b). Furthermore, the KOS UL20-null virus did not form syncytial plaques on 143TK- cells. The observable phenotypes of the KOS UL20-null virus were in agreement with the phenotype described for a PrV virus lacking UL20 (Fuchs et al., 1997), indicating that the previously described phenotype of the R7225 virus was in all likelihood due to the presence of the aberrantly fused gene. In addition, we found that the UL20 protein was required for virus-induced cell-to-cell fusion resulting from syncytial mutations in either gB or gK, implying that UL20 may serve to regulate membrane fusion events in HSV-1 (Foster et al., 2004b). In Chapter IV, we investigate the domains of UL20 that function in gB and/or gK-induced syncytia formation as well as in virion egress.

Characterization of the UL11 Protein

The UL11 ORF encodes a 96 amino acid tegument protein, which is N-terminally myristylated (MacLean, Clark, and McGeoch, 1989) and palmitylated (Loomis et al., 2001). The UL11 protein localizes to nuclear and TGN-derived apparatus derived membranes in infected cells (Baines et al., 1995), but only to the TGN-membranes in non-infected cells (Bowzard et al., 2000). UL11 was shown to specifically interact with the UL16 tegument protein, providing a potential docking mechanism for tegumented capsids onto TGN-membranes(Loomis, Courtney, and Wills, 2003; Vittone et al., 2005). An HSV UL11-null mutant obtained by deletion of most of the UL11 coding region accumulated capsids into the nucleus and un-enveloped capsids in the cytoplasm (Baines and Roizman, 1992b), while a PRV-null virus with the entire UL11 gene deleted showed accumulation of un-enveloped capsids in the cytoplasm of infected cells embedded in tegument-like material (Kopp et al., 2003).

Function of the UL11 Protein in the HSV-1 Lifecycle

Previously, the role of UL11 in HSV-1 virion morphogenesis and egress was described based on the observations of a UL11-null virus obtained by deleting the majority of the UL11 ORF (176 of 291 bps). This virus (R7219) exhibited a major defect in capsid envelopment via budding through the nuclear membrane resulting in an unusual accumulation of intra-nuclear capsids as well as a reduced number of enveloped capsids in the cytoplasm (Baines and Roizman, 1992b).

Characterization of the UL16 Protein

The UL16 ORF encodes a 373 amino acid protein (Nalwanga et al., 1996) that exhibits a late ($\gamma 1$) expression profile (Costa et al., 1985) and requires viral DNA synthesis for its replication. UL15 is a rarity among herpes-virus genes in that it consist of 2 exons and 1 intron that are spliced together (Baines and Roizman, 1992a). The UL16 ORF is located within this intron on the anti-sense strand, and is conserved among herpesviruses (Costa et al., 1985; Wing, Lee, and Huang, 1996).

Function of the UL16 Protein in the HSV-1 Life Cycle

Initial studies of the function of UL16 involving a UL16 deletion mutant (R7210) revealed that UL16 protein was dispensable for HSV-1 replication in cell culture, although the virus did replicate at titres 3 to 10 fold lower than those of the wild type virus (Baines and Roizman, 1991). To investigate the possibility that the UL15 intron acted to downregulate UL16 expression by an antisense mechanism, the growth of viruses as well as UL16 protein production with and without the UL15 intro were compared, with no differences found. However the UL15 splicing event has been shown to be dispensable for virus replication (Baines and Roizman, 1992a). These two findings make the importance of the UL15 intron and splicing events unknown.

The UL16 protein initially appears in the nuclei of infected cells in discrete sites which also contain large amounts of capsid proteins (Nalwanga et al., 1996; Ward, Barker, and Roizman, 1996). These sites, known as assemblons, are believed to be sites at which capsid assembly and/or DNA cleavage and packaging may occur (Ward, Ogle, and Roizman, 1996). These results suggest a possible role for UL16 in intra-nuclear capsid assembly (Nalwanga et al., 1996). Later in the virus life-cycle, UL16 protein becomes associated with peri-nuclear virions and is found as a component of purified virions (Nalwanga et al., 1996). This finding is similar to that of the human cytomegalovirus UL16 homolog, UL94 (Wing, Lee, and Huang, 1996). Interestingly, this finding is not duplicated in HSV-2 (Oshima et al., 1998).

UL16 has been shown to specifically interact with UL11 (Loomis, Courtney, and Wills, 2003; Vittone et al., 2005). This interaction is dependant upon the presence of an acidic amino acid cluster and a di-leucine motif located within the first 50 amino acids of UL11 (Loomis, Courtney, and Wills, 2003). UL11 is associated with TGN-derived vesicles via N-terminal palmitolation and myristylation. The interaction between UL11 on TGN-membranes and the capsid associated UL16 presents a possible pathway for the recruitment of cytoplasmic capsids to TGN-derived membranes for final envelopment. In work presented in Chapter 3, we seek to further elucidate the roles of UL11 and UL16 in virion morphogenesis and egress.

REFERENCES

Adams, H. G., Benson, E. A., Alexander, E. R., Vontver, L. A., Remington, M. A., and Holmes, K. K. (1976). Genital herpetic infection in men and women: clinical course and effect of topical application of adenine arabinoside. *J Infect Dis* **133 Suppl,** A151-9.

Adams, R., Cunningham, C., Davison, M. D., MacLean, C. A., and Davison, A. J. (1998). Characterization of the protein encoded by gene UL49A of herpes simplex virus type 1. *J Gen Virol* **79 (Pt 4),** 813-23.

Alconada, A., Bauer, U., Sodeik, B., and Hoflack, B. (1999). Intracellular traffic of herpes simplex virus glycoprotein gE: characterization of the sorting signals required for its trans-Golgi network localization. *J Virol* **73**(1)**,** 377-87.

Allen, W. P., and Rapp, F. (1982). Concept review of genital herpes vaccines. *J Infect Dis* **145**(3)**,** 413-21.

Alwine, J. C., Steinhart, W. L., and Hill, C. W. (1974). Transcription of herpes simplex type 1 DNA in nuclei isolated from infected HEp-2 and KB cells. *Virology* **60**(1)**,** 302-7.

Amir, J., Harel, L., Smetana, Z., and Varsano, I. (1997). Treatment of herpes simplex gingivostomatitis with aciclovir in children: a randomised double blind placebo controlled study. *Bmj* **314**(7097)**,** 1800-3.

Armstrong, J. A., Pereira, H. G., and Andrewes, C. H. (1961). Observations on the virus of infectious bovine rhinotracheitis, and its affinity with the Herpesvirus group. *Virology* **14,** 276-85.

Astruc, J. (1736). "De Morbis Venereis Libri Sex." G. Cavelier, Paris.

Atkinson, M. A., Barr, S., and Timbury, M. C. (1978). The fine structure of cells infected with temperature-sensitive mutants of herpes simplex virus type 2. *Journal of General Virology* **40,** 103-119.

Babic, N., Klupp, B., Brack, A., Mettenleiter, T. C., Ugolini, G., and Flamand, A. (1996). Deletion of glycoprotein gE reduces the propagation of pseudorabies virus in the nervous system of mice after intranasal inoculation. *Virology* **219**(1)**,** 279-84.

Bader, C., Crumpacker, C. S., Schnipper, L. E., Ransil, B., Clark, J. E., Arndt, K., and Freedberg, I. M. (1978). The natural history of recurrent facial-oral infection with herpes simplex virus. *J Infect Dis* **138**(6)**,** 897-905.

Baghian, A., Huang, L., Newman, S., Jayachandra, S., and Kousoulas, K. G. (1993). Truncation of the carboxy-terminal 28 amino acids of glycoprotein B specified by herpes simplex virus type 1 mutant amb1511-7 causes extensive cell fusion. *Journal of Virology* **67**(4)**,** 2396-401.

Baines, J. D., Jacob, R. J., Simmerman, L., and Roizman, B. (1995). The herpes simplex virus 1 UL11 proteins are associated with cytoplasmic and nuclear membranes and with nuclear bodies of infected cells. *Journal of Virology* **69**(2)**,** 825-33.

Baines, J. D., and Roizman, B. (1991). The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. *Journal of Virology* **65**(2)**,** 938-44.

Baines, J. D., and Roizman, B. (1992a). The cDNA of UL15, a highly conserved herpes simplex virus 1 gene, effectively replaces the two exons of the wild-type virus. *J Virol* **66**(9)**,** 5621-6.

Baines, J. D., and Roizman, B. (1992b). The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. *Journal of Virology* **66**(8)**,** 5168-74.

Baines, J. D., and Roizman, B. (1993). The UL10 gene of herpes simplex virus 1 encodes a novel viral glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells. *Journal of Virology* **67**(3)**,** 1441-52.

Baines, J. D., Ward, P. L., Campadelli-Fiume, G., and Roizman, B. (1991). The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. *Journal of Virology* **65**(12)**,** 6414-24.

Baines, J. D., Wills, E., Jacob, R. J., Pennington, J., and Roizman, B. (2007). Glycoprotein M of herpes simplex virus 1 is incorporated into virions during budding at the inner nuclear membrane. *J Virol* **81**(2)**,** 800-12.

Balan, P., Davis-Poynter, N., Bell, S., Atkinson, H., Browne, H., and Minson, T. (1994). An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *Journal of General Virology* **75**(Pt 6)**,** 1245-58.

Banfield, B. W., Leduc, Y., Esford, L., Visalli, R. J., Brandt, C. R., and Tufaro, F. (1995). Evidence for an interaction of herpes simplex virus with chondroitin sulfate proteoglycans during infection. *Virology* **208**(2)**,** 531-9.

Baringer, J. R., and Swoveland, P. (1973). Recovery of herpes-simplex virus from human trigeminal ganglions. *N Engl J Med* **288**(13)**,** 648-50.

Barker, D. E., and Roizman, B. (1992). The unique sequence of the herpes simplex virus 1 L component contains an additional translated open reading frame designated UL49.5. *J Virol* **66**(1)**,** 562-6.

Barnett, B. C., Dolan, A., Telford, E. A., Davison, A. J., and McGeoch, D. J. (1992). A novel herpes simplex virus gene (UL49A) encodes a putative membrane protein with counterparts in other herpesviruses. *J Gen Virol* **73 (Pt 8),** 2167-71.

Bartoletti, A. M., Tognon, M., Manservigi, R., and Mannini-Palenzona, A. (1985). Characterization of virus obtained from MDBK cells persistently infected with a variant of herpes simplex virus type 1 strain MP [HSV-1(MP)]. *Virology* **141**(2)**,** 306-10.

Bastian, F. O., Rabson, A. S., Yee, C. L., and Tralka, T. S. (1972). Herpesvirus hominis: isolation from human trigeminal ganglion. *Science* **178**(58)**,** 306-7.

Bateman, T. (1814). "A Practical Synopsis of Cutaneous Diseases According to the Arrangement of Dr. Willan." Longman, Hurst, Orme, and Brown, London.

Batterson, W., Furlong, D., and Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII. further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. *J Virol* **45**(1)**,** 397-407.

Batterson, W., and Roizman, B. (1983). Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. *Journal of Virology* **46**(2)**,** 371-7.

Becker, Y., Dym, H., and Sarov, I. (1968). Herpes simplex virus DNA. *Virology* **36**(2)**,** 184-92.

Binder, P. S. (1977). Herpes simplex keratitis. *Surv Ophthalmol* **21**(4)**,** 313-31.

Black, F. L. (1975). Infectious diseases in primitive societies. *Science* **187**(4176)**,** 515-8.

Black, F. L., Hierholzer, W. J., Pinheiro, F., Evans, A. S., Woodall, J. P., Opton, E. M., Emmons, J. E., West, B. S., Edsall, G., Downs, W. G., and Wallace, G. D. (1974). Evidence for persistence of infectious agents in isolated human populations. *Am J Epidemiol* **100**(3)**,** 230-50.

Bond, V. C., and Person, S. (1984). Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. *Virology* **132**(2)**,** 368-76.

Bond, V. C., Person, S., and Warner, S. C. (1982). The isolation and characterization of mutants of herpes simplex virus type 1 that induce cell fusion. *Journal of General Virology* **61 (Pt 2),** 245-54.

Bowzard, J. B., Visalli, R. J., Wilson, C. B., Loomis, J. S., Callahan, E. M., Courtney, R. J., and Wills, J. W. (2000). Membrane targeting properties of a herpesvirus tegument protein-retrovirus Gag chimera. *J Virol* **74**(18)**,** 8692-9.

Brack, A. R., Dijkstra, J. M., Granzow, H., Klupp, B. G., and Mettenleiter, T. C. (1999). Inhibition of virion maturation by simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. *J Virol* **73**(7)**,** 5364-72.

Brown, Z. A., Benedetti, J., Ashley, R., Burchett, S., Selke, S., Berry, S., Vontver, L. A., and Corey, L. (1991). Neonatal herpes simplex virus infection in relation to asymptomatic maternal infection at the time of labor. *N Engl J Med* **324**(18)**,** 1247-52.

Browne, H., Bell, S., and Minson, T. (2004). Analysis of the requirement for glycoprotein m in herpes simplex virus type 1 morphogenesis. *J Virol* **78**(2)**,** 1039-41.

Browne, H., Bell, S., Minson, T., and Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* **70**(7)**,** 4311-6.

Buddingh, G. J., Schrum, D. I., Lanier, J. C., and Guidry, D. J. (1953). Studies of the natural history of herpes simplex infections. *Pediatrics* **11**(6)**,** 595-610.

Burnet, F. M., and Williams, S. W. (1939). Herpes simplex: new point of view. *Med J Aust* **1,** 637-640.

Bzik, D. J., Fox, B. A., DeLuca, N. A., and Person, S. (1984). Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. *Virology* **137**(1)**,** 185-90.

Cai, W., Person, S., Warner, S. C., Zhou, J., and DeLuca, N. A. (1987). Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. *Journal of Virology* **61,** 714-721.

Cai, W. H., Gu, B., and Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *Journal of Virology* **62**(8)**,** 2596-604.

Cai, W. Z., Person, S., DebRoy, C., and Gu, B. H. (1988). Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1. An analysis of linker insertion mutants. *Journal of Molecular Biology* **201**(3)**,** 575-88.

Cairns, T. M., Landsburg, D. J., Whitbeck, J. C., Eisenberg, R. J., and Cohen, G. H. (2005). Contribution of cysteine residues to the structure and function of herpes simplex virus gH/gL. *Virology* **332**(2)**,** 550-62.

Campadelli-Fiume, G., Arsenakis, M., Farabegoli, F., and Roizman, B. (1988). Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. *Journal of Virology* **62**(1)**,** 159-67.

Campadelli-Fiume, G., Farabegoli, F., Di, G. S., and Roizman, B. (1991). Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. *Journal of Virology* **65**(3)**,** 1589-95.

Campbell, M. E., Palfreyman, J. W., and Preston, C. M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. *J Mol Biol* **180**(1)**,** 1-19.

Card, J. P., Whealy, M. E., Robbins, A. K., and Enquist, L. W. (1992). Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. *J Virol* **66**(5)**,** 3032-41.

Carfi, A., Willis, S. H., Whitbeck, J. C., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Wiley, D. C. (2001). Herpes simplex virus glycoprotein D bound to the human receptor HveA. *Mol Cell* **8**(1)**,** 169-79.

Challberg, M. D. (1986). A method for identifying the viral genes required for herpesvirus DNA replication. *Proc Natl Acad Sci U S A* **83**(23)**,** 9094-8.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**(5192)**,** 1865-9.

Chang, Y. E., Menotti, L., Filatov, F., Campadelli-Fiume, G., and Roizman, B. (1998). UL27.5 is a novel gamma2 gene antisense to the herpes simplex virus 1 gene encoding glycoprotein B. *J Virol* **72**(7)**,** 6056-64.

Chang, Y. E., Van Sant, C., Krug, P. W., Sears, A. E., and Roizman, B. (1997). The null mutant of the U(L)31 gene of herpes simplex virus 1: construction and phenotype in infected cells. *J Virol* **71**(11)**,** 8307-15.

Chou, J., and Roizman, B. (1986). The terminal a sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. *J Virol* **57**(2)**,** 629-37.

Church, G. A., and Wilson, D. W. (1997). Study of herpes simplex virus maturation during a synchronous wave of assembly. *J Virol* **71**(5)**,** 3603-12.

Claesson-Welsh, L., and Spear, P. G. (1986). Oligomerization of herpes simplex virus glycoprotein B. *Journal of Virology* **60,** 803-806.

Claesson-Welsh, L., and Spear, P. G. (1987). Amino-terminal sequence, synthesis, and membrane insertion of glycoprotein B of herpes simplex virus type 1. *Journal of Virology* **61,** 1-7.

Clements, J. B., Watson, R. J., and Wilkie, N. M. (1977). Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. *Cell* **12**(1)**,** 275-85.

Cocchi, F., Menotti, L., Di Ninni, V., Lopez, M., and Campadelli-Fiume, G. (2004). The herpes simplex virus JMP mutant enters receptor-negative J cells through a novel pathway independent of the known receptors nectin1, HveA, and nectin2. *J Virol* **78**(9)**,** 4720-9.

Cocchi, F., Menotti, L., Mirandola, P., Lopez, M., and Campadelli-Fiume, G. (1998). The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. *J Virol* **72**(12)**,** 9992-10002.

Cohen, J. I., and Nguyen, H. (1997). Varicella-zoster virus glycoprotein I is essential for growth of virus in Vero cells. *J Virol* **71**(9)**,** 6913-20.

Conley, A. J., Knipe, D. M., Jones, P. C., and Roizman, B. (1981). Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. *J Virol* **37**(1)**,** 191-206.

Corey, L. (1982). The diagnosis and treatment of genital herpes. *Jama* **248**(9)**,** 1041-9.

Corey, L., Adams, H. G., Brown, Z. A., and Holmes, K. K. (1983). Genital herpes simplex virus infections: clinical manifestations, course, and complications. *Ann Intern Med* **98**(6)**,** 958-72.

Corey, L., Holmes, K., Benedetti, J., and Critchlow, C. (1981). Clinical course of genital herpes: Implications for therapeutic trials. *In* "The human herpesviruses, an interdisciplinary perspective" (W. R. Dowdle, A. J. Nahmias, and R. F. Schinazi, Eds.), pp. 496-502. Elsevier, Amsterdam.

Costa, R. H., Draper, K. G., Kelly, T. J., and Wagner, E. K. (1985). An unusual spliced herpes simplex virus type 1 transcript with sequence homology to Epstein-Barr virus DNA. *J Virol* **54**(2)**,** 317-28.

Costanzo, F., Campadelli-Fiume, G., Foa-Tomasi, L., and Cassai, E. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. *J Virol* **21**(3)**,** 996-1001.

Craig, J. M., Macauley, J. C., Weller, T. H., and Wirth, P. (1957). Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease. *Proc Soc Exp Biol Med* **94**(1)**,** 4-12.

Darlington, R. W., and Moss, L. H. D. (1968). Herpesvirus envelopment. *Journal of Virology* **2**(1)**,** 48-55.

Davison, M. D., Rixon, F. J., and Davison, A. J. (1992). Identification of genes encoding two capsid proteins (VP24 and VP26) of herpes simplex virus type 1. *J Gen Virol* **73 (Pt 10),** 2709-13.

Deb, S., and Doelberg, M. (1988). A 67-base-pair segment from the Ori-S region of herpes simplex virus type 1 encodes origin function. *J Virol* **62**(7)**,** 2516-9.

Debroy, C., Pederson, N., and Person, S. (1985). Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. *Virology* **145**(1)**,** 36-48.

Deiss, L. P., Chou, J., and Frenkel, N. (1986). Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. *J Virol* **59**(3)**,** 605-18.

Desai, P., Sexton, G. L., McCaffery, J. M., and Person, S. (2001). A null mutation in the gene encoding the herpes simplex virus type 1 UL37 polypeptide abrogates virus maturation. *J Virol* **75**(21)**,** 10259-71.

Desai, P. J. (2000). A null mutation in the UL36 gene of herpes simplex virus type 1 results in accumulation of unenveloped DNA-filled capsids in the cytoplasm of infected cells. *J Virol* **74**(24)**,** 11608-18.

Dietz, P., Klupp, B. G., Fuchs, W., Kollner, B., Weiland, E., and Mettenleiter, T. C. (2000). Pseudorabies virus glycoprotein K requires the UL20 gene product for processing. *J Virol* **74**(11)**,** 5083-90.

Dingwell, K. S., Brunetti, C. R., Hendricks, R. L., Tang, Q., Tang, M., Rainbow, A. J., and Johnson, D. C. (1994). Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. *Journal of Virology* **68**(2)**,** 834-45.

Dingwell, K. S., Doering, L. C., and Johnson, D. C. (1995). Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. *Journal of Virology* **69**(11)**,** 7087-98.

Dingwell, K. S., and Johnson, D. C. (1998). The herpes simplex virus gE-gI complex facilitates cell-to-cell spread and binds to components of cell junctions. *J Virol* **72**(11)**,** 8933-42.

Dixon, R. A., and Schaffer, P. A. (1980). Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. *J Virol* **36**(1)**,** 189-203.

Dubin, G., and Jiang, H. (1995). Expression of herpes simplex virus type 1 glycoprotein L (gL) in transfected mammalian cells: evidence that gL is not independently anchored to cell membranes. *J Virol* **69**(7)**,** 4564-8.

Eberle, F., Dubreuil, P., Mattei, M. G., Devilard, E., and Lopez, M. (1995). The human PRR2 gene, related to the human poliovirus receptor gene (PVR), is the true homolog of the murine MPH gene. *Gene* **159**(2)**,** 267-72.

Eisenberg, R. J., Ponce de Leon, M., Friedman, H. M., Fries, L. F., Frank, M. M., Hastings, J. C., and Cohen, G. H. (1987). Complement component C3b binds directly to purified glycoprotein C of herpes simplex virus types 1 and 2. *Microb Pathog* **3**(6)**,** 423-35.

Elgadi, M. M., and Smiley, J. R. (1999). Picornavirus internal ribosome entry site elements target RNA cleavage events induced by the herpes simplex virus virion host shutoff protein. *J Virol* **73**(11)**,** 9222-31.

Enquist, L. W., Husak, P. J., Banfield, B. W., and Smith, G. A. (1998). Infection and spread of alphaherpesviruses in the nervous system. *Adv Virus Res* **51,** 237-347.

Epstein, M. A. (1962). Observations on the mode of release of herpes virus from infected HeLa cells. *J Cell Biol* **12,** 589-97.

Epstein, M. A., Achong, B. G., and Barr, Y. M. (1964). Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. *Lancet* **15,** 702-3.

Everett, R. D., Orr, A., and Elliott, M. (1991). High level expression and purification of herpes simplex virus type 1 immediate early polypeptide Vmw110. *Nucleic Acids Res* **19**(22)**,** 6155-61.

Faber, S. W., and Wilcox, K. W. (1986). Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. *Nucleic Acids Res* **14**(15)**,** 6067-83.

Falke, D., Siegert, R., and Vogell, W. (1959). [Electron microscopic findings on the problem of double membrane formation in herpes simplex virus]. *Arch Gesamte Virusforsch* **9,** 484-96.

Farnsworth, A., Goldsmith, K., and Johnson, D. C. (2003). Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm. *J Virol* **77**(15)**,** 8481-94.

Fenwick, M., Morse, L. S., and Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XI. Apparent clustering of functions effecting rapid inhibition of host DNA and protein synthesis. *J Virol* **29**(2)**,** 825-7.

Fenwick, M. L., and Walker, M. J. (1978). Suppression of the synthesis of cellular macromolecules by herpes simplex virus. *J Gen Virol* **41**(1)**,** 37-51.

Fleming, D. T., McQuillan, G. M., Johnson, R. E., Nahmias, A. J., Aral, S. O., Lee, F. K., and St Louis, M. E. (1997). Herpes simplex virus type 2 in the United States, 1976 to 1994. *N Engl J Med* **337**(16)**,** 1105-11.

Foa-Tomasi, L., Avitabile, E., Boscaro, A., Brandimarti, R., Gualandri, R., Manservigi, R., Dall'Olio, F., Serafini-Cessi, F., and Fiume, G. C. (1991). Herpes simplex virus (HSV) glycoprotein H is partially processed in a cell line that expresses the glycoprotein and fully processed in cells infected with deletion or ts mutants in the known HSV glycoproteins. *Virology* **180**(2)**,** 474-82.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N., and Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J Virol* **66**(1)**,** 341-8.

Forrester, A. J., Sullivan, V., Simmons, A., Blacklaws, B. A., Smith, G. L., Nash, A. A., and Minson, A. C. (1991). Induction of protective immunity with antibody to herpes simplex virus type 1 glycoprotein H (gH) and analysis of the immune response to gH expressed in recombinant vaccinia virus. *J Gen Virol* **72 (Pt 2),** 369-75.

Foster, T. P., Alvarez, X., and Kousoulas, K. G. (2003). Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion. *J Virol* **77**(1)**,** 499-510.

Foster, T. P., Melancon, J. M., Baines, J. D., and Kousoulas, K. G. (2004a). The Herpes Simplex Virus Type 1 UL20 Protein Modulates Membrane Fusion Events during Cytoplasmic Virion Morphogenesis and Virus-Induced Cell Fusion. *J Virol* **78**(10)**,** 5347-57.

Foster, T. P., Melancon, J. M., and Kousoulas, K. G. (2001). An alpha-helical domain within the carboxyl terminus of herpes simplex virus type 1 (HSV-1) glycoprotein B (gB) is associated with cell fusion and resistance to heparin inhibition of cell fusion. *Virology* **287**(1)**,** 18-29.

Foster, T. P., Melancon, J. M., Olivier, T. L., and Kousoulas, K. G. (2004b). Herpes simplex virus type-1 (HSV-1) glycoprotein K (gK) and the UL20 protein are interdependent for intracellular trafficking and trans-Golgi network localization. *J Virol***,** (In Press).

Frame, M. C., Marsden, H. S., and McGeoch, D. J. (1986). Novel herpes simplex virus type 1 glycoproteins identified by antiserum against a synthetic oligopeptide from the predicted product of gene US4. *J Gen Virol* **67 (Pt 4),** 745-51.

Frenkel, N., Locker, H., Batterson, W., Hayward, G. S., and Roizman, B. (1976). Anatomy of herpes simplex virus DNA. VI. Defective DNA originates from the S component. *J Virol* **20**(2)**,** 527-31.

Frenkel, N., Schirmer, E. C., Wyatt, L. S., Katsafanas, G., Roffman, E., Danovich, R. M., and June, C. H. (1990). Isolation of a new herpesvirus from human CD4+ T cells. *Proc Natl Acad Sci U S A* **87**(2)**,** 748-52.

Friedman, H. M., Cohen, G. H., Eisenberg, R. J., Seidel, C. A., and Cines, D. B. (1984). Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. *Nature* **309**(5969)**,** 633-5.

Friedman, H. M., Wang, L., Fishman, N. O., Lambris, J. D., Eisenberg, R. J., Cohen, G. H., and Lubinski, J. (1996). Immune evasion properties of herpes simplex virus type 1 glycoprotein gC. *J Virol* **70**(7)**,** 4253-60.

Frink, R. J., Anderson, K. P., and Wagner, E. K. (1981). Herpes simplex virus type 1 HindIII fragment L encodes spliced and complementary mRNA species. *J Virol* **39**(2)**,** 559-72.

Frink, R. J., Eisenberg, R., Cohen, G., and Wagner, E. K. (1983). Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. *J Virol* **45**(2)**,** 634-47.

Fuchs, W., Klupp, B. G., Granzow, H., and Mettenleiter, T. C. (1997). The UL20 gene product of pseudorabies virus functions in virus egress. *J Virol* **71**(7)**,** 5639-46.

Fuchs, W., Klupp, B. G., Granzow, H., Osterrieder, N., and Mettenleiter, T. C. (2002). The interacting UL31 and UL34 gene products of pseudorabies virus are involved in egress from the host-cell nucleus and represent components of primary enveloped but not mature virions. *J Virol* **76**(1)**,** 364-78.

Furlong, D., Swift, H., and Roizman, B. (1972). Arrangement of herpesvirus deoxyribonucleic acid in the core. *J Virol* **10**(5)**,** 1071-4.

Gage, P. J., Levine, M., and Glorioso, J. C. (1993). Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B. *Journal of Virology* **67**(4)**,** 2191-201.

Gelman, I. H., and Silverstein, S. (1987). Herpes simplex virus immediate-early promoters are responsive to virus and cell trans-acting factors. *J Virol* **61**(7)**,** 2286-96.

Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Spear, P. G. (1998). Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**(5369)**,** 1618-20.

Gershon, A. A., Sherman, D. L., Zhu, Z., Gabel, C. A., Ambron, R. T., and Gershon, M. D. (1994). Intracellular transport of newly synthesized varicella-zoster virus: final envelopment in the trans-Golgi network. *J Virol* **68**(10)**,** 6372-90.

Ghiasi, H., Nesburn, A. B., Cai, S., and Wechsler, S. L. (1998). The US5 open reading frame of herpes simplex virus type 1 does encode a glycoprotein (gJ). *Intervirology* **41**(2-3)**,** 91-7.

Gianni, T., Forghieri, C., and Campadelli-Fiume, G. (2006). The herpesvirus glycoproteins B and H.L are sequentially recruited to the receptor-bound gD to effect membrane fusion at virus entry. *Proc Natl Acad Sci U S A* **103**(39)**,** 14572-7.

Gibson, W. (1996). Structure and assembly of the virion. *Intervirology* **39**(5-6)**,** 389-400.

Gibson, W., and Roizman, B. (1972). Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. *J Virol* **10**(5)**,** 1044-52.

Godowski, P. J., and Knipe, D. M. (1986). Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. *Proc Natl Acad Sci U S A* **83**(2)**,** 256-60.

Goodman, J. L., and Engel, J. P. (1991). Altered pathogenesis in herpes simplex virus type 1 infection due to a syncytial mutation mapping to the carboxy terminus of glycoprotein B. *Journal of Virology* **65**(4)**,** 1770-8.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N., and Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *J Virol* **75**(8)**,** 3675-84.

Gruter, W. (1924). Das herpesvirus, seine atiologischeund ung klinische bedeutung. *Munch Med Wschr* **71,** 1058.

Haan, K. M., Lee, S. K., and Longnecker, R. (2001). Different functional domains in the cytoplasmic tail of glycoprotein B are involved in Epstein-Barr virus-induced membrane fusion. *Virology* **290**(1)**,** 106-14.

Hannah, B. P., Heldwein, E. E., Bender, F. C., Cohen, G. H., and Eisenberg, R. J. (2007). Mutational evidence of internal fusion loops in herpes simplex virus glycoprotein B. *J Virol* **81**(9)**,** 4858-65.

Harley, C. A., Dasgupta, A., and Wilson, D. W. (2001). Characterization of herpes simplex virus-containing organelles by subcellular fractionation: role for organelle acidification in assembly of infectious particles. *J Virol* **75**(3)**,** 1236-51.

Harms, J. S., Ren, X., Oliveira, S. C., and Splitter, G. A. (2000). Distinctions between bovine herpesvirus 1 and herpes simplex virus type 1 VP22 tegument protein subcellular associations. *J Virol* **74**(7)**,** 3301-12.

Harris, S. L., Frank, I., Yee, A., Cohen, G. H., Eisenberg, R. J., and Friedman, H. M. (1990). Glycoprotein C of herpes simplex virus type 1 prevents complement-mediated cell lysis and virus neutralization. *J Infect Dis* **162**(2)**,** 331-7.

Heine, J. W., Honess, R. W., Cassai, E., and Roizman, B. (1974). Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. *J Virol* **14**(3)**,** 640-51.

Heldwein, E. E., Lou, H., Bender, F. C., Cohen, G. H., Eisenberg, R. J., and Harrison, S. C. (2006). Crystal structure of glycoprotein B from herpes simplex virus 1. *Science* **313**(5784)**,** 217-20.

Herold, B. C., Visalli, R. J., Susmarski, N., Brandt, C. R., and Spear, P. G. (1994). Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. *Journal of General Virology* **75,** 1211-22.

Highlander, S. L., Goins, W. F., Person, S., Holland, T. C., Levine, M., and Glorioso, J. C. (1991). Oligomer formation of the gB glycoprotein of herpes simplex virus type 1. *J Virol* **65**(8)**,** 4275-83.

Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. *Bionformatics* **14,** 378-379.

Hofmann, K., and Stoffel, W. (1993). TMbase-database of membrane spanning segments. *J. Biol. Chem.* **377,** 166.

Homa, F. L., Purifoy, D. J., Glorioso, J. C., and Levine, M. (1986). Molecular basis of the glycoprotein C-negative phenotypes of herpes simplex virus type 1 mutants selected with a virus-neutralizing monoclonal antibody. *J Virol* **58**(2)**,** 281-9.

Honess, R. W., and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* **14**(1)**,** 8-19.

Hook, L. M., Lubinski, J. M., Jiang, M., Pangburn, M. K., and Friedman, H. M. (2006). Herpes simplex virus type 1 and 2 glycoprotein C prevents complement-mediated neutralization induced by natural immunoglobulin M antibody. *J Virol* **80**(8)**,** 4038-46.

Huber, M. T., and Compton, T. (1998). The human cytomegalovirus UL74 gene encodes the third component of the glycoprotein H-glycoprotein L-containing envelope complex. *J Virol* **72**(10)**,** 8191-7.

Huemer, H. P., Larcher, C., van Drunen Littel-van den Hurk, S., and Babiuk, L. A. (1993). Species selective interaction of Alphaherpesvirinae with the "unspecific" immune system of the host. *Arch Virol* **130**(3-4)**,** 353-64.

Huemer, H. P., Nowotny, N., Crabb, B. S., Meyer, H., and Hubert, P. H. (1995). gp13 (EHV-gC): a complement receptor induced by equine herpesviruses. *Virus Res* **37**(2)**,** 113-26.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A. C., and Johnson, D. C. (1992a). A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. *Journal of Virology* **66**(4)**,** 2240-50.

Hutchinson, L., Goldsmith, K., Snoddy, D., Ghosh, H., Graham, F. L., and Johnson, D. C. (1992b). Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. *J Virol* **66**(9)**,** 5603-9.

Hutchinson, L., and Johnson, D. C. (1995). Herpes simplex virus glycoprotein K promotes egress of virus particles. *Journal of Virology* **69**(9)**,** 5401-13.

Hutchinson, L., Roop-Beauchamp, C., and Johnson, D. C. (1995). Herpes simplex virus glycoprotein K is known to influence fusion of infected cells, yet is not on the cell surface. *Journal of Virology* **69**(7)**,** 4556-63.

Igarashi, K., Fawl, R., Roller, R. J., and Roizman, B. (1993). Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. *J Virol* **67**(4)**,** 2123-32.

Isaacs, S. N., Kotwal, G. J., and Moss, B. (1992). Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. *Proc Natl Acad Sci U S A* **89**(2)**,** 628-32.

Ishov, A. M., and Maul, G. G. (1996). The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. *J Cell Biol* **134**(4)**,** 815-26.

Jacob, R. J., Morse, L. S., and Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol* **29**(2)**,** 448-57.

Jacobs, L., Mulder, W. A., Van Oirschot, J. T., Gielkens, A. L., and Kimman, T. G. (1993). Deleting two amino acids in glycoprotein gI of pseudorabies virus decreases virulence and neurotropism for pigs, but does not affect immunogenicity. *J Gen Virol* **74 (Pt 10),** 2201-6.

Jacobson, J. G., Martin, S. L., and Coen, D. M. (1989). A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. *Journal of Virology* **63**(4)**,** 1839-43.

Jayachandra, S., Baghian, A., and Kousoulas, K. G. (1997). Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space. *Journal of Virology* **71**(7)**,** 5012-24.

Johnson, D. C., Burke, R. L., and Gregory, T. (1990). Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. *Journal of Virology* **64**(6)**,** 2569-76.

Johnson, D. C., and Feenstra, V. (1987). Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. *Journal of Virology* **61**(7)**,** 2208-16.

Johnson, D. C., Frame, M. C., Ligas, M. W., Cross, A. M., and Stow, N. D. (1988). Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. *J Virol* **62**(4)**,** 1347-54.

Johnson, D. C., and Spear, P. G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *Journal of Virology* **43**(3)**,** 1102-12.

Johnson, D. C., Webb, M., Wisner, T. W., and Brunetti, C. (2001). Herpes simplex virus gE/gI sorts nascent virions to epithelial cell junctions, promoting virus spread. *J Virol* **75**(2)**,** 821-33.

Jons, A., Dijkstra, J. M., and Mettenleiter, T. C. (1998). Glycoproteins M and N of pseudorabies virus form a disulfide-linked complex. *J Virol* **72**(1)**,** 550-7.

Jons, A., Granzow, H., Kuchling, R., and Mettenleiter, T. C. (1996). The UL49.5 gene of pseudorabies virus codes for an O-glycosylated structural protein of the viral envelope. *J Virol* **70**(2)**,** 1237-41.

Kalinyak, J. E., Fleagle, G., and Docherty, J. J. (1977). Incidence and distribution of herpes simplex virus types 1 and 2 from genital lesions in college women. *J Med Virol* **1**(3)**,** 175-81.

Karr, B. M., and Read, G. S. (1999). The virion host shutoff function of herpes simplex virus degrades the 5' end of a target mRNA before the 3' end. *Virology* **264**(1)**,** 195-204.

Katan, M., Haigh, A., Verrijzer, C. P., van der Vliet, P. C., and O'Hare, P. (1990). Characterization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex. *Nucleic Acids Res* **18**(23)**,** 6871-80.

Kaufman, R. H., Gardner, H. L., Rawls, W. E., Dixon, R. E., and Young, R. L. (1973). Clinical features of herpes genitalis. *Cancer Res* **33**(6)**,** 1446-51.

Kieff, E. D., Bachenheimer, S. L., and Roizman, B. (1971). Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. *J Virol* **8**(2)**,** 125-32.

Kimura, H., Wang, Y., Pesnicak, L., Cohen, J. I., Hooks, J. J., Straus, S. E., and Williams, R. K. (1998). Recombinant varicella-zoster virus glycoproteins E and I: immunologic responses and clearance of virus in a guinea pig model of chronic uveitis. *J Infect Dis* **178**(2)**,** 310-7.

Klupp, B. G., Fuchs, W., Granzow, H., Nixdorf, R., and Mettenleiter, T. C. (2002). Pseudorabies virus UL36 tegument protein physically interacts with the UL37 protein. *J Virol* **76**(6)**,** 3065-71.

Klupp, B. G., Granzow, H., and Mettenleiter, T. C. (2000). Primary envelopment of pseudorabies virus at the nuclear membrane requires the UL34 gene product. *J Virol* **74**(21)**,** 10063-73.

Klupp, B. G., Granzow, H., Mundt, E., and Mettenleiter, T. C. (2001). Pseudorabies virus UL37 gene product is involved in secondary envelopment. *J Virol* **75**(19)**,** 8927-36.

Klupp, B. G., Nixdorf, R., and Mettenleiter, T. C. (2000). Pseudorabies virus glycoprotein M inhibits membrane fusion. *J Virol* **74**(15)**,** 6760-8.

Knipe, D. M., Batterson, W., Nosal, C., Roizman, B., and Buchan, A. (1981). Molecular genetics of herpes simplex virus. VI. Characterization of a temperature-sensitive mutant defective in the expression of all early viral gene products. *J Virol* **38**(2)**,** 539-47.

Knipe, D. M., Ruyechan, W. T., and Roizman, B. (1979). Molecular genetics of herpes simplex virus. III. Fine mapping of a genetic locus determining resistance to phosphonoacetate by two methods of marker transfer. *J Virol* **29**(2)**,** 698-704.

Knipe, D. M., Senechek, D., Rice, S. A., and Smith, J. L. (1987). Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. *J Virol* **61**(2)**,** 276-84.

Knopf, C. W. (1986). Nucleotide sequence of the DNA polymerase gene of herpes simplex virus type 1 strain Angelotti. *Nucleic Acids Res* **14**(20)**,** 8225-6.

Kopp, M., Granzow, H., Fuchs, W., Klupp, B., and Mettenleiter, T. C. (2004). Simultaneous deletion of pseudorabies virus tegument protein UL11 and glycoprotein M severely impairs secondary envelopment. *J Virol* **78**(6)**,** 3024-34.

Kopp, M., Granzow, H., Fuchs, W., Klupp, B. G., Mundt, E., Karger, A., and Mettenleiter, T. C. (2003). The pseudorabies virus UL11 protein is a virion component involved in secondary envelopment in the cytoplasm. *J Virol* **77**(9)**,** 5339-51.

Korsager, B., Spencer, E. S., Mordhorst, C. H., and Andersen, H. K. (1975). Herpesvirus hominis infections in renal transplant recipients. *Scand J Infect Dis* **7**(1)**,** 11-9.

Kotwal, G. J., Isaacs, S. N., McKenzie, R., Frank, M. M., and Moss, B. (1990). Inhibition of the complement cascade by the major secretory protein of vaccinia virus. *Science* **250**(4982)**,** 827-30.

Koyano, S., Mar, E. C., Stamey, F. R., and Inoue, N. (2003). Glycoproteins M and N of human herpesvirus 8 form a complex and inhibit cell fusion. *J Gen Virol* **84**(Pt 6)**,** 1485-91.

Kristie, T. M., and Roizman, B. (1984). Separation of sequences defining basal expression from those conferring alpha gene recognition within the regulatory domains of herpes simplex virus 1 alpha genes. *Proc Natl Acad Sci U S A* **81**(13)**,** 4065-9.

Kristie, T. M., and Roizman, B. (1986). DNA-binding site of major regulatory protein alpha 4 specifically associated with promoter-regulatory domains of alpha genes of herpes simplex virus type 1. *Proc Natl Acad Sci U S A* **83**(13)**,** 4700-4.

Kristie, T. M., and Sharp, P. A. (1990). Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV alpha-trans-activator protein. *Genes Dev* **4**(12B)**,** 2383-96.

Kritas, S. K., Pensaert, M. B., and Mettenleiter, T. C. (1994). Role of envelope glycoproteins gI, gp63 and gIII in the invasion and spread of Aujeszky's disease virus in the olfactory nervous pathway of the pig. *J Gen Virol* **75 (Pt 9),** 2319-27.

Kudelova, M., Kostal, M., Cervenakova, L., Rajcani, J., and Kaerner, H. C. (1991). Pathogenicity and latency competence for rabbits of the herpes simplex virus type 1 ANGpath gC and gE defective mutants. *Acta Virol* **35**(5)**,** 438-49.

Kwon, B., Kim, B. S., Cho, H. R., Park, J. E., and Kwon, B. S. (2003). Involvement of tumor necrosis factor receptor superfamily(TNFRSF) members in the pathogenesis of inflammatory diseases. *Exp Mol Med* **35**(1)**,** 8-16.

Kwong, A. D., and Frenkel, N. (1987). Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. *Proc Natl Acad Sci U S A* **84**(7)**,** 1926-30.

Kwong, A. D., Kruper, J. A., and Frenkel, N. (1988). Herpes simplex virus virion host shutoff function. *J Virol* **62**(3)**,** 912-21.

La Boissiere, S., Hughes, T., and O'Hare, P. (1999). HCF-dependent nuclear import of VP16. *Embo J* **18**(2)**,** 480-9.

Ladin, B. F., Blankenship, M. L., and Ben-Porat, T. (1980). Replication of herpesvirus DNA. V. Maturation of concatemeric DNA of pseudorabies virus to genome length is related to capsid formation. *J Virol* **33**(3)**,** 1151-64.

Ladin, B. F., Ihara, S., Hampl, H., and Ben-Porat, T. (1982). Pathway of assembly of herpesvirus capsids: an analysis using DNA+ temperature-sensitive mutants of pseudorabies virus. *Virology* **116**(2)**,** 544-61.

Lake, C. M., Molesworth, S. J., and Hutt-Fletcher, L. M. (1998). The Epstein-Barr virus (EBV) gN homolog BLRF1 encodes a 15-kilodalton glycoprotein that cannot be authentically processed unless it is coexpressed with the EBV gM homolog BBRF3. *J Virol* **72**(7)**,** 5559-64.

Lam, Q., Smibert, C. A., Koop, K. E., Lavery, C., Capone, J. P., Weinheimer, S. P., and Smiley, J. R. (1996). Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. *Embo J* **15**(10)**,** 2575-81.

Laquerre, S., Person, S., and Glorioso, J. C. (1996). Glycoprotein B of herpes simplex virus type 1 oligomerizes through the intermolecular interaction of a 28-amino-acid domain. *J Virol* **70**(3)**,** 1640-50.

Lee, G. T., Para, M. F., and Spear, P. G. (1982). Location of the structural genes for glycoproteins gD and gE and for other polypeptides in the S component of herpes simplex virus type 1 DNA. *J Virol* **43**(1)**,** 41-9.

Lee, J. Y., Irmiere, A., and Gibson, W. (1988). Primate cytomegalovirus assembly: evidence that DNA packaging occurs subsequent to B capsid assembly. *Virology* **167**(1)**,** 87-96.

Leopardi, R., Ward, P. L., Ogle, W. O., and Roizman, B. (1997). Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and viral DNA requires posttranslational modification by the U(L)13 proteinkinase. *J Virol* **71**(2)**,** 1133-9.

Ligas, M. W., and Johnson, D. C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. *Journal of Virology* **62**(5)**,** 1486-94.

Locker, H., Frenkel, N., and Halliburton, I. (1982). Structure and expression of class II defective herpes simplex virus genomes encoding infected cell polypeptide number 8. *J Virol* **43**(2)**,** 574-93.

Lockshon, D., and Galloway, D. A. (1986). Cloning and characterization of oriL2, a large palindromic DNA replication origin of herpes simplex virus type 2. *J Virol* **58**(2)**,** 513-21.

Long, D., Wilcox, W. C., Abrams, W. R., Cohen, G. H., and Eisenberg, R. J. (1992). Disulfide bond structure of glycoprotein D of herpes simplex virus types 1 and 2. *J Virol* **66**(11)**,** 6668-85.

Longnecker, R., Chatterjee, S., Whitley, R. J., and Roizman, B. (1987). Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. *Proceedings of the National Academy of Sciences of the United States of America* **84**(12)**,** 4303-7.

Loomis, J. S., Bowzard, J. B., Courtney, R. J., and Wills, J. W. (2001). Intracellular trafficking of the UL11 tegument protein of herpes simplex virus type 1. *J Virol* **75**(24)**,** 12209-19.

Loomis, J. S., Courtney, R. J., and Wills, J. W. (2003). Binding partners for the UL11 tegument protein of herpes simplex virus type 1. *J Virol* **77**(21)**,** 11417-24.

Lopez, C., Pellett, P., Stewart, J., Goldsmith, C., Sanderlin, K., Black, J., Warfield, D., and Feorino, P. (1988). Characteristics of human herpesvirus-6. *J Infect Dis* **157**(6)**,** 1271-3.

Lopez, M., Cocchi, F., Menotti, L., Avitabile, E., Dubreuil, P., and Campadelli-Fiume, G. (2000). Nectin2alpha (PRR2alpha or HveB) and nectin2delta are low-efficiency mediators for entry of herpes simplex virus mutants carrying the Leu25Pro substitution in glycoprotein D. *J Virol* **74**(3)**,** 1267-74.

Lubinski, J., Wang, L., Mastellos, D., Sahu, A., Lambris, J. D., and Friedman, H. M. (1999). In vivo role of complement-interacting domains of herpes simplex virus type 1 glycoprotein gC. *J Exp Med* **190**(11)**,** 1637-46.

Lubinski, J. M., Wang, L., Soulika, A. M., Burger, R., Wetsel, R. A., Colten, H., Cohen, G. H., Eisenberg, R. J., Lambris, J. D., and Friedman, H. M. (1998). Herpes simplex virus type 1 glycoprotein gC mediates immune evasion in vivo. *J Virol* **72**(10)**,** 8257-63.

Mach, M., Kropff, B., Dal Monte, P., and Britt, W. (2000). Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73). *J Virol* **74**(24)**,** 11881-92.

Machtiger, N. A., Pancake, B. A., Eberle, R., Courtney, R. J., Tevethia, S. S., and Schaffer, P. A. (1980). Herpes simplex virus glycoproteins: isolation of mutants resistant to immune cytolysis. *J. Virol.* **34,** 336-346.

MacLean, C. A., Clark, B., and McGeoch, D. J. (1989). Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristylated. *Journal of General Virology* **70**(Pt 12)**,** 3147-57.

MacLean, C. A., Dolan, A., Jamieson, F. E., and McGeoch, D. J. (1992). The myristylated virion proteins of herpes simplex virus type 1: investigation of their role in the virus life cycle. *Journal of General Virology* **73**(Pt 3)**,** 539-47.

MacLean, C. A., Efstathiou, S., Elliott, M. L., Jamieson, F. E., and McGeoch, D. J. (1991). Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. *Journal of General Virology* **72**(Pt 4)**,** 897-906.

MacLean, C. A., Robertson, L. M., and Jamieson, F. E. (1993). Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role in vivo. *J Gen Virol* **74 (Pt 6),** 975-83.

Manservigi, R., Spear, P. G., and Buchan, A. (1977). Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **74**(9)**,** 3913-7.

Markovitz, N. S., Filatov, F., and Roizman, B. (1999). The U(L)3 protein of herpes simplex virus 1 is translated predominantly from the second in-frame methionine codon and is subject to at least two posttranslational modifications. *J Virol* **73**(10)**,** 8010-8.

Maul, G. G., Ishov, A. M., and Everett, R. D. (1996). Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. *Virology* **217**(1)**,** 67-75.

Mauri, D. N., Ebner, R., Montgomery, R. I., Kochel, K. D., Cheung, T. C., Yu, G. L., Ruben, S., Murphy, M., Eisenberg, R. J., Cohen, G. H., Spear, P. G., and Ware, C. F. (1998). LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* **8**(1)**,** 21-30.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E., and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69,** 1531-1574.

McGeoch, D. J., Dolan, A., Donald, S., and Rixon, F. J. (1985). Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *J Mol Biol* **181**(1)**,** 1-13.

McLauchlan, J., and Rixon, F. J. (1992). Characterization of enveloped tegument structures (L particles) produced by alphaherpesviruses: integrity of the tegument does not depend on the presence of capsid or envelope. *Journal of General Virology* **73**(Pt 2)**,** 269-76.

McNabb, D. S., and Courtney, R. J. (1992). Characterization of the large tegument protein (ICP1/2) of herpes simplex virus type 1. *Virology* **190**(1)**,** 221-32.

Melancon, J. M., Luna, R. E., Foster, T. P., and Kousoulas, K. G. (2005). Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment. *J Virol* **79**(1)**,** 299-313.

Mettenleiter, T. C. (2000). Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis--state of the art, June 1999. *Vet Res* **31**(1)**,** 99-115.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J Virol* **76**(4)**,** 1537-47.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* **106**(2)**,** 167-80.

Mettenleiter, T. C. (2006). Intriguing interplay between viral proteins during herpesvirus assembly or: the herpesvirus assembly puzzle. *Vet Microbiol* **113**(3-4)**,** 163-9.

Mettenleiter, T. C., Schreurs, C., Zuckermann, F., and Ben-Porat, T. (1987a). Role of pseudorabies virus glycoprotein gI in virus release from infected cells. *J Virol* **61**(9)**,** 2764-9.

Mettenleiter, T. C., Zsak, L., Kaplan, A. S., Ben-Porat, T., and Lomniczi, B. (1987b). Role of a structural glycoprotein of pseudorabies in virus virulence. *J Virol* **61**(12)**,** 4030-2.

Milne, R. S., Connolly, S. A., Krummenacher, C., Eisenberg, R. J., and Cohen, G. H. (2001). Porcine HveC, a member of the highly conserved HveC/nectin 1 family, is a functional alphaherpesvirus receptor. *Virology* **281**(2)**,** 315-28.

Minson, A. C., Hodgman, T. C., Digard, P., Hancock, D. C., Bell, S. E., and Buckmaster, E. A. (1986). An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization. *J Gen Virol* **67 (Pt 6),** 1001-13.

Mo, C., and Holland, T. C. (1997). Determination of the transmembrane topology of herpes simplex virus type 1 glycoprotein K. *Journal of Biological Chemistry* **272**(52)**,** 33305-11.

Mocarski, E. S., and Roizman, B. (1982). Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. *Cell* **31**(1)**,** 89-97.

Montgomerie, J. Z., Becroft, D. M., Croxson, M. C., Doak, P. B., and North, J. D. (1969). Herpes-simplex-virus infection after renal transplantation. *Lancet* **2**(7626)**,** 867-71.

Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87,** 427-36.

Morgan, C., Rose, H. M., Holden, M., and Jones, E. P. (1959). Electron microscopic observations on the development of herpes simplex virus. *J Exp Med* **110,** 643-56.

Morgan, C., Rose, H. M., and Mednis, B. (1968). Electron microscopy of herpes simplex virus. I. Entry. *J Virol* **2**(5)**,** 507-16.

Mori, Y., Yang, X., Akkapaiboon, P., Okuno, T., and Yamanishi, K. (2003). Human herpesvirus 6 variant A glycoprotein H-glycoprotein L-glycoprotein Q complex associates with human CD46. *J Virol* **77**(8)**,** 4992-9.

Mossman, K. L., Sherburne, R., Lavery, C., Duncan, J., and Smiley, J. R. (2000). Evidence that herpes simplex virus VP16 is required for viral egress downstream of the initial envelopment event. *J Virol* **74**(14)**,** 6287-99.

Mulder, W. A., Jacobs, L., Priem, J., Kok, G. L., Wagenaar, F., Kimman, T. G., and Pol, J. M. (1994). Glycoprotein gE-negative pseudorabies virus has a reduced capability to infect second- and third-order neurons of the olfactory and trigeminal routes in the porcine central nervous system. *J Gen Virol* **75 (Pt 11),** 3095-106.

Muller, M. T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. *J Virol* **61**(3)**,** 858-65.

Nahmias, A. J., Josey, W. E., Naib, Z. M., Luce, C. F., and Guest, B. A. (1970). Antibodies to Herpesvirus hominis types 1 and 2 in humans. II. Women with cervical cancer. *Am J Epidemiol* **91**(6)**,** 547-52.

Nahmias, A. J., Keyserling, H., and Kerrick, G. M. (1983). Herpes simplex. *In* "Infectious diseases of the fetus and newborn infant" (J. S. Remington, and J. O. Klein, Eds.), pp. 636-678. WB Saunders, Philadelphia.

Nahmias, A. J., Keyserling, H., and Lee, F. K. (1989). Herpes simplex viruses 1 and 2. 3rd ed. *In* "Viral infections of humans : epidemiology and control" (A. S. Evans, Ed.), pp. 393-417. Plenum Medical Book Co., New York.

Nahmias, A. J., Lee, F. K., and Beckman-Nahmias, S. (1990). Sero-epidemiological and - sociological patterns of herpes simplex virus infection in the world. *Scand J Infect Dis Suppl* **69,** 19-36.

Nalwanga, D., Rempel, S., Roizman, B., and Baines, J. D. (1996). The UL 16 gene product of herpes simplex virus 1 is a virion protein that colocalizes with intranuclear capsid proteins. *Virology* **226**(2)**,** 236-42.

Nazerian, K. (1974). DNA configuration in the core of Marek's disease virus. *J Virol* **13**(5)**,** 1148-50.

Neidhardt, H., Schroder, C. H., and Kaerner, H. C. (1987). Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infectivity. *Journal of Virology* **61**(2)**,** 600-3.

Newcomb, W. W., Homa, F. L., Thomsen, D. R., Booy, F. P., Trus, B. L., Steven, A. C., Spencer, J. V., and Brown, J. C. (1996). Assembly of the herpes simplex virus capsid: characterization of intermediates observed during cell-free capsid formation. *J Mol Biol* **263**(3)**,** 432-46.

Newcomb, W. W., Trus, B. L., Booy, F. P., Steven, A. C., Wall, J. S., and Brown, J. C. (1993). Structure of the herpes simplex virus capsid. Molecular composition of the pentons and the triplexes. *J Mol Biol* **232**(2)**,** 499-511.

Nicholson, P., Addison, C., Cross, A. M., Kennard, J., Preston, V. G., and Rixon, F. J. (1994). Localization of the herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires the abundant scaffolding protein VP22a. *J Gen Virol* **75 (Pt 5),** 1091-9.

Nicola, A. V., McEvoy, A. M., and Straus, S. E. (2003). Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. *J Virol* **77**(9)**,** 5324-32.

Nicola, A. V., and Straus, S. E. (2004). Cellular and viral requirements for rapid endocytic entry of herpes simplex virus. *J Virol* **78**(14)**,** 7508-17.

Nishioka, Y., and Silverstein, S. (1977). Degradation of cellular mRNA during infection by herpes simplex virus. *Proc Natl Acad Sci U S A* **74**(6)**,** 2370-4.

Nishioka, Y., and Silverstein, S. (1978a). Alterations in the protein synthetic apparatus of Friend erythroleukemia cells infected with vesicular stomatitis virus or herpes simplex virus. *J Virol* **25**(1)**,** 422-6.

Nishioka, Y., and Silverstein, S. (1978b). Requirement of protein synthesis for the degradation of host mRNA in Friend erythroleukemia cells infected wtih herpes simplex virus type 1. *J Virol* **27**(3)**,** 619-27.

Novotny, M. J., Parish, M. L., and Spear, P. G. (1996). Variability of herpes simplex virus 1 gL and anti-gL antibodies that inhibit cell fusion but not viral infectivity. *Virology* **221**(1)**,** 1-13.

Ojala, P. M., Sodeik, B., Ebersold, M. W., Kutay, U., and Helenius, A. (2000). Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. *Mol Cell Biol* **20**(13)**,** 4922-31.

Olson, J. K., and Grose, C. (1998). Complex formation facilitates endocytosis of the varicella-zoster virus gE:gI Fc receptor. *J Virol* **72**(2)**,** 1542-51.

Olson, L. C., Buescher, E. L., Artenstein, M. S., and Parkman, P. D. (1967). Herpesvirus infections of the human central nervous system. *N Engl J Med* **277**(24)**,** 1271-7.

Oroskar, A. A., and Read, G. S. (1987). A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. *J Virol* **61**(2)**,** 604-6.

Oshima, S., Daikoku, T., Shibata, S., Yamada, H., Goshima, F., and Nishiyama, Y. (1998). Characterization of the UL16 gene product of herpes simplex virus type 2. *Arch Virol* **143**(5)**,** 863-80.

Pellett, P. E., Kousoulas, K. G., Pereira, L., and Roizman, B. (1985). Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. *Journal of Virology* **53**(1)**,** 243-53.

Perdue, M. L., Cohen, J. C., Randall, C. C., and O'Callaghan, D. J. (1976). Biochemical studies of the maturation of herpesvirus nucleocapsid species. *Virology* **74**(1)**,** UNKNOWN.

Perdue, M. L., Kemp, M. C., Randall, C. C., and O'Callaghan, D. J. (1974). Studies of the molecular anatomy of the L-M cell strain of equine herpes virus type 1: proteins of the nucleocapsid and intact virion. *Virology* **59**(1)**,** 201-16.

Pertel, P. E. (2002). Human herpesvirus 8 glycoprotein B (gB), gH, and gL can mediate cell fusion. *J Virol* **76**(9)**,** 4390-400.

Pertel, P. E., and Spear, P. G. (1996). Modified entry and syncytium formation by herpes simpelx virus type 1 mutants selected for resistance to heparin inhibition. *Virology* **226,** 22-33.

Pogue-Geile, K. L., Lee, G. T., Shapira, S. K., and Spear, P. G. (1984). Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. *Virology* **136**(1)**,** 100-9.

Polcicova, K., Goldsmith, K., Rainish, B. L., Wisner, T. W., and Johnson, D. C. (2005). The extracellular domain of herpes simplex virus gE is indispensable for efficient cell-to-cell spread: evidence for gE/gI receptors. *J Virol* **79**(18)**,** 11990-2001.

Polvino-Bodnar, M., Orberg, P. K., and Schaffer, P. A. (1987). Herpes simplex virus type 1 oriL is not required for virus replication or for the establishment and reactivation of latent infection in mice. *J Virol* **61**(11)**,** 3528-35.

Post, L. E., Mackem, S., and Roizman, B. (1981). Regulation of alpha genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. *Cell* **24**(2)**,** 555-65.

Purifoy, D. J., Lewis, R. B., and Powell, K. L. (1977). Identification of the herpes simplex virus DNA polymerase gene. *Nature* **269**(5629)**,** 621-3.

Purves, F. C., Longnecker, R. M., Leader, D. P., and Roizman, B. (1987). Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture. *J Virol* **61**(9)**,** 2896-901.

Quinlan, M. P., Chen, L. B., and Knipe, D. M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell* **36**(4)**,** 857-68.

Rafield, L. F., and Knipe, D. M. (1984). Characterization of the major mRNAs transcribed from the genes for glycoprotein B and DNA-binding protein ICP8 of herpes simplex virus type 1. *Journal of Virology* **49**(3)**,** 960-9.

Ramaswamy, R., and Holland, T. C. (1992). In vitro characterization of the HSV-1 UL53 gene product. *Virology* **186**(2)**,** 579-87.

Read, G. S., and Frenkel, N. (1983). Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of alpha (immediate early) viral polypeptides. *J Virol* **46**(2)**,** 498-512.

Read, G. S., Karr, B. M., and Knight, K. (1993). Isolation of a herpes simplex virus type 1 mutant with a deletion in the virion host shutoff gene and identification of multiple forms of the vhs (UL41) polypeptide. *J Virol* **67**(12)**,** 7149-60.

Reeves, W. C., Corey, L., Adams, H. G., Vontver, L. A., and Holmes, K. K. (1981). Risk of recurrence after first episodes of genital herpes. Relation to HSV type and antibody response. *N Engl J Med* **305**(6)**,** 315-9.

Reynolds, A. E., Liang, L., and Baines, J. D. (2004). Conformational changes in the nuclear lamina induced by herpes simplex virus type 1 require genes U(L)31 and U(L)34. *J Virol* **78**(11)**,** 5564-75.

Reynolds, A. E., Ryckman, B. J., Baines, J. D., Zhou, Y., Liang, L., and Roller, R. J. (2001). U(L)31 and U(L)34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids. *J Virol* **75**(18)**,** 8803-17.

Reynolds, A. E., Wills, E. G., Roller, R. J., Ryckman, B. J., and Baines, J. D. (2002). Ultrastructural localization of the herpes simplex virus type 1 UL31, UL34, and US3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids. *J Virol* **76**(17)**,** 8939-52.

Rice, S. A., Long, M. C., Lam, V., and Spencer, C. A. (1994). RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection. *J Virol* **68**(2)**,** 988-1001.

Rixon, F. J., Addison, C., McGregor, A., Macnab, S. J., Nicholson, P., Preston, V. G., and Tatman, J. D. (1996). Multiple interactions control the intracellular localization of the herpes simplex virus type 1 capsid proteins. *J Gen Virol* **77 (Pt 9),** 2251-60.

Rixon, F. J., Addison, C., and McLauchlan, J. (1992). Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus type 1-infected cells. *Journal of General Virology* **73**(Pt 2)**,** 277-84.

Roberts, S. R., Ponce de Leon, M., Cohen, G. H., and Eisenberg, R. J. (1991). Analysis of the intracellular maturation of the herpes simplex virus type 1 glycoprotein gH in infected and transfected cells. *Virology* **184**(2)**,** 609-24.

Roizman, B., Bartha, A., and Biggs, P. M. (1973). Editorial: Provisional labels for herpesviruses. *J Gen Virol* **20**(3)**,** 417-9.

Roizman, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C., and Studdert, M. J. (1992). The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Arch Virol* **123**(3-4)**,** 425-49.

Roizman, B., and Furlong, D. (1974). The replication of herpesviruses. *In* "Comprehensive virology" (H. Fraenkel-Conrat, and R. R. Wagner, Eds.), Vol. 3, pp. 229-403. Plenum Press, New York,.

Roizman, B., and Knipe, D. M. (2001). Herpes Simplex Viruses and Their Replication. Third edition ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2399-2459. 2 vols. Lippincott-Williams & Wilkins, Philadelphia, PA.

Roizman, B., and Pellett, P. (2001). The Family *Herpesviridae*: A Brief Introduction. Third edition ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2381-2397. 2 vols. Lippincott-Williams & Wilkins, Philadelphia, PA.

Roizman, B., and Sears, A. E. (2001). Herpes Simplex Viruses and Their Replication. Third edition ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2399-2459. 2 vols. Lippincott-Williams & Wilkins, Philadelphia, PA.

Roller, R. J., Zhou, Y., Schnetzer, R., Ferguson, J., and DeSalvo, D. (2000). Herpes simplex virus type 1 U(L)34 gene product is required for viral envelopment. *J Virol* **74**(1)**,** 117-29.

Roop, C., Hutchinson, L., and Johnson, D. C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. *Journal of Virology* **67**(4)**,** 2285-97.

Rowe, W. P., Hartley, J. W., Waterman, S., Turner, H. C., and Huebner, R. J. (1956). Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids. *Proc Soc Exp Biol Med* **92**(2)**,** 418-24.

Ruyechan, W. T., Morse, L. S., Knipe, D. M., and Roizman, B. (1979). Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. *J. Virol.* **29,** 677-697.

Sahu, A., Isaacs, S. N., Soulika, A. M., and Lambris, J. D. (1998). Interaction of vaccinia virus complement control protein with human complement proteins: factor I-mediated degradation of C3b to iC3b1 inactivates the alternative complement pathway. *J Immunol* **160**(11)**,** 5596-604.

Salahuddin, S. Z., Ablashi, D. V., Markham, P. D., Josephs, S. F., Sturzenegger, S., Kaplan, M., Halligan, G., Biberfeld, P., Wong-Staal, F., Kramarsky, B., and et al. (1986). Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* **234**(4776)**,** 596-601.

Sanders, P. G., Wilkie, N. M., and Davison, A. J. (1982). Thymidine kinase deletion mutants of herpes simplex virus type 1. *Journal of General Virology* **63**(2)**,** 277-95.

Sarmiento, M., Haffey, M., and Spear, P. G. (1979). Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B2) in virion infectivity. *J Virol* **29**(3)**,** 1149-58.

Scott, E. S., and O'Hare, P. (2001). Fate of the inner nuclear membrane protein lamin B receptor and nuclear lamins in herpes simplex virus type 1 infection. *J Virol* **75**(18)**,** 8818-30.

Segal, A. L., Katcher, A. H., Brightman, V. J., and Miller, M. F. (1974). Recurrent herpes labialis, recurrent aphthous ulcers, and the menstrual cycle. *J Dent Res* **53**(4)**,** 797-803.

Shieh, M. T., WuDunn, D., Montgomery, R. I., Esko, J. D., and Spear, P. G. (1992). Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *Journal of Cell Biology* **116**(5)**,** 1273-81.

Ship, II, Miller, M. F., and Ram, C. (1977). A retrospective study of recurrent herpes labialis (RHL) in a professional population, 1958-1971. *Oral Surg Oral Med Oral Pathol* **44**(5)**,** 723-30.

Shukla, D., Dal Canto, M. C., Rowe, C. L., and Spear, P. G. (2000). Striking similarity of murine nectin-1alpha to human nectin-1alpha (HveC) in sequence and activity as a glycoprotein D receptor for alphaherpesvirus entry. *J Virol* **74**(24)**,** 11773-81.

Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999). A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* **99**(1)**,** 13-22.

Sinclair, M. C., McLauchlan, J., Marsden, H., and Brown, S. M. (1994). Characterization of a herpes simplex virus type 1 deletion variant (1703) which under-produces Vmw63 during immediate early conditions of infection. *J Gen Virol* **75 (Pt 5),** 1083-9.

Skepper, J. N., Whiteley, A., Browne, H., and Minson, A. (2001). Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment --> deenvelopment --> reenvelopment pathway. *J Virol* **75**(12)**,** 5697-702.

Smibert, C. A., Popova, B., Xiao, P., Capone, J. P., and Smiley, J. R. (1994). Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. *J Virol* **68**(4)**,** 2339-46.

Smiley, J. R., Duncan, J., and Howes, M. (1990). Sequence requirements for DNA rearrangements induced by the terminal repeat of herpes simplex virus type 1 KOS DNA. *J Virol* **64**(10)**,** 5036-50.

Smith, M. G. (1956). Propagation in tissue cultures of a cytopathogenic virus from human salivary gland virus (SGV) disease. *Proc Soc Exp Biol Med* **92**(2)**,** 424-30.

Sodeik, B., Ebersold, M. W., and Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**(5)**,** 1007-21.

Spear, P. G. (1993). Membrane fusion induced by herpes simplex virus. *In* "Viral fusion mechanisms" (J. Bentz, Ed.), pp. 201-232. CRC Press., Boca Raton, Fla.

Spear, P. G., Eisenberg, R. J., and Cohen, G. H. (2000). Three classes of cell surface receptors for alphaherpesvirus entry. *Virology* **275**(1)**,** 1-8.

Spear, P. G., and Longnecker, R. (2003). Herpesvirus entry: an update. *J Virol* **77**(19)**,** 10179-85.

Spencer, J. V., Newcomb, W. W., Thomsen, D. R., Homa, F. L., and Brown, J. C. (1998). Assembly of the herpes simplex virus capsid: preformed triplexes bind to the nascent capsid. *J Virol* **72**(5)**,** 3944-51.

Spengler, M., Niesen, N., Grose, C., Ruyechan, W. T., and Hay, J. (2001). Interactions among structural proteins of varicella zoster virus. *Arch Virol Suppl*(17)**,** 71-9.

Spruance, S. L., and Crumpacker, C. S. (1982). Topical 5 percent acyclovir in polyethylene glycol for herpes simplex labialis. Antiviral effect without clinical benefit. *Am J Med* **73**(1A)**,** 315-9.

Spruance, S. L., Crumpacker, C. S., Schnipper, L. E., Kern, E. R., Marlowe, S., Arndt, K. A., and Overall, J. C., Jr. (1984). Early, patient-initiated treatment of herpes labialis with topical 10% acyclovir. *Antimicrob Agents Chemother* **25**(5)**,** 553-5.

Spruance, S. L., Overall, J. C., Jr., Kern, E. R., Krueger, G. G., Pliam, V., and Miller, W. (1977). The natural history of recurrent herpes simplex labialis: implications for antiviral therapy. *N Engl J Med* **297**(2)**,** 69-75.

Steven, A. C., and Spear, P. G. (1997). *In* "Structural Biology of Viruses" (W. Chiu, R. M. Burnett, and R. L. Garcea, Eds.), pp. 312-351. Oxford University Press, New York.

Stow, N. D. (1982). Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. *Embo J* **1**(7)**,** 863-7.

Stow, N. D., and Stow, E. C. (1986). Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *J Gen Virol* **67 (Pt 12),** 2571-85.

Subramanian, R. P., and Geraghty, R. J. (2007). Herpes simplex virus type 1 mediates fusion through a hemifusion intermediate by sequential activity of glycoproteins D, H, L, and B. *Proc Natl Acad Sci U S A* **104**(8)**,** 2903-8.

Sullivan, V., and Smith, G. L. (1987). Expression and characterization of herpes simplex virus type 1 (HSV-1) glycoprotein G (gG) by recombinant vaccinia virus: neutralization of HSV-1 infectivity with anti-gG antibody. *J Gen Virol* **68 (Pt 10),** 2587-98.

Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A., and Takai, Y. (1999). Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein. *J Cell Biol* **145**(3)**,** 539-49.

Takai, Y., and Nakanishi, H. (2003). Nectin and afadin: novel organizers of intercellular junctions. *J Cell Sci* **116**(Pt 1)**,** 17-27.

Tal-Singer, R., Peng, C., Ponce, D. L. M., Abrams, W. R., Banfield, B. W., Tufaro, F., Cohen, G. H., and Eisenberg, R. J. (1995). Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. *Journal of Virology* **69**(7)**,** 4471-83.

Thomsen, D. R., Newcomb, W. W., Brown, J. C., and Homa, F. L. (1995). Assembly of the herpes simplex virus capsid: requirement for the carboxyl-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes. *J Virol* **69**(6)**,** 3690-703.

Tirabassi, R. S., and Enquist, L. W. (1998). Role of envelope protein gE endocytosis in the pseudorabies virus life cycle. *J Virol* **72**(6)**,** 4571-9.

Tirabassi, R. S., Townley, R. A., Eldridge, M. G., and Enquist, L. W. (1997). Characterization of pseudorabies virus mutants expressing carboxy-terminal truncations of gE: evidence for envelope incorporation, virulence, and neurotropism domains. *J Virol* **71**(9)**,** 6455-64.

Tognon, M., Manservigi, R., Cavrini, V., and Campadelli-Fiume, G. (1984). Characterization of a herpes simplex virus type 1 mutant resistant to benzhydrazone, a selective inhibitor of herpesvirus glycosylation. *Proc Natl Acad Sci U S A* **81**(8)**,** 2440-3.

Tran, L. C., Kissner, J. M., Westerman, L. E., and Sears, A. E. (2000). A herpes simplex virus 1 recombinant lacking the glycoprotein G coding sequences is defective in entry through apical surfaces of polarized epithelial cells in culture and in vivo. *Proc Natl Acad Sci U S A* **97**(4)**,** 1818-22.

Trus, B. L., Booy, F. P., Newcomb, W. W., Brown, J. C., Homa, F. L., Thomsen, D. R., and Steven, A. C. (1996). The herpes simplex virus procapsid: structure, conformational changes upon maturation, and roles of the triplex proteins VP19c and VP23 in assembly. *J Mol Biol* **263**(3)**,** 447-62.

Uprichard, S. L., and Knipe, D. M. (1996). Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. *J Virol* **70**(3)**,** 1969-80.

van Genderen, I. L., Brandimarti, R., Torrisi, M. R., Campadelli, G., and van Meer, G. (1994). The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. *Virology* **200**(2)**,** 831-6.

Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., and Pringle, C. R. (2000). "Virus taxonomy : classification and nomenclature of viruses : seventh report of the International Committee on Taxonomy of Viruses." Academic Press, San Diego.

Varmuza, S. L., and Smiley, J. R. (1985). Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. *Cell* **41**(3)**,** 793-802.

Vidal, J. B. (1873). *Ann Dermatol Syphiligr* **1,** 350.

Vittone, V., Diefenbach, E., Triffett, D., Douglas, M. W., Cunningham, A. L., and Diefenbach, R. J. (2005). Determination of interactions between tegument proteins of herpes simplex virus type 1. *J Virol* **79**(15)**,** 9566-71.

Vlazny, D. A., Kwong, A., and Frenkel, N. (1982). Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. *Proc Natl Acad Sci U S A* **79**(5)**,** 1423-7.

Wagner, E. K. (1985). Individual HSV transcripts: Characterisation of specific genes. *In* "The Herpesviruses" (B. Roizman, Ed.), pp. 45-104. Plenum Press, New York.

Ward, P. L., Avitabile, E., Campadelli-Fiume, G., and Roizman, B. (1998). Conservation of the architecture of the Golgi apparatus related to a differential organization of microtubules in polykaryocytes induced by syn- mutants of herpes simplex virus 1. *Virology* **241**(2)**,** 189-99.

Ward, P. L., Barker, D. E., and Roizman, B. (1996). A novel herpes simplex virus 1 gene, UL43.5, maps antisense to the UL43 gene and encodes a protein which colocalizes in nuclear structures with capsid proteins. *J Virol* **70**(5)**,** 2684-90.

Ward, P. L., Campadelli-Fiume, G., Avitabile, E., and Roizman, B. (1994). Localization and putative function of the UL20 membrane protein in cells infected with herpes simplex virus 1. *Journal of Virology* **68**(11)**,** 7406-17.

Ward, P. L., Ogle, W. O., and Roizman, B. (1996). Assemblons: nuclear structures defined by aggregation of immature capsids and some tegument proteins of herpes simplex virus 1. *J Virol* **70**(7)**,** 4623-31.

Warner, M. S., Geraghty, R. J., Martinez, W. M., Montgomery, R. I., Whitbeck, J. C., Xu, R., Eisenberg, R. J., Cohen, G. H., and Spear, P. G. (1998). A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. *Virology* **246**(1)**,** 179-89.

Watson, R. J., Weis, J. H., Salstrom, J. S., and Enquist, L. W. (1982). Herpes simplex virus type-1 glycoprotein D gene: nucleotide sequence and expression in Escherichia coli. *Science* **218**(4570)**,** 381-4.

Weller, S. K., Spadaro, A., Schaffer, J. E., Murray, A. W., Maxam, A. M., and Schaffer, P. A. (1985). Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. *Mol Cell Biol* **5**(5)**,** 930-42.

Weller, T. H., and Stoddard, M. B. (1952). Intranuclear inclusion bodies in cultures of human tissue inoculated with varicella vesicle fluid. *J Immunol* **68**(3)**,** 311-9.

Whealy, M. E., Card, J. P., Robbins, A. K., Dubin, J. R., Rziha, H. J., and Enquist, L. W. (1993). Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. *J Virol* **67**(7)**,** 3786-97.

Whitley, R., Arvin, A., Prober, C., Corey, L., Burchett, S., Plotkin, S., Starr, S., Jacobs, R., Powell, D., Nahmias, A., and et al. (1991). Predictors of morbidity and mortality in neonates with herpes simplex virus infections. The National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group. *N Engl J Med* **324**(7)**,** 450-4.

Whitley, R. J. (2001). Herpes Simplex Viruses. Third edition ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2461-2509. 2 vols. Lippincott-Williams & Wilkins, Philadelphia, PA.

Whitley, R. J., Levin, M., Barton, N., Hershey, B. J., Davis, G., Keeney, R. E., Whelchel, J., Diethelm, A. G., Kartus, P., and Soong, S. J. (1984). Infections caused by herpes simplex virus in the immunocompromised host: natural history and topical acyclovir therapy. *J Infect Dis* **150**(3)**,** 323-9.

Whitley, R. J., Soong, S. J., Dolin, R., Galasso, G. J., Ch'ien, L. T., and Alford, C. A. (1977). Adenine arabinoside therapy of biopsy-proved herpes simplex encephalitis. National Institute of Allergy and Infectious Diseases collaborative antiviral study. *N Engl J Med* **297**(6)**,** 289-94.

Whitley, R. J., Soong, S. J., Hirsch, M. S., Karchmer, A. W., Dolin, R., Galasso, G., Dunnick, J. K., and Alford, C. A. (1981). Herpes simplex encephalitis: vidarabine therapy and diagnostic problems. *N Engl J Med* **304**(6)**,** 313-8.

Wildy, P. (1973). Herpes: history and classification. *In* "The herpesviruses" (A. S. Kaplan, Ed.), pp. 1-25. Academic Press, New York.

Wildy, P., and Watson, D. H. (1962). Electron microscopic studies on the architecture of animal viruses. *Cold Spring Harb Symp Quant Biol* **27,** 25-47.

Wing, B. A., Lee, G. C., and Huang, E. S. (1996). The human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. *J Virol* **70**(6)**,** 3339-45.

Wisner, T., Brunetti, C., Dingwell, K., and Johnson, D. C. (2000). The extracellular domain of herpes simplex virus gE is sufficient for accumulation at cell junctions but not for cell-to-cell spread. *J Virol* **74**(5)**,** 2278-87.

Wu, C. A., Nelson, N. J., McGeoch, D. J., and Challberg, M. D. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. *J Virol* **62**(2)**,** 435-43.

Wu, S. X., Zhu, X. P., and Letchworth, G. J. (1998). Bovine herpesvirus 1 glycoprotein M forms a disulfide-linked heterodimer with the U(L)49.5 protein. *J Virol* **72**(4)**,** 3029-36.

WuDunn, D., and Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *Journal of Virology* **63**(1)**,** 52-8.

Yoon, M., Zago, A., Shukla, D., and Spear, P. G. (2003). Mutations in the N termini of herpes simplex virus type 1 and 2 gDs alter functional interactions with the entry/fusion receptors HVEM, nectin-2, and 3-O-sulfated heparan sulfate but not with nectin-1. *J Virol* **77**(17)**,** 9221-31.

Zago, A., and Spear, P. G. (2003). Differences in the N termini of herpes simplex virus type 1 and 2 gDs that influence functional interactions with the human entry receptor Nectin-2 and an entry receptor expressed in Chinese hamster ovary cells. *J Virol* **77**(17)**,** 9695-9.

Zelus, B. D., Stewart, R. S., and Ross, J. (1996). The virion host shutoff protein of herpes simplex virus type 1: messenger ribonucleolytic activity in vitro. *J Virol* **70**(4)**,** 2411-9.

Zhou, Z. H., Chen, D. H., Jakana, J., Rixon, F. J., and Chiu, W. (1999). Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J Virol* **73**(4)**,** 3210-8.

Zhou, Z. H., He, J., Jakana, J., Tatman, J. D., Rixon, F. J., and Chiu, W. (1995). Assembly of VP26 in herpes simplex virus-1 inferred from structures of wild-type and recombinant capsids. *Nat Struct Biol* **2**(11)**,** 1026-30.

Zhu, Q., and Courtney, R. J. (1994). Chemical cross-linking of virion envelope and tegument proteins of herpes simplex virus type 1. *Virology* **204**(2)**,** 590-9.

Zhu, Z., Gershon, M. D., Hao, Y., Ambron, R. T., Gabel, C. A., and Gershon, A. A. (1995). Envelopment of varicella-zoster virus: targeting of viral glycoproteins to the trans-Golgi network. *J Virol* **69**(12)**,** 7951-9.

Zsak, L., Zuckermann, F., Sugg, N., and Ben-Porat, T. (1992). Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. *J Virol* **66**(4)**,** 2316-25.

Zuckermann, F. A., Mettenleiter, T. C., Schreurs, C., Sugg, N., and Ben-Porat, T. (1988). Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. *J Virol* **62**(12)**,** 4622-6.

CHAPTER 2
THE UL20 PROTEIN FUNCTIONS PRECEDE AND ARE REQUIRED FOR UL11 FUNCTIONS IN HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) CYTOPLASMIC VIRION ENVELOPMENT

Introduction

Herpes simplex virus type-1 (HSV-1) morphogenesis occurs in multiple stages within infected cells. Initially, the virion capsid assembles within the nucleus and the virion acquires an initial envelope by budding into the perinuclear spaces (Roizman and Sears, 2001). Subsequently, these enveloped virions fuse with the outer nuclear lamellae leading to the accumulation of unenveloped capsids into the cytoplasm. Within the cytoplasm, a number of additional tegument proteins attach to the capsid and the fully tegumented capsids bud into cytoplasmic vesicles, which mostly likely originate from the *trans*-Golgi network (TGN). Enveloped virions are ultimately secreted to extracellular spaces through the utilization of cellular vesicular trafficking systems (Browne et al., 1996; Granzow et al., 2001; Harley, Dasgupta, and Wilson, 2001; Mettenleiter, 2002; Skepper et al., 2001; Zhu et al., 1995). The process by which the tegumented cytoplasmic capsids bud into TGN-derived vesicles is not well-understood. The prevalent model calls for specific interactions among viral tegument proteins and membrane proteins and glycoproteins embedded within TGN membranes as key factors that drive cytoplasmic virion envelopment. This model is supported by evidence that specific mutations within tegument proteins and multiple membrane proteins and glycoproteins inhibit cytoplasmic envelopment (Mettenleiter, 2004; Mettenleiter, 2006). Apparently, multiple glycoproteins may be concurrently involved in cytoplasmic virion envelopment. Simultaneous absence of both gM and gE, or gM and the gE cytoplasmic tail result in inhibition of cytoplasmic envelopment for pseudorabies virus (PRV) (Brack et al., 1999; Brack et al., 2000); however, deletion of gM or gE does not appear to affect HSV-1 cytoplasmic envelopment. In contrast, deletion of both HSV-1 gD and gE cause accumulation of capsids into the cytoplasm of

infected cells presumably due to loss of contacts with tegument proteins (Mettenleiter, 2004; Mettenleiter, 2006). These results suggest that PRV and HSV-1 cytoplasmic envelopment may rely on different repertoires of protein-protein interactions to drive cytoplasmic virion envelopment.

Of particular interest to these investigations are the membrane proteins: UL11 (Baines and Roizman, 1992b; Kopp et al., 2004; Kopp et al., 2003), UL20 (Baines et al., 1991; Foster et al., 2004b; Fuchs et al., 1997; Melancon, Foster, and Kousoulas, 2004b), and UL53 (gK) (Foster and Kousoulas, 1999; Hutchinson and Johnson, 1995; Jayachandra, Baghian, and Kousoulas, 1997), which are known to be important determinants of cytoplasmic envelopment for both PRV and HSV-1. The UL11 gene encodes a 96 amino acid tegument protein, which is N-terminally myristylated (MacLean, Clark, and McGeoch, 1989) and palmitylated (Loomis et al., 2001). The UL11 protein localizes to nuclear and TGN-derived apparatus derived membranes in infected cells (Baines et al., 1995), but only to the TGN-membranes in non-infected cells (Bowzard et al., 2000). UL11 was shown to specifically interact with the UL16 tegument protein, providing a potential docking mechanism for tegumented capsids onto TGN-membranes(Loomis, Courtney, and Wills, 2003; Vittone et al., 2005). An HSV UL11-null mutant obtained by deletion of most of the UL11 coding region accumulated capsids into the nucleus and unenveloped capsids in the cytoplasm (Baines and Roizman, 1992b), while a PRV-null virus with the entire UL11 gene deleted showed accumulation of unenveloped capsids in the cytoplasm of infected cells embedded in tegument-like material (Kopp et al., 2003).

The UL20 and UL53 (gK) genes encode multipass transmembrane proteins of 222 and 338 amino acids, respectively, and are conserved in all alphaherpesviruses (Debroy, Pederson, and Person, 1985; MacLean et al., 1991; Ramaswamy and Holland, 1992). UL20p and gK localize to TGN membranes after endocytosis from cell surfaces (Foster et al., 2004b). UL20p and gK are essential for cytoplasmic virion morphogenesis, since mutant viruses lacking either gK or UL20p accumulate capsids

within the cytoplasm that are unable to acquire envelopes by budding into TGN-associated membranes (Dietz et al., 2000; Foster, Alvarez, and Kousoulas, 2003; Foster et al., 2004b; Fuchs et al., 1997; Melancon, Foster, and Kousoulas, 2004b). Furthermore, UL20p is essential for virus-induced cell fusion caused by either gB or gK syncytial mutations, and it is necessary for gK cell-surface expression. Recently, our laboratory has shown that gK and UL20 interact and that this interaction is essential for their co-transport and membrane fusion and virion morphogenesis functions (Foster et al., 2004d; Melancon et al., 2005).

The purpose of the present investigations was two-fold: 1) To revisit the role of UL11 in virion morphogenesis and egress by constructing a new recombinant UL11-null virus in the HSV-1(F) genetic background that could be directly compared to our previously constructed gK-null and UL20-null viruses. 2) To investigate whether UL11 and the gK/UL20 heterodimer functioned synergistically or independently of each other in the late stages of cytoplasmic virion morphogenesis. The bacterial artificial chromosome (BAC) cloned HSV-1 (F) viral genome was used to generated UL11-single null and UL11/UL20-double null viruses. Characterization of replication and ultrastructural characteristics of these recombinant viruses revealed that UL11 played an important role in cytoplasmic virion envelopment. Furthermore, although UL11 and UL20 were independently transported and localized at the TGN, UL20 functions preceded and were required for UL11 functions in cytoplasmic virion envelopment.

Materials and Methods

Cells, viruses, and plasmids

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-

BRL; Grand Island, N.Y.), supplemented with 10% fetal calf serum and antibiotics. The UL19- and UL20-complementing cell line G5 was a gift of P. Desai (Johns Hopkins Medical Center) (Desai et al., 1993). A plasmid encoding UL11-GFP was a gift of J Wills (Loomis, Courtney, and Wills, 2003). Construction of both a UL20-3xFLAG plasmid and a gK-V5 plasmid was described previously (Foster et al., 2004d). Cell line Fd20-1 constitutively expressing UL20 was constructed in this laboratory (Melancon, Foster, and Kousoulas, 2004b).

Construction of HSV-1 mutants with deletions of the UL11, and/or UL20 genes (pYEbac102, pYEbac102ΔUL11, pYEbac102ΔUL20, and pYEBac102ΔUL11ΔUL20)

Insertion-deletion mutagenesis of pYEbac102 DNA was accomplished in *Escherichia coli* with the λ *gam recE recT* (GET) recombination system (Narayanan et al., 1999; Orford et al., 2000) as described previously for mutagenesis of the KSHV genome (Luna et al., 2004). Electrocompetent YEBAC102 *Escherichia coli* DH10B cells were transformed with plasmid pGETrec, which contains the genes encoding *recE*, *recT*, and bacteriophage λ *gam*, grown on plates containing chloramphenicol (12.5 μg/ml) and ampicillin (100 μg/ml). Individual colonies were picked and grown overnight in Luria-Bertani (LB) medium containing chloramphenicol and ampicillin. The next day, the culture was inoculated into 250 ml of LB containing chloramphenicol and ampicillin until an optical density at 600 nm of 0.4 was reached. Addition of L-arabinose to a final concentration of 0.2% (wt/vol) and further incubation for 40 min induced expression of the *recE*, *recT* and λ *gam* genes from plasmid pGETrec. The cells were then harvested and made electrocompetent.

For the ΔUL11-Kan mutation, a PCR fragment containing a kanamycin resistance (Kan) gene cassette flanked by ~50 bp of viral sequences on both sides was used for recombination to construct pYEbacΔUL11, containing the Kan gene cassette within the targeted UL11 genomic region.

Specifically, the Kan gene cassette was inserted 94 nucleotides downstream of the UL11 ATG codon, to avoid interruption of the UL12 open reading frame (ORF) that overlaps with the 5' terminus of the UL11 gene by 87 nucleotides. The remaining coding sequence of the UL11 gene was deleted up to three nucleotides past the TAA termination codon. The construction of the ΔUL20-GFP-Zeo mutation was described previously (Melancon et al., 2005). For the ΔUL11ΔUL20 mutation, a PCR fragment containing either a kanamycin resistance (Kan) gene cassette or a GFP-Zeocin resistance cassette flanked by ~50 bp of viral sequences on both sides was used for recombination to construct pYEbac102ΔUL11ΔUL20. Briefly, 40μl of electrocompetent DH10B cells harboring both pYEbac102 and pGETrec were electroporated with 200 ng of each PCR product to delete the target gene(s) (UL11 or UL20) with standard electroporation parameters (1.8 kV/cm, 200 Ω, and 25 μF). Following electroporation, cells were grown in 1 ml of LB for 60 min and subsequently streaked onto LB agar plates containing chloramphenicol (12.5 μg/ml) as well as either kanamycin (50 μg/ml) or zeocin (25 μg/ml). Mutant pYEbac102 DNA containing a deletion in the UL11 or UL20 gene was isolated from bacterial colonies, and a second round of electroporation was performed to remove plasmid pGETrec. Following electroporation, cells were grown on agar plates containing chloramphenicol as well as either kanamycin or zeocin.

Confirmation of the targeted mutations in pYEbac102 DNA

HSV-1 BAC DNAs (pYEbac102, pYEbac102ΔUL11, and pYEbac102ΔUL11ΔUL20 were purified from 500 ml of BAC cultures with the Qiagen large-construct kit (Qiagen; Valencia, Calif.). PCR primers were designed that lie outside both UL11 and UL20. A PCR test was performed to verify that the desired mutations were present. All mutations were sequenced to verify the presence of the desired mutations in the BACs. To verify that no spurious mutations were introduced into the viral

genome of the individual BAC mutants during mutagenesis or during the transfection procedure, each null mutation was complimented by expression vectors containing the appropriate gene or genes. Each complemented virus was verified to have the plaque phenotype characteristics of the wild-type BAC YEbac102.

Transfection of HSV-1 BAC DNAs

Transient transfection of cells with BAC DNAs was performed with Lipofectamine 2000 (Invitrogen). VERO cells (pYEbacΔUL11) and Fd20-1 cells (pYEbacΔUL11ΔUL20) were grown to 95% confluency in six-well plates. Cells were transfected with BAC DNA mixed with Lipofectamine 2000 in Opti-MEM medium as recommended by the manufacturer (Invitrogen). After 6 h of incubation at 37°C, the medium was removed from the transfected cells, cells were washed with phosphate buffered saline, and subsequent fresh Dulbecco's modified Eagle's medium with 10% fetal calf serum was added. At 72 h post transfection, virus stocks were collected.

One-step growth kinetics of YEbac102 mutants

Analysis of one-step growth kinetics was as described previously (Foster, Alvarez, and Kousoulas, 2003; Foster, Rybachuk, and Kousoulas, 2001). Briefly, each virus at an MOI of 2 was adsorbed to approximately 6 x 10^5 Vero cells at 4°C for 1 h. Thereafter, warm medium was added, and virus was allowed to penetrate for 2 h at 37°C. Any remaining extra-cellular virus was inactivated by low-pH treatment (0.1Mglycine, pH 3.0). Cells and supernatants were harvested immediately thereafter (0 h) or after 4, 8, 12, 18, 24, or 36 h of incubation at 37°C. Virus titers were determined by endpoint titration of virus stocks on Vero cells for pYEbacΔUL11 or G5 cells for pYEbac102ΔUL11ΔUL20.

Electron microscopy

Cell monolayers were infected with the indicated virus at an MOI of 3. All cells were prepared for transmission electron microscopy (TEM) examination 24 h post infection. Infected cells were fixed in a mixture of 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Following treatment with 1% OsO_4 and dehydration in an ethanol series, the samples were embedded in Epon-Araldyte resin and polymerized at 70°C. Thin sections were made on an MTXL ultratome (RMC Products), stained with 5% uranyl acetate and citrate/nitrate/acetate lead, and observed with a Zeiss 10 transmission electron microscope as described previously (Foster et al., 2004b; Melancon, Foster, and Kousoulas, 2004b).

Confocal microscopy

To determine intracellular localization of UL20 and gK in the presence or absence of UL11, Vero cells were grown on cover slips in 6 well plates to 95% confluency and then transfected with UL11, UL11 and UL20, UL11 and gK, or UL11, UL20 and gK using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. After 6 h of incubation at 37°C, the medium was removed from the transfected cells, cells were washed with phosphate buffered saline, and subsequent fresh Dulbecco's modified Eagle's medium with 10% fetal calf serum was added. At 48h post-transfection cells were washed with TBS and fixed with electron microscopy grade 3% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 15 minutes, washed twice with PBS-50 mM glycine, and permeabilized with 1.0% Triton X-100. Monolayers were subsequently blocked for 1 h with 7% normal goat serum and 7% BSA in TBS (TBS-blocking buffer) before incubation for 2h with either anti-GFP (Immunology Consultants Laboratory, Newberg, OR) for detection of UL11, anti-FLAG (Sigma Chemical) for detection of UL20, anti-V5 (Invitrogen, Carlsbad, CA) for detection of gK, anti-TGN 46

(Serotec, Raleigh, NC) for detection of the Trans-Golgi network, or a combination of these antibodies as indicated. Alternatively, to determine the intracellular localization of UL11 and UL20 during an active virus infection, 143 TK^- cells were prepared as described above with the exception that 24 h post transfection the cells were infected with YEbac102ΔUL11ΔUL20 at an MOI of 2. At 48 h post-infection, the cells were prepared as described above.

To visualize specific fluorescence, secondary antibodies were added to the cells. Briefly, cells were washed thoroughly and Alexafluor 488, Alexafluor 594, and Alexafluor 647 at a 1:750 dilution were added and incubated for 1 h. Cells were washed again and mounted on slides. The fluorescence was then visualized using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Exton, PA) fitted with a CS APO 63x Leica objective (1.4 numerical aperture). Individual optical sections in the *z*-axis, averaged 6 times, were collected at the indicated zoom in series in the different channels at 1024 x 1024 pixel resolution as described previously (Foster et al., 2004b; Foster et al., 2004d; Foster, Rybachuk, and Kousoulas, 2001; Fuchs et al., 2002a). Images were compiled and rendered in Adobe Photoshop.

Results

Construction of the HSV-1 BACs pYEbac102ΔUL11 and pYEbac102ΔUL11ΔUL20

The complete HSV-1(F) genome has been cloned into a bacterial artificial chromosome (BAC; pYEbac102) enabling the genetic manipulation of the HSV-1 genome in *E. coli*. (Tanaka et al., 2003). Previously, we utilized the BAC-based GET homologous recombination system to construct deletions within the gB, UL20 and gK genes in *E. coli* (Melancon et al., 2005). A similar strategy was utilized to construct a HSV-1 BAC with most of the UL11 ORF deleted. Deletion of the UL11 gene was accomplished using specific oligonucleotide primers (Table 2.1). Specifically, the UL11 deletion

encompassed 194 bps of the UL11 open reading frame (ORF), while 97 bps of the 5' UL11 ORF remained intact. This deletion was engineered to ensure that the UL12 ORF, which overlaps with the UL11 ORF by 86 bps, was unaffected (see Materials Methods). A similar deletion of the UL11 gene was constructed in the pYEbac102ΔUL20 genetic background.

For construction of the ΔUL11-Kan mutation, primers A and B were used for homologous recombination and positioned to remove a 198 bp region of the UL11 gene upon insertion of the kanamycin cassette extending from nucleotide 96 of UL11 to 3 bases past the UL11 stop codon (Table 2.1; Fig 2.1). The UL11 deletion was constructed so that it does not disrupt the UL12 ORF, which overlaps the 5' end of UL11 by 85 bases (see Materials & Methods). An identical UL11 insertion/deletion was constructed on both the wild type pYEbac102 genetic background as well as on the pYEbac102 ΔUL20 genetic background. A similar methodology was previously used to construct a recombinant virus carrying a deletion of the UL20 gene after insertion of a GFP-Zeocin gene cassette (ΔUL20-GFPZeo)(Foster et al., 2004b). This construction deleted a 353 bp region of the UL20 gene extending from the UL20 ATG to the HpaI site located within the UL20 ORF (Table 2.1; Fig.2. 1). Each pYEbac102 mutant construct contains one or more of the insertion-deletion mutations mentioned above: pYEbac102ΔUL11 contains the ΔUL11-Kan mutation; pYEbacΔUL20 contains the ΔUL20-GFPZeo mutation; and pYEbac102ΔUL11ΔUL20 contains both the ΔUL11-Kan and the ΔUL20-GFPZeo mutations (Fig. 2.1).

PCR-based confirmation of the pYEbac102**ΔUL11 and pYE**bac102**ΔUL11ΔUL20 genotypes**

The YEbac102-based genomic constructs were tested for the presence of the engineered insertion-deletion mutations via diagnostic PCR. Primers a and b (Table 2.1; Fig. 2.1), located outside and bracketing the UL11 gene, amplified the predicted 794 bp UL11 DNA fragments from pYEbac102 and

Table 2.1: Primer designation. Oligonucleotide primers used for GET recombination. HSV-1 homologous regions are denoted by uppercase letters, lowercase letters signify sequences which bind to the marker gene.

Primers

Primer designation	Name	Sequence	Purpose and product size (bp)
A	3' UL11-Kan	5'-GGGTTTTTTAAAAACGACACGCGTGCGACCGTATACAGAAATTGTTTTGGcggttgatgagagctttgttgtaggtggac-3'	UL11 GET recombination (1208)
B	5' UL11-Kan	5'-AACGTCCTCATCACCGACGACGGGGAGGTCGTCTCGCTGACCGCCCACGACTagccacgttgtgtctcaaaatctctgatgtta-3'	
C	3' UL20-GFPZeo	5'-CACGGACATCCCCCAAAACACGGGCGCCGACAACGGCAGACGATCCCTCTTGATGTTagacatgataagatacattgatgagtttgg-3'	UL20 GET recombination (2025)
D	5' UL20 GFPZeo	5'-CTGACGTAAGCGACCCTTTGCGGTTTCGGTCTCCCCACCTCCACCGCACACCCCgtccgttacataacttacggtaaatgg	
a	3' UL11-Rev	5'-GCACCAGCGCGGAGGAGGGC-3'	Diagnostic PCR for wt UL11 (794) or ΔUL11-Kan (1701)
b	5'UL11-For	5'-ATTGTACGCCCAAGATACAACACCG-3'	
c	3' UL20-Rev	5'-ACGGCCTTATCAAAACACTCGCCTC-3'	Diagnostic PCR for wt UL20 (909) or ΔUL20-GFPZeo (2469)
d	5' UL20-For	5'-GAAGGCTGCGGCCTCGTTCCAG-3'	

pYEbac102ΔUL20 (Fig. 2.2; lanes 1, 2). Primers c and d (Table 1; Fig 1), located outside and bracketing the UL20 gene, amplified the predicted 909 bp UL20 DNA fragments from pYEbac102 and pYEbac102ΔUL11 (Fig. 2.2; lanes 5, 7). In contrast, diagnostic PCR against the pYEbac102ΔUL11 and pYEbac102ΔUL11ΔUL20 DNAs using primers a and b (Table 2.1; Fig 2.1) produced PCR-amplified DNA fragment of 1608 bp, as predicted due to the insertion of the kanamycin gene cassette (Fig. 2.2; lanes 3, 4). Primers c and d (Table 2.1; Fig. 2.1) located outside and bracketing the UL20 gene amplified

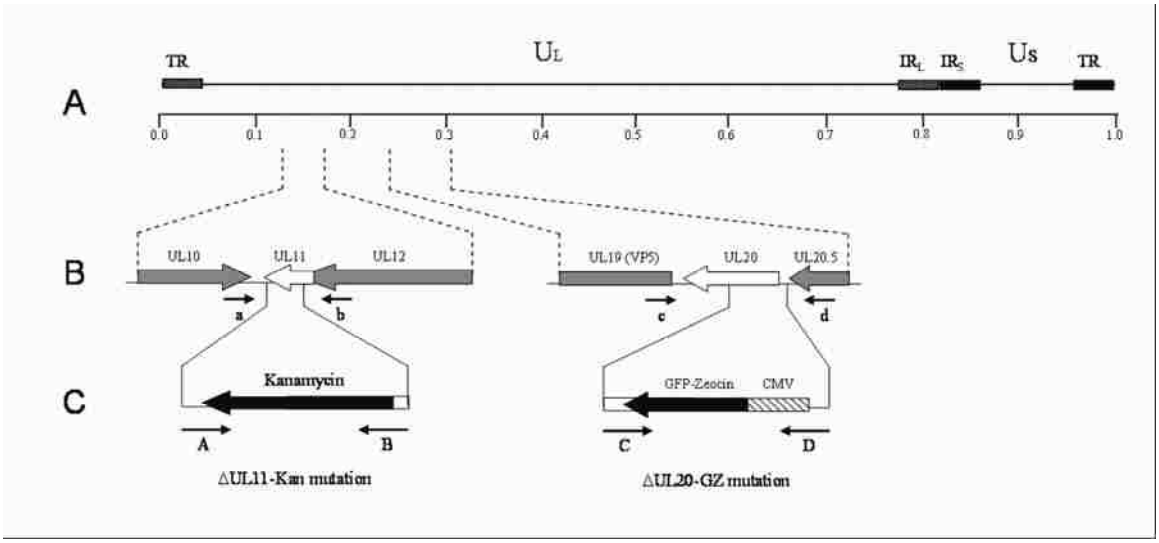


Figure 2.1: Construction of mutant YEbac102s. Schematic of the strategy for the construction of pYEbac102 mutant BACs. (A) The top line represents the prototypic arrangement of the HSV-1 genome, with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (B) Shown below are the expanded genomic regions of the UL11 and UL20 ORFs, the approximate locations of the genomic sites to which insertion of the marker genes was targeted, and the primers used in diagnostic PCR to confirm the presence of each mutation. (C) PCR fragments containing the kanamycin resistance or GFP-Zeocin resistance gene cassette flanked by approximately 50 bp of viral sequences on both sides were used for targeted GET recombination in *E.* coli to construct pYEbac102 mutant BACs with insertion-deletion mutations in the UL11 and/or UL20 ORFs, respectively. The approximate locations of the primers used in amplification of each PCR fragment are also shown.

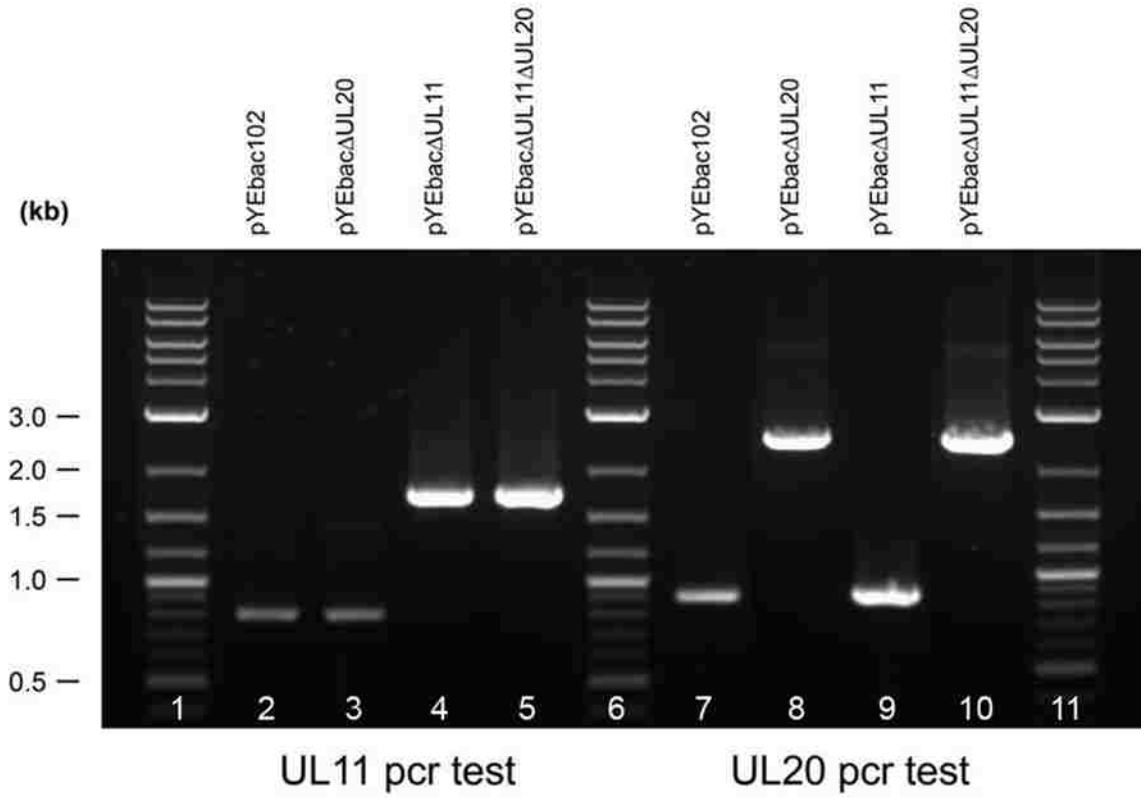


Figure 2.2: PCR diagnostics. PCR-based diagnostic analysis of pYEbac102ΔUL11 and pYEbac102ΔUL20 mutants. Oligonucleotide primers a, b (Table 1) were utilized to amplify DNA fragments containing the Kan inserted gene cassette. (i) Amplification with primers a and b produced the predicted 1701 size DNA fragment for the pYEbac102ΔUL11 and pYEbac102ΔUL11ΔUL20 genomes consistent with the insertion of the kan gene cassette, and the predicted 794 bp size fragment for the pYEbac102 and pYEbac102ΔUL20 controls. (ii) Amplification with primers c and d (Table 1) produced the predicted 2,469 bp size DNA fragment for the pYEbac102ΔUL20 and pYEbac102ΔUL11ΔUL20 consistent with insertion of the GZ gene cassette, and the 999 bp predicted DNA fragment for the pYEbac102 and pYEbac102ΔUL11ΔUL20 controls.

the predicted 2,469 bp DNA fragment from pYEbac102ΔUL20 and pYEbac102ΔUL11ΔUL20 due to the insertion of the GFP-Zeocin gene cassette (Fig. 2.2; lanes 6, 8).

Production of infectious virus from pYEbac102-based constructs

To generate virus stocks from the mutant pYEbac102 constructs, transient transfection of individual BAC DNAs was performed into Vero cells, or the Fd20-1 cell line, which is transformed with the UL20

gene and was shown to efficiently complement UL20-null viruses (Melancon, Foster, and Kousoulas, 2004b). Specifically, pYEbac102 and pYEbac102ΔUL11 were transfected into Vero cells, while pYEbac102ΔUL20 and pYEbac102ΔUL11ΔUL20 were transfected into the UL20 complementing Fd20-1 cells (Melancon, Foster, and Kousoulas, 2004b). For all transfection experiments, virus plaques became visible 72 hours post transfection, and virus stocks were collected at appropriate points exhibiting maximum cytopathic effects.

Plaque morphology and replication kinetics of HSV-1 YEbac102 mutants

As we have noted previously (Melancon et al., 2005), construction of mutant HSV-1 viruses using the YEbac102 plasmid allows for the rapid generation of recombinant viruses carrying desired mutations without the need for extensive plaque purification, which is normally needed when recombinant viruses are produced via classical homologous recombination in cell culture. To assess and compare the effect of the deletion of the UL11 and UL20 genes in the context of the same viral HSV-1(F) genome on cell-to-cell spread, the plaque morphologies of the YEbac102, YEbac102ΔUL11, YEbacΔUL20 and YEbac102ΔUL11ΔUL20 viruses were examined in Vero cells in the presence or absence of complementation by either the UL11 or UL20 proteins provided in trans via transient expression (Fig. 2.3). Complementation experiments were performed by transfection of UL20, UL11 or both UL20 and UL11 expression plasmids followed by infection with the ΔUL11, ΔUL20, or ΔUL11ΔUL20 viruses at 24 hours post transfection. As expected, the wild type YEbac102 produced large plaques on all infected cells (Fig 2.3: A, B). Infection of non-complementing cells with either YEbac102ΔUL20 or YEbac102ΔUL20ΔUL11 resulted in very small plaques containing on average 1 to 2 cells (Fig 2.3: E, G), while infection of non-complementing cells with the pYEbac102ΔUL11 virus resulted in plaques, which were approximately one-third the average size of the wild-type virus (Fig. 2. 3, C).

Complementation of the YEbac102ΔUL11, YEbac102ΔUL20, or YEbac102ΔUL11/UL20 by UL11, UL20 or UL20+UL11, respectively, produced viral plaques similar in size to those of the parental wild-type virus (Fig. 2.3: D, F, H). Furthermore, complementation of the YEbacΔUL20ΔUL11 with the UL20 alone produced a viral plaque similar in size to that of the YEbacΔUL11 virus (not shown). Complementation of the mutant viruses to wild-type plaque phenotypes was observed at a rate of 60-70% for all mutant viruses, indicating the absence of any secondary mutations that could account for the observed mutant phenotypes.

To examine the effect of the various mutations on virus replication, Vero cells were infected at an MOI of 2 with either the wild-type or each mutant virus. Virus stocks were prepared at 0, 4, 8, 12, 18, 24, and 36 h.p.i. and titrated in triplicate onto complementing cells (Fig. 2.4). The kinetics of both YEbac102ΔUL20 and YEbac102ΔUL11ΔUL20 were similar to each other and substantially slower than that of the YEbac102 virus with maximum titers of more than 3-logs reduced in comparison to the YEbac102 at 36 h.p.i. The replication kinetics of the YEbac102ΔUL11 virus was reduced in comparison to the YEbac102, with maximum titers reduced by more than one-log at 36 h.p.i.

Ultrastructural characterization of the YEbac102ΔUL11 and YEbac102ΔUL11ΔUL20 mutant viruses

The ultrastructural phenotypes of the Ybac102ΔUL20, YEbac102ΔUL11 and YEbac102ΔUL11ΔUL20 viruses relative to the YEbac102 parental virus were investigated utilizing transmission electron microscopy at 24 h.p.i. As expected, the YEbac102 virus exhibited no apparent defects in virion egress as exemplified by the presence of fully enveloped virions extracellularly, as well as the presence of fully enveloped virions intracellularly (Melancon et al., 2005)(not shown). Unlike the wild-type virus, ultrastructural visualization of YEbac102ΔUL11, YEbac102ΔUL20, and

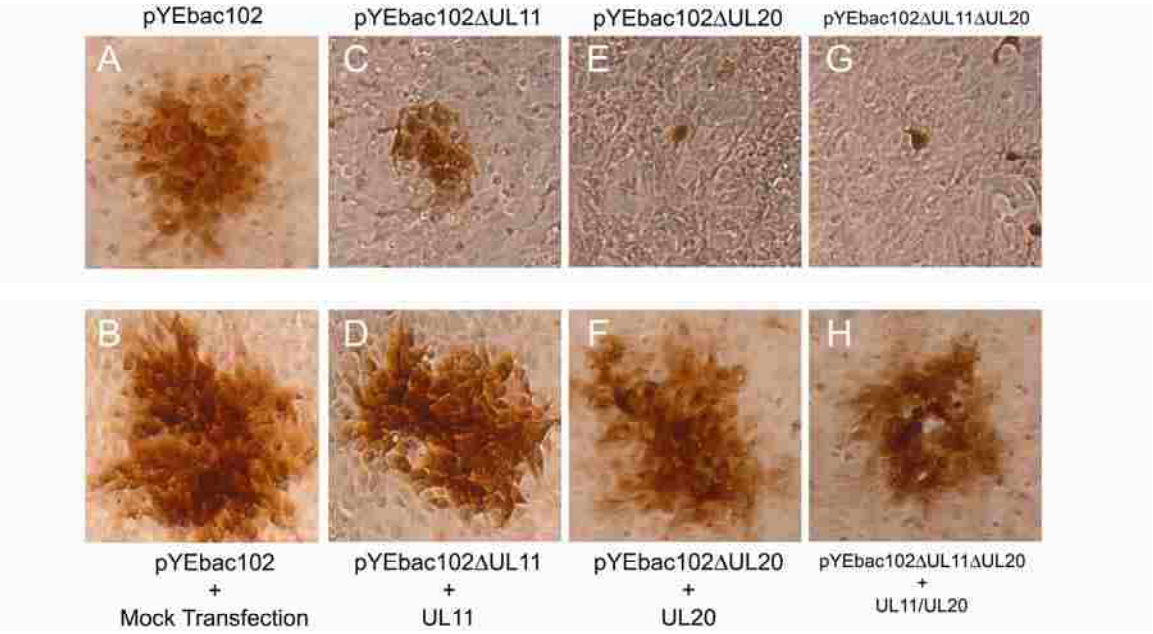


Figure 2.3: Plaque morphology of mutant viruses. Plaque phenotypes of UL11-, UL20-, and UL11/UL20-double null viruses under complementing and non-complementing conditions. Vero cell monolayers were either mock transfected or transfected with plasmids expressing UL11, UL20, or both UL11 and UL20. Transfected cells were infected 24 hrs post transfection with the corresponding viruses, pYEbac102, pYEbac102ΔUL11, pYEbac102ΔUL20 and pYEbac102ΔUL11ΔUL20. Individual viral plaques were visualized at 24 hrs post-infection by immunohistochemistry.

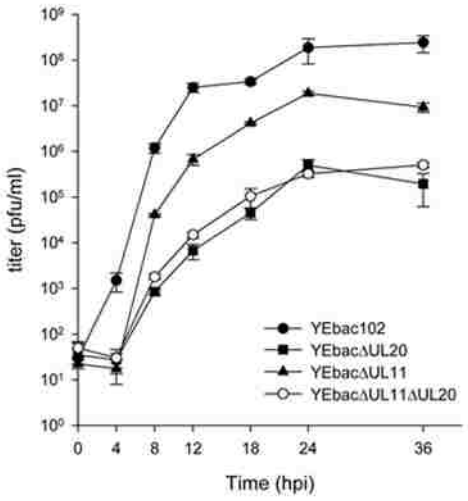


Figure 2.4: Viral replication kinetics. Comparison of the viral replication characteristics of YEbac102 (●), YEbac102ΔUL20 (■), YEbac102ΔUL11 (▲), and YEbac102ΔUL11ΔUL20 (○) on Vero cells. One-step growth kinetics of infections virus production was calculated after infection at an MOI of 2 followed by incubation at 37°C.

YEbac102ΔUL11ΔUL20 infected Vero cells revealed cytoplasmic defects in virion envelopment. The YEbac102ΔUL11 mutant produced largely unenveloped capsids in the cytoplasm embedded within morphologically darker stained areas that may be caused by the accumulation of tegument proteins (Fig. 2.5A). This ultrastructural phenotype appeared to be dissimilar to that of a previously constructed HSV-1(F)-null virus (Baines and Roizman, 1992b), in as much, there was no noticeable accumulation of capsids within nuclei of infected cells. In contrast, the YEbac102□UL11 ultrastructural morphology was similar to that produced by a pseudorabies (PRV) recombinant virus that lacked the UL11 homologous gene, although the tegument-like staining material surrounding the unenveloped capsids did not appear to be as concentrated as in the PRV case(Kopp et al., 2003). As reported previously (Melancon et al., 2005), the YEbac102ΔUL20 mutant virus produced unenveloped capsids in the cytoplasm as well as aberrantly enveloped virions (Fig. 2.5B). Ultrastructural examination of Vero cells

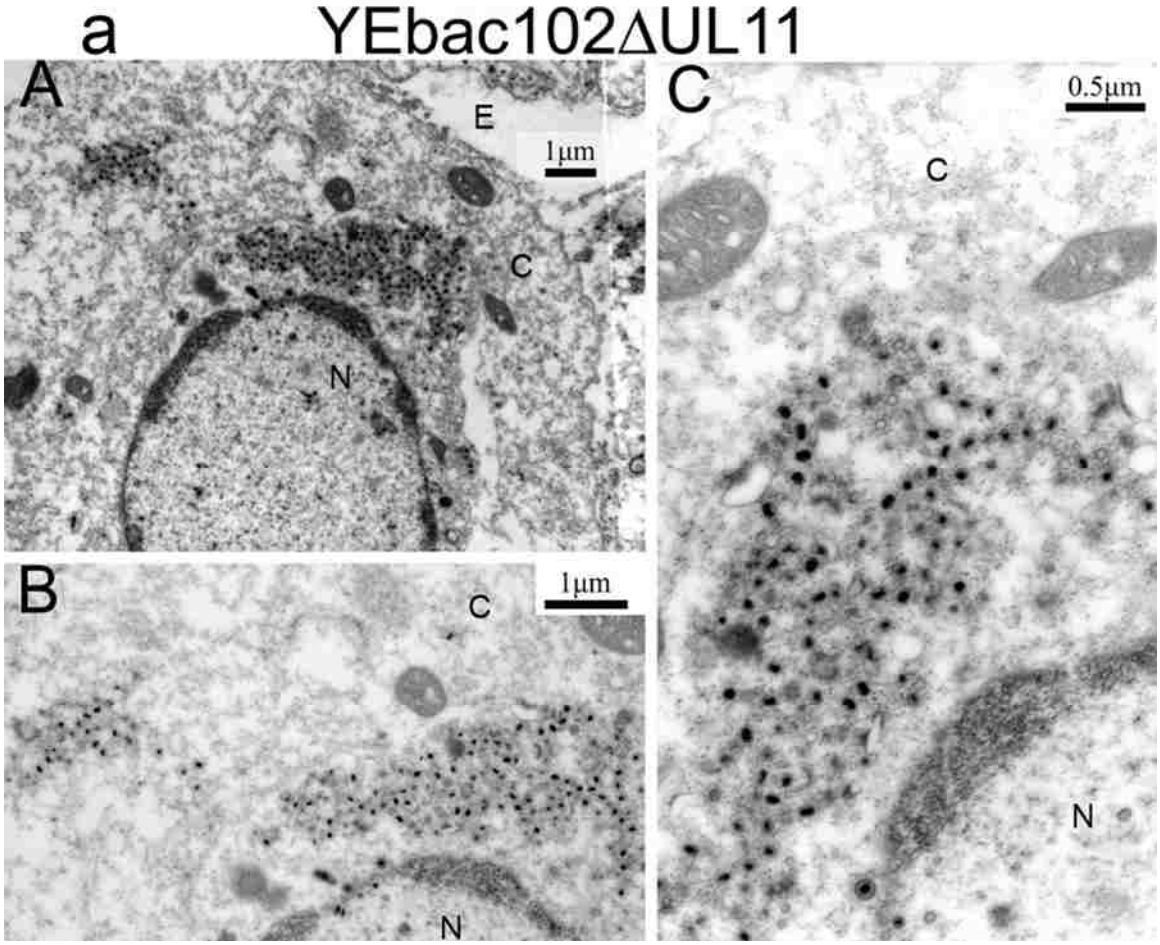
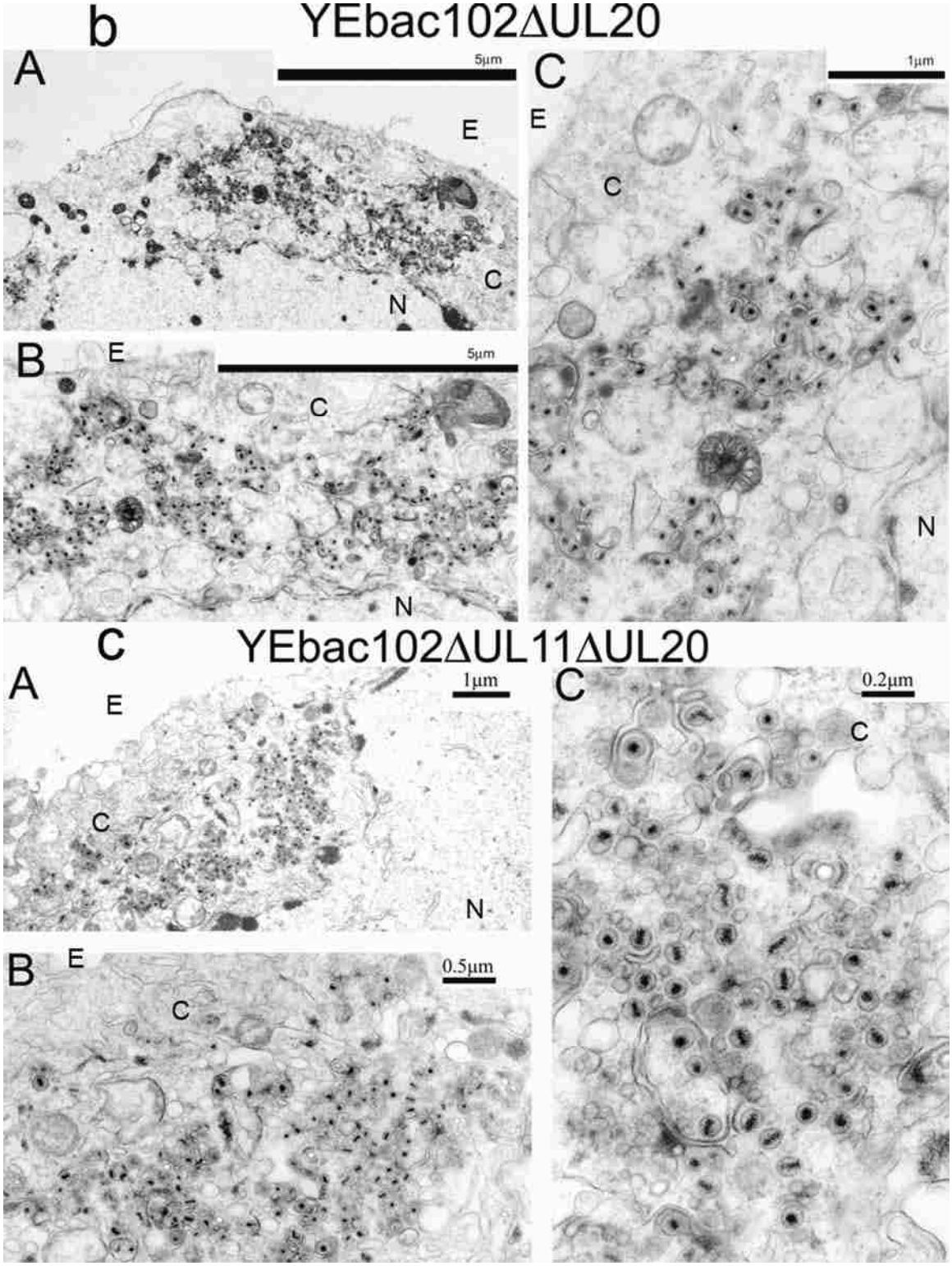


Figure 2.5: Ultrastructural characterization of mutant viruses. Ultrastructural morphology of the YEbac102ΔUL11 (a), YEbac102ΔUL20 (b), and YEbac102ΔUL11ΔUL20 (c) viruses. a) Confluent cell monolayers were infected with the YEbac102ΔUL11 virus at an MOI of 2, incubated for 24 hrs at 37°C, and prepared for transmission electron microscopy. Panel A: low magnification of an (c) infected cell. Panels B, C: higher magnifications of cell shown in panel A. b) Confluent cell monolayers were infected with the YEbac102ΔUL20 at an MOI of 2, incubated for 24 hrs at 37°C, and prepared for transmission electron microscopy. Panel A: low magnification of an (c) infected cell. Panels B, C: higher magnifications of cell shown in panel A**.** c) Confluent cell monolayers were infected with the YEbac102ΔUL11ΔUL20 virus at an MOI of 2, incubated for 24 hrs at 37°C, and prepared for transmission electron microscopy. Panel A: low magnification of an (c) infected cell. Panels B, C: higher magnifications of cell shown in panel A. Nuclear (N),cytoplasmic (C) and extracellular (E) spaces are marked, as well as a bar shown the relative magnification scale.


b
YEbac102ΔUL20
A
5μm
E
C
N
B
E
5μm
C
N
C
1μm
E
C
N
C
YEbac102ΔUL11ΔUL20
A
1μm
E
C
N
B
E
0.5μm
C
C
0.2μm
C

infected with the YEbac102ΔUL11ΔUL20 double-null virus revealed virion morphogenetic defects that were quite similar to the UL20-null and different to that of the UL11-null virus (Fig. 2.5C).

UL11 and UL20 are independently transported to the TGN

It has been previously reported that UL11 localizes to the TGN (Loomis et al., 2001). In agreement with these findings, transient expression of UL11 in Vero cells resulted in localization of the UL11 protein in the TGN (Fig.2. 6). Previously, we showed that gK or UL20 alone remained at the rough endoplasmic reticulum when expressed alone. However, co-expression of gK and UL20 resulted in the TGN localization of both proteins strongly suggesting that these two proteins interact and that this interaction is necessary for the coordinate transport to the TGN (Foster et al., 2004b). To determine whether either UL20 or gK expression was able to alter the TGN localization of the UL11 protein, the UL11 gene was transiently expressed in Vero cells concurrently with either the UL20 or gK gene alone or with both gK and UL20 genes. UL11 localized to the TGN in the presence of either UL20, gK or both UL20 and gK (Fig. 2.7). Additional experimentation was performed to ascertain whether the presence or absence of the UL20 or gK could affect the TGN localization of the UL11 protein in the context of other viral proteins. In these experiments, 143 TK- cells were transfected with either the UL11, UL20, or both UL11 and UL20 and 24 hours post transfection, cells were infected with the YEbac102ΔUL11ΔUL20 virus. Again, the UL11 protein localized efficiently to the TGN (Fig. 2.8).

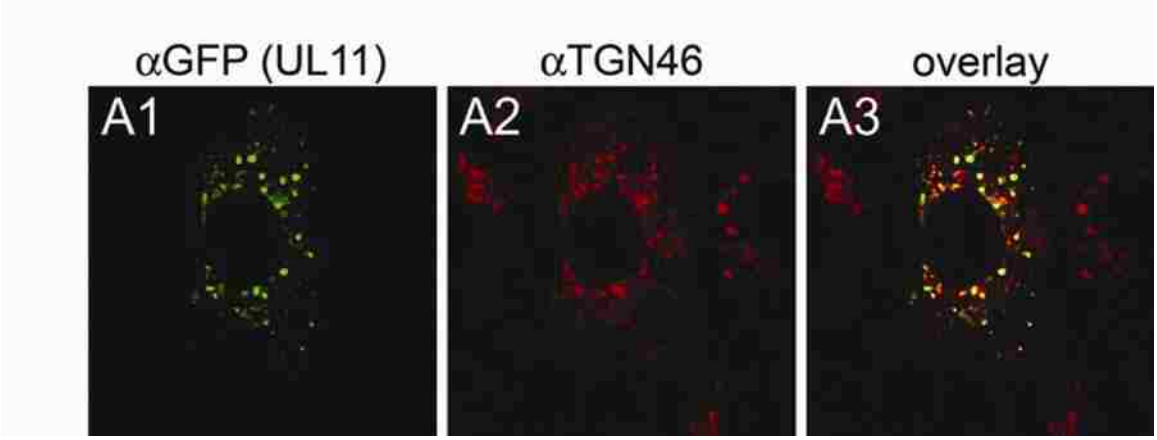


Figure 2.6: Confocal microscopy. Digital images of confocal micrographs showing UL11 localization. Vero cell monolayers were transfected with the UL11-GFP expressing plasmid. At 24 hrs post transfection, cells were washed thoroughly, fixed, and stained with the anti-GFP antibody (A1), or stained with the Golgi specific marker TGN46 (A2) and fluorescence was visualized by confocal microscopy. Magnification, x63; zoom x2.

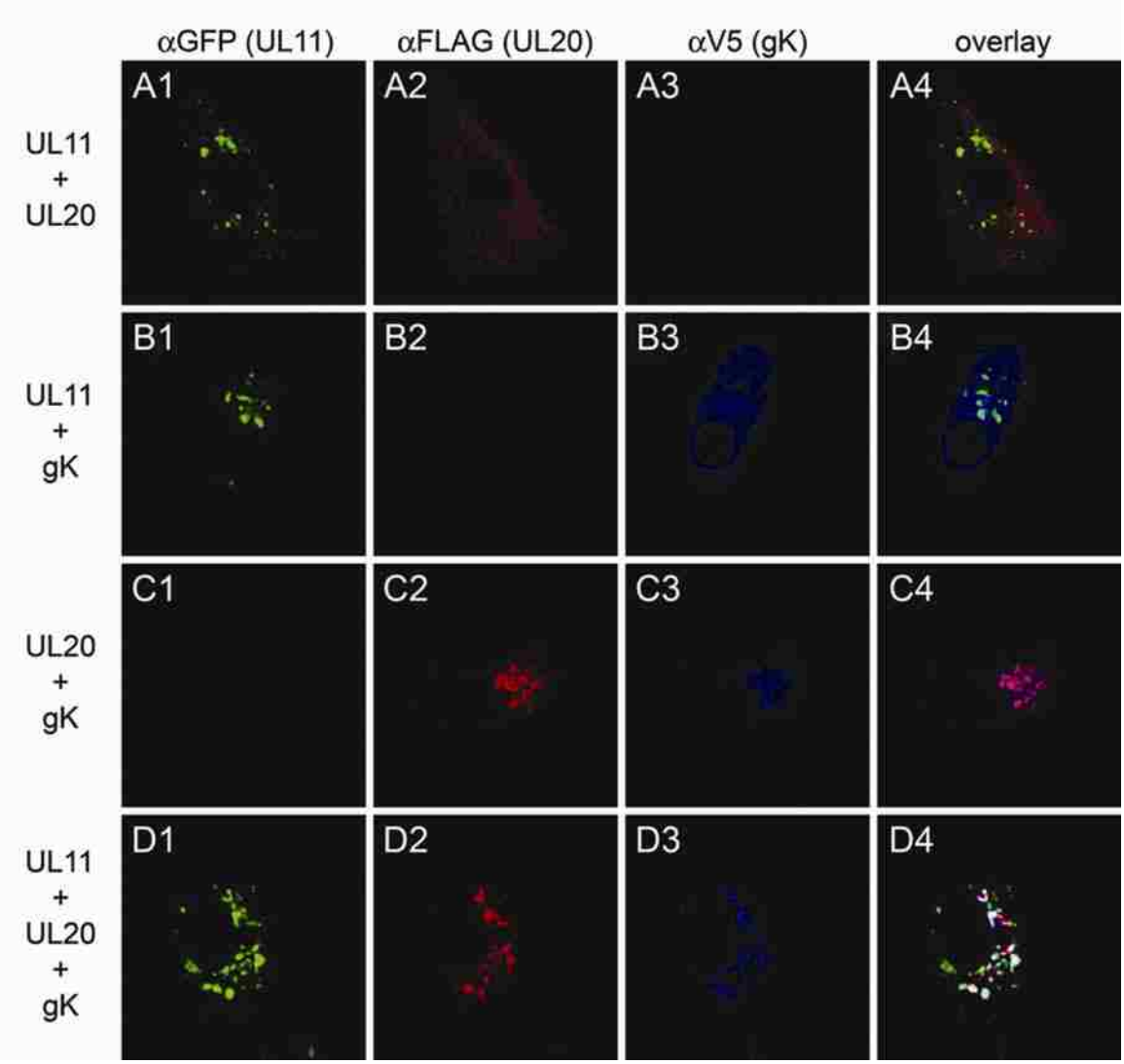


Figure 2.7: Confocal microscopy. Comparison of UL11 intracellular localization in the presence or absence of gK/UL20. Vero cells were transfected with a combination of plasmids expressing UL11-GFP, UL20, and/or gK. At 24 hrs post transfection, cells were washed thoroughly, fixed, and prepared for confocal microscopy after staining with the appropriate conditions and antibodies. UL11 was stained with the anti-GFP antibody, UL20 with the anti-FLAG antibody and gK with the anti-V5 antibody. (A) UL11+UL20 localization. (B) UL11+gK localization. (C) UL20+gK localization. (D) UL11+UL20+gK localization. Magnification, x63; zoom x2.

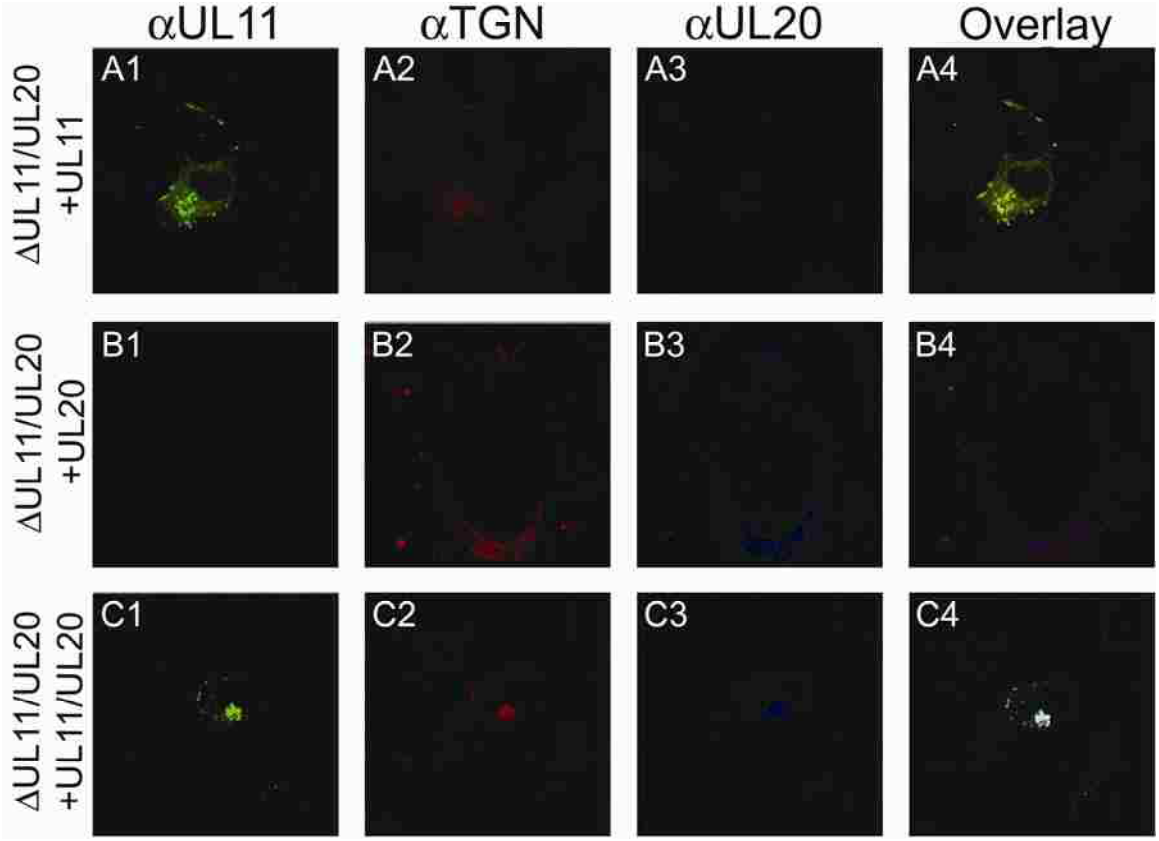


Figure 2.8: Confocal microscopy. Intracellular localization of the UL11 and UL20 proteins in virus-infected cells. Vero cells were transfected with plasmids expressing UL11-GFP, UL20, or both. At 24 hrs post-transfection, cells were infected at an MOI of 2 with YEbac102ΔUL11ΔUL20. At 24 hrs post infection, cells were washed thoroughly, fixed, stained with appropriate antibodies, and prepared for confocal microscopy. (A) UL11 localization. (B) UL20 localization. (C) UL11+UL20 localization. Magnification, x63; zoom x2.

Discussion

HSV-1 cytoplasmic virion envelopment is thought to be mediated by complex interactions among tegument proteins and viral glycoproteins embedded in the TGN membranes. A number of viral glycoproteins have been shown to either interact with tegument proteins or otherwise affect cytoplasmic envelopment. In this manuscript, we examined potential physical and functional relationships between the UL11 protein and the gK/UL20 heterodimer in cytoplasmic virion envelopment. Specifically, it was of interest to compare the ultrastructural phenotype of a newly constructed UL11-null virus in the HSV-1(F) background with that of the gK-null and UL20-null viruses and to determine whether UL11 and the gK/UL20 heterodimer functioned in a dependent or independent manner to each other. The salient features of our results are: 1) The HSV-1 UL11 protein functioned exclusively in cytoplasmic envelopment as evidenced by the accumulation of cytoplasmic capsids in cells infected with the HSV-1(F) UL11-null virus in a manner similar to the previously described PRV UL11-null virus(Kopp et al., 2003); 2) The UL20-null and UL11-null plaque morphology, as well as their ultrastructural phenotypes were substantially different; 3) A double UL20/UL11-null virus produced plaques and ultrastructural morphologies closely resembling those of the UL20-null viral phenotype; 4) The UL11/UL20- double null virus replicated with replication kinetics similar to that of the UL20-null virus; 5) UL11 and UL20 were transported independently of each other in transient transfection experiments. These results led to the conclusion that UL20 and UL11 transport and localize at the TGN independently of each other; however UL20 must function at a virion morphogenetic step occurring prior to and required for UL11 function.

Recently, we constructed and characterized deletion mutants of the gK, UL20, and gB genes utilizing the HSV-1(F) genome cloned into a bacterial artificial chromosome (BAC) system (Melancon et al., 2005). The HSV-1 gK-null and UL20-null viruses produced smaller plaques than the original gK-

null and UL20-null viruses, which were constructed via classical recombination/deletion methodologies in the HSV-1(KOS) genetic background (Foster et al., 2004b; Jayachandra, Baghian, and Kousoulas, 1997). This apparent very small plaque phenotype could be due to the difference in viral strains. Alternatively, we favor the hypothesis that the BAC-assisted construction of mutant viruses allowed the rapid generation of mutant genomes in E. coli substantially lowering the possibility that compensatory mutations that increase plaque size may be selected after serial passage of the KOS gK or UL20-null viruses. Appreciably, the very small plaque phenotype of the UL20/gK null viruses exemplify that the gK/UL20 proteins are essential for virus spread.

To compare the role of UL11 in virion morphogenesis, The YEbac102ΔUL11 (ΔUL11) virus was produced using our previously described BAC-assisted mutagenesis (Luna et al., 2004; Melancon et al., 2005). The ΔUL11 virus was constructed by deleting most of the UL11 ORF taking care not to interrupt the UL12 ORF that overlaps with the UL11 ORF by 87 bps. Previously, a similar UL11-null virus, R7219, carrying a UL11 deletion of 176 bps was constructed using classical recombination experiments (Baines and Roizman, 1992b). In comparison, the ΔUL11 virus carried an additional deletion of 18 bps. Overall, the new ΔUL11virus confirmed previous findings with the R7219 virus, since both viruses exhibited a significant reduction in virus replication. However, the R7219 virus exhibited a major defect in capsid envelopment via budding through the nuclear membrane resulting in an unusual accumulation of intra-nuclear capsids as well as a reduced number of enveloped capsids in the cytoplasm (Baines and Roizman, 1992b). In contrast to these findings, the ΔUL11virus accumulated capsids in the cytoplasm of infected cells that failed to acquire cytoplasmic envelopes. There was no apparent defect in nuclear egress as evidenced by the lack of accumulation of capsids into the nucleus or incomplete nuclear budding processes. Importantly, the ΔUL11 virus accumulated capsids in the cytoplasm of infected cells in large aggregates surrounded by electron-dense material, which may be derived by tegument proteins.

A similar ultrastructural phenotype was observed for the pseudorabies (PRV) UL11-null virus, with the exception that capsid aggregates were embedded within strongly electron-dense structures of uniform density attributed to the accumulation of capsid proteins (Kopp et al., 2003). Therefore, the HSV-1(F) UL11 gene functions in cytoplasmic envelopment in a similar manner to that of the PRV UL11 gene.

The UL11 protein is anchored to TGN membranes through an N-terminal myristylate anchor. In addition, the UL11 protein contains a di-Leucine and an acidic amino acid motif, which are known to be involved in physical interactions with the UL16 protein, as well as recycling from the plasma membranes to TGN. Deletion of either motif abrogates UL16 interaction and plasma membrane TGN recycling (Loomis et al., 2001; Loomis, Courtney, and Wills, 2003). The ΔUL11 virus is predicted to code for the first 32 amino acids of the UL11 protein effectively retaining the di-Leucine motif, while deleting the entire acidic motif. In contrast, the R7219 virus is predicted to code for the first 39 amino acids of the UL11 protein, effectively retaining the di-Leucine motif as well as three of the seven amino acids of the acidic acid motif. Thus, it can be predicted that both UL11 peptides coded by the ΔUL11 and R7219 viruses could be myristylated and anchored to TGN membranes. However, the ΔUL11 protein could not interact with the UL16 protein since the entire acidic motif is deleted. In contrast, it is possible that the R7219 UL11 protein may be able to interact with the UL16 protein since the di-Leucine motif is intact and three of the 7 acidic amino acids remain with the protein. It has been reported that the UL16 tegument protein is present on intranuclear assemblons and a structural component of mature virions (Nalwanga et al., 1996). Therefore, these subtle differences in the UL11 amino terminal peptides may account for the reported delay of nuclear egress for the R7219 virus, which may be caused by an aberrant interaction of the UL11 amino terminal peptide encoded by the R7219 virus with UL16 at the nuclear membrane.

The ΔUL11 ultrastructural phenotype is similar to the PRV UL11-null virus, with the exception that PRV-UL11-null virus formed capsids within a highly uniform electron dense material, which appeared to be derived from tegument proteins. In contrast, the ΔUL11 appeared to have a diffuse electron dense material surrounding the unenveloped capsids. The 32 amino acid UL11 peptide coded by the UL11-null virus retains the site of myristilation at its N- terminus, and thus, could be anchored to the TGN membranes. The PRV-UL11-null virus does not code for any UL11-derived peptide, since the UL11 initiation codon was altered by site-directed mutagenesis(Kopp et al., 2003). Therefore, the ΔUL11 amino terminal peptide within TGN membranes as well as in the cytoplasm may interact with other tegument proteins preventing the more pronounced aggregation of tegument proteins produced by the PRV UL-11-null virus.

To address potential synergistic effects between the UL11 and UL20 genes in cytoplasmic envelopment, we constructed the ΔUL20/UL11 double-null virus. Surprisingly, the ΔUL20/UL11 double-null virus exhibited replication characteristics very similar to the UL20-null virus. Similarly, the ultrastructural phenotype of the double-null virus was largely similar to the UL20-null virus, without any apparent contribution by the UL11-null mutation. Additional studies in transient expression systems revealed that UL11 and UL20/gK were transported to TGN independently of each other indicating that they did not physically or otherwise functionally interact in either cells expressing only the UL11 and UL20/gK genes, or in cells infected with virus. Taken together, these results suggest that the UL11 functions in cytoplasmic envelopment are totally dependent on UL20 expression. One way to interpret this apparent dependence of UL11 on UL20 is that the UL20 protein functions in an earlier cytoplasmic envelopment step with UL11, which is required for UL11 function. However, this prediction is contrary to the observation that the UL11-null virions accumulate capsids with aberrant tegument-derived structures, apparently distal to TGN-membranes. One interpretation of these results is that in the absence

of UL20 protein there is an irreversible attachment to TGN membranes that cannot be overcome by the UL11-null mutation. In contrast, the UL11-null mutation may allow initial binding of tegumented capsids to membranes followed by capsid release forming the observed aggregates of capsids embedded in tegument-like material. In this instance, the known physical interaction of UL11 with UL16 may in part, be responsible for the observed defect in tegument protein accumulation in the cytoplasm, since in the absence of UL11, UL16 as well as other tegument proteins that may interact with UL11 would not bind to TGN membranes, but remain free in the cytoplasm. An alternative scenario is based on the observation that the cellular intracytoplasmic membranes seemed to be restructured in the absence of the PRV UL11 indicating that UL11 may play some stabilization role in Golgi-TGN membranes in infected cells (Kopp et al., 2003). In this regard, it is possible that gK/UL20 may play a similar stabilization role for TGN membranes and specifically at capsid budding sites. In Vero cells Golgi stacks are largely fragmented during infection (Campadelli et al., 1993), which would argue against overall Golgi-TGN stabilization roles for either gK or UL20. However, concurrent action of gK/UL20 and UL11 at TGN budding sites may occur with gK/UL20 playing a dominant role over UL11.

The UL20/gK heterodimer is of paramount importance in cytoplasmic envelopment, since deletion of either gene causes profound accumulation of cytoplasmic capsids and reduction of viral titers by more than three logs, while viral plaques are greatly diminished in size. It is important to determine if the UL20/gK functions precedes those of other viral glycoproteins that are known to function in cytoplasmic virion envelopment such as gE/gI and gM. In this regard, an investigation of the phenotype of double mutants carrying deletions in more than one gene may shed some light in the sequence of events that control cytoplasmic envelopment.

REFERENCES

Baines, J. D., Jacob, R. J., Simmerman, L., and Roizman, B. (1995). The herpes simplex virus 1 UL11 proteins are associated with cytoplasmic and nuclear membranes and with nuclear bodies of infected cells. *Journal of Virology* **69**(2)**,** 825-33.

Baines, J. D., and Roizman, B. (1992). The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. *Journal of Virology* **66**(8)**,** 5168-74.

Baines, J. D., Ward, P. L., Campadelli-Fiume, G., and Roizman, B. (1991). The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. *Journal of Virology* **65**(12)**,** 6414-24.

Bowzard, J. B., Visalli, R. J., Wilson, C. B., Loomis, J. S., Callahan, E. M., Courtney, R. J., and Wills, J. W. (2000). Membrane targeting properties of a herpesvirus tegument protein-retrovirus Gag chimera. *J Virol* **74**(18)**,** 8692-9.

Brack, A. R., Dijkstra, J. M., Granzow, H., Klupp, B. G., and Mettenleiter, T. C. (1999). Inhibition of virion maturation by simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. *J Virol* **73**(7)**,** 5364-72.

Brack, A. R., Klupp, B. G., Granzow, H., Tirabassi, R., Enquist, L. W., and Mettenleiter, T. C. (2000). Role of the cytoplasmic tail of pseudorabies virus glycoprotein E in virion formation. *J Virol* **74**(9)**,** 4004-16.

Browne, H., Bell, S., Minson, T., and Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* **70**(7)**,** 4311-6.

Campadelli, G., Brandimarti, R., Di, L. C., Ward, P. L., Roizman, B., and Torrisi, M. R. (1993). Fragmentation and dispersal of Golgi proteins and redistribution of glycoproteins and glycolipids processed through the Golgi apparatus after infection with herpes simplex virus 1. *Proceedings of the National Academy of Sciences of the United States of America* **90**(7)**,** 2798-802.

Debroy, C., Pederson, N., and Person, S. (1985). Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. *Virology* **145**(1)**,** 36-48.

Desai, P., DeLuca, N. A., Glorioso, J. C., and Person, S. (1993). Mutations in herpes simplex virus type 1 genes encoding VP5 and VP23 abrogate capsid formation and cleavage of replicated DNA. *Journal of Virology* **67**(3)**,** 1357-64.

Dietz, P., Klupp, B. G., Fuchs, W., Kollner, B., Weiland, E., and Mettenleiter, T. C. (2000). Pseudorabies virus glycoprotein K requires the UL20 gene product for processing. *J Virol* **74**(11)**,** 5083-90.

Foster, T. P., Alvarez, X., and Kousoulas, K. G. (2003). Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion. *J Virol* **77**(1)**,** 499-510.

Foster, T. P., and Kousoulas, K. G. (1999). Genetic analysis of the role of herpes simplex virus type 1 glycoprotein K in infectious virus production and egress. *J Virol* **73**(10)**,** 8457-68.

Foster, T. P., Melancon, J. M., Baines, J. D., and Kousoulas, K. G. (2004a). The Herpes Simplex Virus Type 1 UL20 Protein Modulates Membrane Fusion Events during Cytoplasmic Virion Morphogenesis and Virus-Induced Cell Fusion. *J Virol* **78**(10)**,** 5347-57.

Foster, T. P., Melancon, J. M., Olivier, T. L., and Kousoulas, K. G. (2004b). Herpes simplex virus type 1 glycoprotein K and the UL20 protein are interdependent for intracellular trafficking and trans-Golgi network localization. *J Virol* **78**(23)**,** 13262-77.

Foster, T. P., Rybachuk, G. V., and Kousoulas, K. G. (2001). Glycoprotein K specified by herpes simplex virus type 1 is expressed on virions as a Golgi complex-dependent glycosylated species and functions in virion entry. *J Virol* **75**(24)**,** 12431-8.

Fuchs, W., Klupp, B. G., Granzow, H., Hengartner, C., Brack, A., Mundt, A., Enquist, L. W., and Mettenleiter, T. C. (2002). Physical interaction between envelope glycoproteins E and M of pseudorabies virus and the major tegument protein UL49. *J Virol* **76**(16)**,** 8208-17.

Fuchs, W., Klupp, B. G., Granzow, H., and Mettenleiter, T. C. (1997). The UL20 gene product of pseudorabies virus functions in virus egress. *J Virol* **71**(7)**,** 5639-46.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N., and Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *J Virol* **75**(8)**,** 3675-84.

Harley, C. A., Dasgupta, A., and Wilson, D. W. (2001). Characterization of herpes simplex virus-containing organelles by subcellular fractionation: role for organelle acidification in assembly of infectious particles. *J Virol* **75**(3)**,** 1236-51.

Hutchinson, L., and Johnson, D. C. (1995). Herpes simplex virus glycoprotein K promotes egress of virus particles. *Journal of Virology* **69**(9)**,** 5401-13.

Jayachandra, S., Baghian, A., and Kousoulas, K. G. (1997). Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space. *Journal of Virology* **71**(7)**,** 5012-24.

Kopp, M., Granzow, H., Fuchs, W., Klupp, B., and Mettenleiter, T. C. (2004). Simultaneous deletion of pseudorabies virus tegument protein UL11 and glycoprotein M severely impairs secondary envelopment. *J Virol* **78**(6)**,** 3024-34.

Kopp, M., Granzow, H., Fuchs, W., Klupp, B. G., Mundt, E., Karger, A., and Mettenleiter, T. C. (2003). The pseudorabies virus UL11 protein is a virion component involved in secondary envelopment in the cytoplasm. *J Virol* **77**(9)**,** 5339-51.

Loomis, J. S., Bowzard, J. B., Courtney, R. J., and Wills, J. W. (2001). Intracellular trafficking of the UL11 tegument protein of herpes simplex virus type 1. *J Virol* **75**(24)**,** 12209-19.

Loomis, J. S., Courtney, R. J., and Wills, J. W. (2003). Binding partners for the UL11 tegument protein of herpes simplex virus type 1. *J Virol* **77**(21)**,** 11417-24.

Luna, R. E., Zhou, F., Baghian, A., Chouljenko, V., Forghani, B., Gao, S. J., and Kousoulas, K. G. (2004). Kaposi's sarcoma-associated herpesvirus glycoprotein K8.1 is dispensable for virus entry. *J Virol* **78**(12)**,** 6389-98.

MacLean, C. A., Clark, B., and McGeoch, D. J. (1989). Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristylated. *Journal of General Virology* **70**(Pt 12)**,** 3147-57.

MacLean, C. A., Efstathiou, S., Elliott, M. L., Jamieson, F. E., and McGeoch, D. J. (1991). Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. *Journal of General Virology* **72**(Pt 4)**,** 897-906.

Melancon, J. M., Foster, T. P., and Kousoulas, K. G. (2004). Genetic analysis of the herpes simplex virus type 1 UL20 protein domains involved in cytoplasmic virion envelopment and virus-induced cell fusion. *J Virol* **78**(14)**,** 7329-43.

Melancon, J. M., Luna, R. E., Foster, T. P., and Kousoulas, K. G. (2005). Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment. *J Virol* **79**(1)**,** 299-313.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J Virol* **76**(4)**,** 1537-47.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* **106**(2)**,** 167-80.

Mettenleiter, T. C. (2006). Intriguing interplay between viral proteins during herpesvirus assembly or: the herpesvirus assembly puzzle. *Vet Microbiol* **113**(3-4)**,** 163-9.

Nalwanga, D., Rempel, S., Roizman, B., and Baines, J. D. (1996). The UL 16 gene product of herpes simplex virus 1 is a virion protein that colocalizes with intranuclear capsid proteins. *Virology* **226**(2)**,** 236-42.

Narayanan, K., Williamson, R., Zhang, Y., Stewart, A. F., and Ioannou, P. A. (1999). Efficient and precise engineering of a 200 kb beta-globin human/bacterial artificial chromosome in

E. coli DH10B using an inducible homologous recombination system. *Gene Ther* **6**(3)**,** 442-7.

Orford, M., Nefedov, M., Vadolas, J., Zaibak, F., Williamson, R., and Ioannou, P. A. (2000). Engineering EGFP reporter constructs into a 200 kb human beta-globin BAC clone using GET Recombination. *Nucleic Acids Res* **28**(18)**,** E84.

Ramaswamy, R., and Holland, T. C. (1992). In vitro characterization of the HSV-1 UL53 gene product. *Virology* **186**(2)**,** 579-87.

Roizman, B., and Sears, A. E. (2001). Herpes Simplex Viruses and Their Replication. Third edition ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2399-2459. 2 vols. Lippincott-Williams & Wilkins, Philadelphia, PA.

Skepper, J. N., Whiteley, A., Browne, H., and Minson, A. (2001). Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment --> deenvelopment --> reenvelopment pathway. *J Virol* **75**(12)**,** 5697-702.

Tanaka, M., Kagawa, H., Yamanashi, Y., Sata, T., and Kawaguchi, Y. (2003). Construction of an excisable bacterial artificial chromosome containing a full-length infectious clone of herpes simplex virus type 1: viruses reconstituted from the clone exhibit wild-type properties in vitro and in vivo. *J Virol* **77**(2)**,** 1382-91.

Vittone, V., Diefenbach, E., Triffett, D., Douglas, M. W., Cunningham, A. L., and Diefenbach, R. J. (2005). Determination of interactions between tegument proteins of herpes simplex virus type 1. *J Virol* **79**(15)**,** 9566-71.

Zhu, Z., Gershon, M. D., Hao, Y., Ambron, R. T., Gabel, C. A., and Gershon, A. A. (1995). Envelopment of varicella-zoster virus: targeting of viral glycoproteins to the trans-Golgi network. *J Virol* **69**(12)**,** 7951-9.

CHAPTER 3
HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) UL16 IS REQUIRED FOR EFFICIENT NUCLEAR EGRESS AND CYTOPLASMIC VIRION ENVELOPMENT

Introduction

Herpes simplex virus type-1 (HSV-1) morphogenesis occurs in multiple stages within infected cells. Initially, the virion capsid assembles within the nucleus and the virion acquires an initial envelope by budding into the perinuclear spaces (Roizman and Sears, 2001). Subsequently, these enveloped virions fuse with the outer nuclear lamellae leading to the accumulation of unenveloped capsids into the cytoplasm. Within the cytoplasm, a number of additional tegument proteins attach to the capsid, and the fully tegumented capsids bud into cytoplasmic vesicles, which most likely originate from the *trans*-Golgi network (TGN). Enveloped virions are ultimately secreted to extracellular spaces through the utilization of cellular vesicular trafficking systems (Browne et al., 1996; Granzow et al., 2001; Harley, Dasgupta, and Wilson, 2001; Mettenleiter, 2002; Skepper et al., 2001; Zhu et al., 1995). The process by which the tegumented cytoplasmic capsids bud into TGN-derived vesicles is not well-understood. The prevalent model calls for specific interactions among viral tegument proteins and the cytoplasmic portions of membrane proteins and glycoproteins embedded within TGN membranes as key factors that drive cytoplasmic virion envelopment. This model is supported by evidence that specific mutations within tegument proteins and multiple membrane proteins and glycoproteins inhibit cytoplasmic envelopment (Mettenleiter, 2004; Mettenleiter, 2006).

One pair of viral proteins, which is thought to contribute to cytoplasmic virion envelopment is the tegument protein UL16 and the lipid membrane anchored tegument protein UL11. Specifically, UL16 was shown to specifically interact with UL11 (Loomis, Courtney, and Wills, 2003; Vittone et al., 2005). The UL16 ORF encodes a 373 amino acid protein (Nalwanga et al., 1996) that is associated with intra-nuclear assemblons early in the HSV-1 life cycle. Later it is found associated with peri-nuclear

virions and is a component of purified virions in HSV-1 and HCMV (Nalwanga et al., 1996; Ward, Barker, and Roizman, 1996; Wing, Lee, and Huang, 1996), but not HSV-2 (Oshima et al., 1998). Assemblons are believed to be sites at which capsid assembly and/or DNA cleavage and packaging may occur (Ward, Ogle, and Roizman, 1996), suggesting a possible role for UL16 in these processes. However, studies on a UL16 null virus (R7210) indicate that the UL16 protein was dispensable for HSV-1 replication in cell culture, although the virus did replicate at titers 3 to 10 fold lower than those of the wild type virus (Baines and Roizman, 1991).

The UL11 ORF encodes a 96 amino acid tegument protein, which is N-terminally myristylated (MacLean, Clark, and McGeoch, 1989) and palmitylated (Loomis et al., 2001). The UL11 protein localizes to nuclear and TGN-derived apparatus derived membranes in infected cells (Baines et al., 1995), but only to the TGN-membranes in non-infected cells (Bowzard et al., 2000). UL11 is believed to play a significant role in cytoplasmic envelopment as UL11 deletion mutants display a severe defect at this step in both HSV-1 (Baines and Roizman, 1992b; Fulmer et al., 2007) and PRV (Kopp et al., 2003). The interaction of UL11 with UL16 is dependant upon the presence of an acidic amino acid cluster and a di-leucine motif located within the first 50 amino acids of UL11 (Loomis, Courtney, and Wills, 2003).

The UL16 ORF is encoded on the antisense genomic strand completely embedded within UL15, which is coded by the sense genomic strand. UL15 is a rarity among HSV-1 genes in that it is encoded by two exons separated by an intron. UL16 is encoded within this intron. It was initially thought that the UL15 intron could serve to down-regulate UL16 protein production by an antisense mechanism, but this turned out not to be the case. In addition, the UL15 splicing event itself seems to have no effect on viral growth (Baines and Roizman, 1992a). The initial studies with a UL16-null virus involved deletion of the UL16 gene (Baines and Roizman, 1991). Due to the fact that the significance of the UL15 intron

and UL15 splicing event is not known, for this study, we sought to delete UL16 without disrupting the amino acid coding sequence of UL15. To achieve this, we employed a recently described marker-less recombination system to introduce single point mutations into the HSV-1 bacterial artificial chromosome (BAC), pYEBac102 (Tischer et al., 2006). Using this system we were able to silence the start codon of UL16 without altering the amino acid sequence of UL15.

Materials and Methods

Cells, viruses and plasmids

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL; Grand Island, N.Y.), supplemented with 10% fetal calf serum and antibiotics.

PCR Primer Design

Marker-less RED recombination in *E. coli* is accomplished mostly through a novel idea for the targeting of mutations using specific PCR primers (Tischer et al., 2006) . The 5' end of the first primer contains approximately 40 bps upstream of the site to be mutated, followed by the mutant DNA sequence(s), and followed by an additional 20 bps downstream of the target site. The 3' end of the forward primer anneals to pEPkan-S so that it overlaps an *I-SceI* site. The 5' end of the reverse primer was designed so that it contains the reverse complement of 40 bps downstream of the target site, followed by the reverse complement of the target site, and then an additional 20bps upstream of the target site. The 3' end is designed to anneal to reverse complement sequences downstream of the *AphA1*conferring Kanamycin resistance encoded by pEPkan-S. Using these primers to amplify the *AphA1* gene on pEDkan-S, results in a PCR product that contains identical sequences containing the

desired mutation flanking both an *I-SceI* site and the Kanamycin resistance cassette. Primer sequences used in this study are given in Table 3.1.

Construction of HSV-1 mutants containing deletions of the UL16 or UL11 gene (pYEBacΔUL16, pYEBacΔUL11)

Mutagenesis of pYEbac102 DNA was accomplished in *Escherichia coli* using the RED recombination machinery as described previously (Tischer et al., 2006). To create pYEBacΔUL16 BAC maintenance and mutagenesis were performed in *E. coli* strain EL250 which contains a λ prophage which encodes recombination enzymes Exo, Beta, and Gam under a heat inducible promoter (Lee et al., 2001). EL250 cells were made electrocompetent and were transformed with pYEBac102 to produce EL250-BAC. RED recombination was induced in the EL250-BAC cells and they were once again made electrocompetent. EL250-BAC with RED induced was then transformed with pBAD-*I-SceI*, containing an arabinose inducible *I-SceI* homing endonuclease cassette, and the UL16-Kan PCR product. The transformation was plated on plates containing chloramphenicol (12.5 μg/ml), ampicillin (100 μg/ml), and kanamycin (50 μg/ml). Colonies were screened for correct integration of the UL16-Kan PCR product using the UL16 test primers from Table 1. Clones that were confirmed to have the correct integration were grown from a 1:1000 dilution for 2 hours, followed by addition of .5% final concentration of arabinose and another 1 hour of growth. Cultures were then placed in a 42^{O}C water bath for 30 minutes, followed by a final 1 hour of growth. 100μL of culture was plated on chloramphenicol and ampicillin plates. After 24 hours, colonies were screened for loss of kanamycin resistance. Kanamycin sensitive colonies were prepared and sent for sequencing to confirm the presence of the desired mutation. Construction of pYEBacΔUL11 was described previously (Fulmer et al., 2007). All mutations were confirmed by DNA sequencing.

Table 3.1 Primer Design for RED Recombiniation.

Table 3.1
Primers

Primer designation	Name	Sequence[1]	Purpose
A	3' UL11-Kan	5'-GGGTTTTTTAAAAACGACACGCGTGCGACCGTATACAGAAATTGTTTTGGcggttgatgagagctttgttgtaggtggac-3'	UL11 GET recombination
B	5' UL11-Kan	5'-AACGTCCTCATCACCGACGACGGGGAGGTCGTCTCGCTGACCGCCCACGACTagccacgttgtgtctcaaaatctctgatgtta-3'	
	3' UL16-Mut	5'-GACCAGGCGGCGCCAGGGGCCGCCGGGGTCCCAGCTGCGCTATGCCGGGGGCGGGGGGAGGGCaggatgacgacgataagt aggg -3'	UL16 RED recombination
	5' UL16-Mut	5'-GGCCCCCGCCTCTGGGGTTTGCCCTCCCCCCGCCCCCGGCATAGCGCAGCTGGGACCCCGGCGgcaaccaattaaccaattct gattag-3'	
a	3' UL11-Rev	5'-GCACCAGCGCGGAGGAGGGC-3'	Diagnostic PCR for wt UL11 or ΔUL11-Kan
b	5'UL11-For	5'-ATTGTACGCCCAAGATACAACACCG-3'	
c	3' UL16-Rev	5'- AATCGCCGCCTCTGTGGCATAGTTG -3'	Test PCR for confirmation of correct PCR product integration
d	5' UL16-For	5'- GACGGAGTTGACAATGCGCGCG -3'	

[1]HSV-1 homologous regions are denoted by uppercase letters, lowercase letters signify sequences which bind to the marker gene, point mutations in red.

Transfection of HSV-1 BAC DNAs

Transient transfection of cells with BAC DNAs was performed with Lipofectamine 2000 (Invitrogen) and infectious virus was recovered as detailed previously (Fulmer et al., 2007; Melancon et al., 2005). VERO cells were grown to 95% confluency in six-well plates. Cells were transfected with BAC DNA mixed with Lipofectamine 2000 in Opti-MEM medium as recommended by the manufacturer (Invitrogen). After 6 h of incubation at 37°C, the medium was removed from the transfected cells, cells were washed with phosphate buffered saline, and subsequent fresh Dulbecco's modified Eagle's medium with 10% fetal calf serum was added. At 72 h post transfection, virus stocks were collected.

One-step growth kinetics and plaque morphology of YEbac102 mutants

Analysis of one-step growth kinetics was as described previously (Foster, Alvarez, and Kousoulas, 2003; Foster, Rybachuk, and Kousoulas, 2001). Briefly, each virus at an MOI of 2 was adsorbed to approximately 6 x 10^5 Vero cells at 4°C for 1 h. Thereafter, warm medium was added, and virus was allowed to penetrate for 2 h at 37°C. Any remaining extra-cellular virus was inactivated by low-pH treatment (0.1M glycine, pH 3.0). Cells and supernatants were harvested immediately thereafter (0 h) or after 4, 8, 12, 24, or 36h of incubation at 37°C. Virus titers were determined by endpoint titration of virus stocks on Vero cells.

Analysis of plaque morphology of mutant viruses was as follows. Confluent Vero cell monolayers in 6 well plates were infected with the indicated virus. To visualize the plaques, cells were stained with a polyclonal HRP conjugated HSV-1 antibody as directed by the manufacturer (DakoCytomation), and as described previously (Fulmer et al., 2007; Melancon, Foster, and Kousoulas, 2004b; Melancon et al., 2005). Briefly, cells were washed with PBS to remove methylcellulose media,

and fixed with 4°C methanol for 15 minutes. TBS containing a 1:750 dilution of the polyclonal HSV-1 antibody was added to the cells and placed on a rocker at 4°C for 1 h. Cells were washed with TBS and developed using the VECTOR NovaRED peroxidase substrate kit as directed by the manufacturer (VECTOR, Inc).

Electron microscopy

Cell monolayers were infected with the indicated virus at an MOI of 3. All cells were prepared for transmission electron microscopy (TEM) examination at 24 h post infection. Infected cells were fixed in a mixture of 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Following treatment with 1% OsO_4 and dehydration in an ethanol series, the samples were embedded in Epon-Araldyte resin and polymerized at 70°C. Thin sections were made on an MTXL ultratome (RMC Products), stained with 5% uranyl acetate and citrate/nitrate/acetate lead, and observed with a Zeiss 10 transmission electron microscope as described previously (Foster et al., 2004b; Melancon, Foster, and Kousoulas, 2004b).

Results

Construction of the HSV-1 BAC pYEbacΔUL16

The complete HSV-1(F) genome has been cloned into a bacterial artificial chromosome (BAC; pYEbac102) enabling the genetic manipulation of the HSV-1 genome in *E. coli*. (Tanaka et al., 2003). Previously, we utilized the BAC-based GET homologous recombination system to construct deletions within the gB, UL20 and gK genes in *E. coli* (Melancon et al., 2005). A similar strategy was utilized to construct a HSV-1 BAC with most of the UL11 ORF deleted(Fulmer et al., 2007). A recently described markerless RED recombination system was used to generate the pYEbac102ΔUL16 mutant. This system allowed for the construction of a pYEbac102 mutant with a single point G to A mutation that

silenced the ATG start codon of UL16, while leaving the amino acid sequence of the overlapping UL15 gene intact. pYEbac102ΔUL16 contains the ΔUL16 mutation and pYEbac102ΔUL11 contains the ΔUL11 mutation (Figure 3.1).

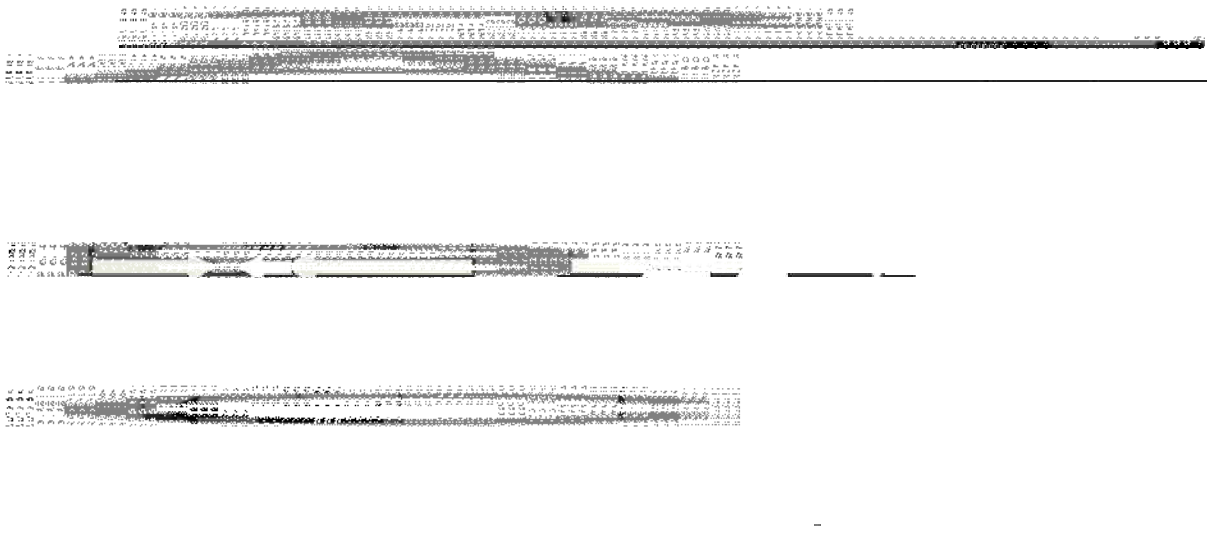


Figure 3.1: Schematic of the strategy for the construction of mutant BACs ΔUL16 and ΔUL11 YEbac102. (A) The top line represents the prototypic arrangement of the HSV-1 genome, with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (B) Shown below are the expanded genomic regions of the UL11 and UL16 ORFs. (C) PCR fragments containing the kanamycin resistance gene cassette flanked by approximately 50 bp of viral sequences on both sides were used for targeted GET recombination in *E.* coli to construct pYEbac102 mutant UL11 BACs with insertion-deletion mutations in the UL11 ORF. The approximate locations of the primers used in amplification of each PCR fragment are also shown. For construction of the ΔUL16 mutant, a single point mutation was introduced to silence the UL16 ORF, shown in red.

Production of infectious virus from pYEbac102-based constructs

To generate virus stocks from the mutant pYEbac102 constructs, transient transfection of individual BAC DNAs was performed into Vero cells (see Materials and Methods). For all transfection experiments, virus plaques became visible 72 hours post transfection, and virus stocks were collected.

Plaque morphology and replication kinetics of HSV-1 YEbac**102 mutants**

As we have noted previously, construction of mutant HSV-1 viruses using the YEbac102 plasmid allows for the rapid generation of recombinant viruses carrying desired mutations without the need for extensive plaque purification, which is normally needed when recombinant viruses are produced via classical homologous recombination in cell culture (Melancon et al., 2005). To assess and compare the effect of the deletion of the UL16 and UL11 genes in the context of the same viral HSV-1(F) genomic background on cell-to-cell spread, the plaque morphologies of the YEbac102, YEbac102ΔUL16, and YEbacΔUL11viruses were examined in Vero cells (Fig. 3.2). As expected, the wild-type YEbac102 produced large plaques on infected cells. In contrast, the YEbac102ΔUL16 and YEbac102ΔUL11 viruses produced plaques approximately one third the size of the wild-type virus. To ensure this phenotype was due only to the deletion of the intended gene, and not some other spurious mutation, YEbacΔUL16 and YEbac102ΔUL11 rescued viruses were also examined. The YEbac102ΔUL16 rescue was achieved by designing primers for the RED recombination system that simply reversed the original mutation. The YEbac102ΔUL11 rescue was achieved by transient complementation of the mutant virus with UL11 on Vero cells as described previously for the YEbac102ΔUL11 virus (Fulmer et al., 2007). Rescue of the YEbac102ΔUL16 mutation produced the expected wild-type morphology, indicating that the UL16 viral plaque phenotype was as in the case of UL11, solely due to the introduced UL16 silencing mutation (Fig. 3.2).

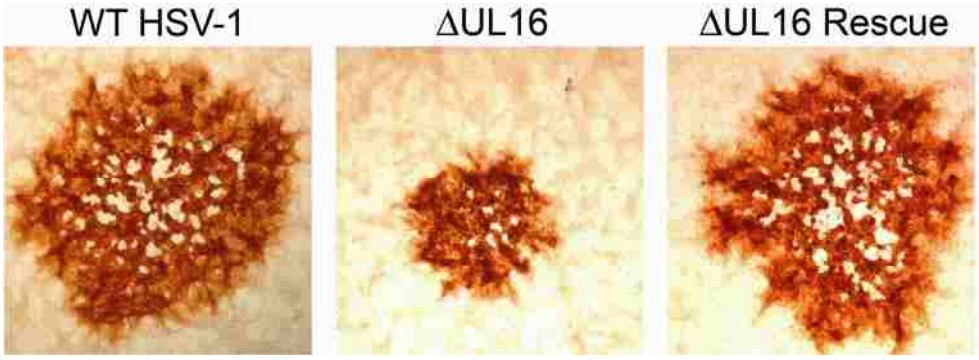


Figure 3.2: Plaque morphology of mutant viruses. Plaque morphology of wild-type YEbac102 (A), ΔUL16 (B), ΔUL11 (C), ΔUL16 rescue (D), and ΔUL11 rescue (E). Viruses were infected on VERO cell monolayers, and plaques were visualized by immunohistochemistry 24 h.p.i.

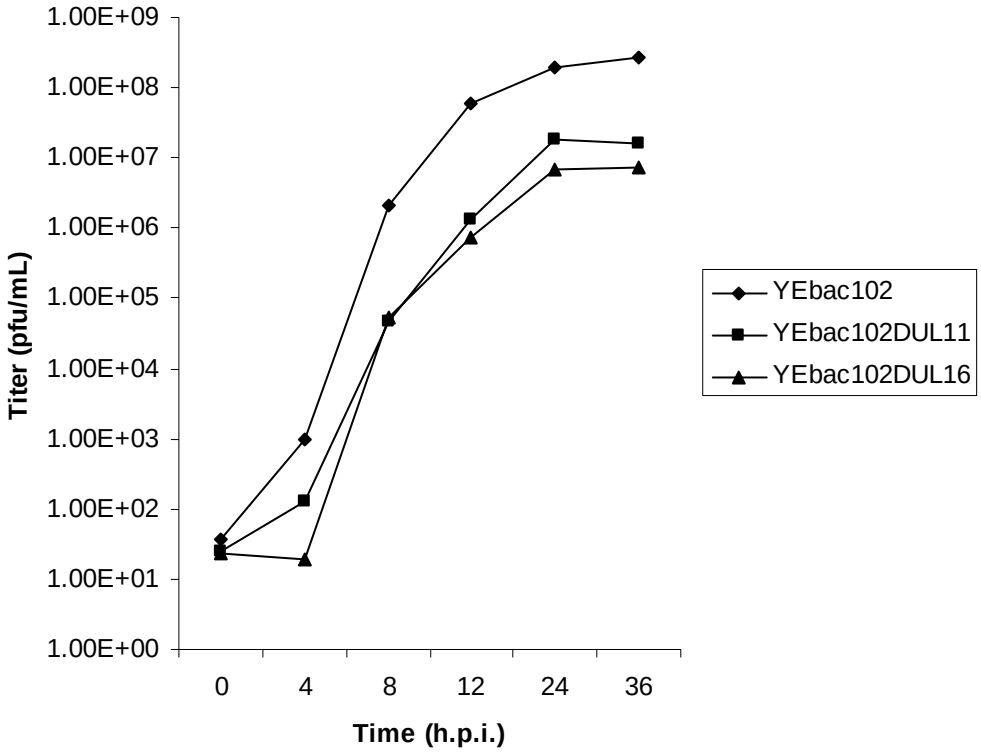


Figure 3.3: Viral replication kinetics. Comparison of the viral replication characteristics of YEbac102 (♦), YEbac102ΔUL11 (■), and YEbac102ΔUL16 (▲) on Vero cells. One-step growth kinetics of infections virus production was calculated after infection at an MOI of 2 followed by incubation at 37°C.

To examine the effect of the various mutations on virus replication, Vero cells were infected at an MOI of 2 with either the wild-type or each mutant virus. Virus stocks were prepared at 0, 4, 8, 12, 24, and 36 h.p.i and titrated in triplicate onto VERO cells. Viral titers of ΔUL16 and ΔUL11 were similar to each other, with both viruses exhibiting more than a log reduction in growth when compared to the wild-type virus, YEbac102 (Fig. 3.3).

Ultrastructural characterization of the YEbac102ΔUL16 and YEbac102ΔUL11 mutant viruses

The ultrastructural phenotypes of the Ybac102ΔUL16, and YEbac102ΔUL11 viruses relative to the YEbac102 parental virus were investigated utilizing transmission electron microscopy at 24 h.p.i. As expected, the YEbac102 virus exhibited no apparent defects in virion egress as exemplified by the presence of fully enveloped virions extracellularly, as well as the presence of fully enveloped virions intracellularly (Melancon et al., 2005)(not shown). Additionally, the YEbac102ΔUL11 virus exhibited a phenotype similar to that described in previous experiments, notably, accumulations of largely unenveloped capsids in the cytoplasm associated with a more electron dense material presumed to be composed of tegument proteins (Fulmer et al., 2007)(not shown). In contrast, the YEbac102ΔUL16 virus exhibited a phenotype suggesting a block in nuclear egress. Uncommon accumulation of nucleocapsids were found in the nucleus, while a low number of cytoplasmic virions and extracellular fully enveloped virions were also noted (Fig. 3.4).

Discussion

HSV-1 cytoplasmic virion envelopment is thought to be mediated by complex interactions among tegument proteins and viral glycoproteins embedded in the TGN membranes. A number of viral glycoproteins have been shown to either interact with tegument proteins or otherwise affect cytoplasmic envelopment. Likewise, specific tegument proteins have been implicated in cytoplasmic virion

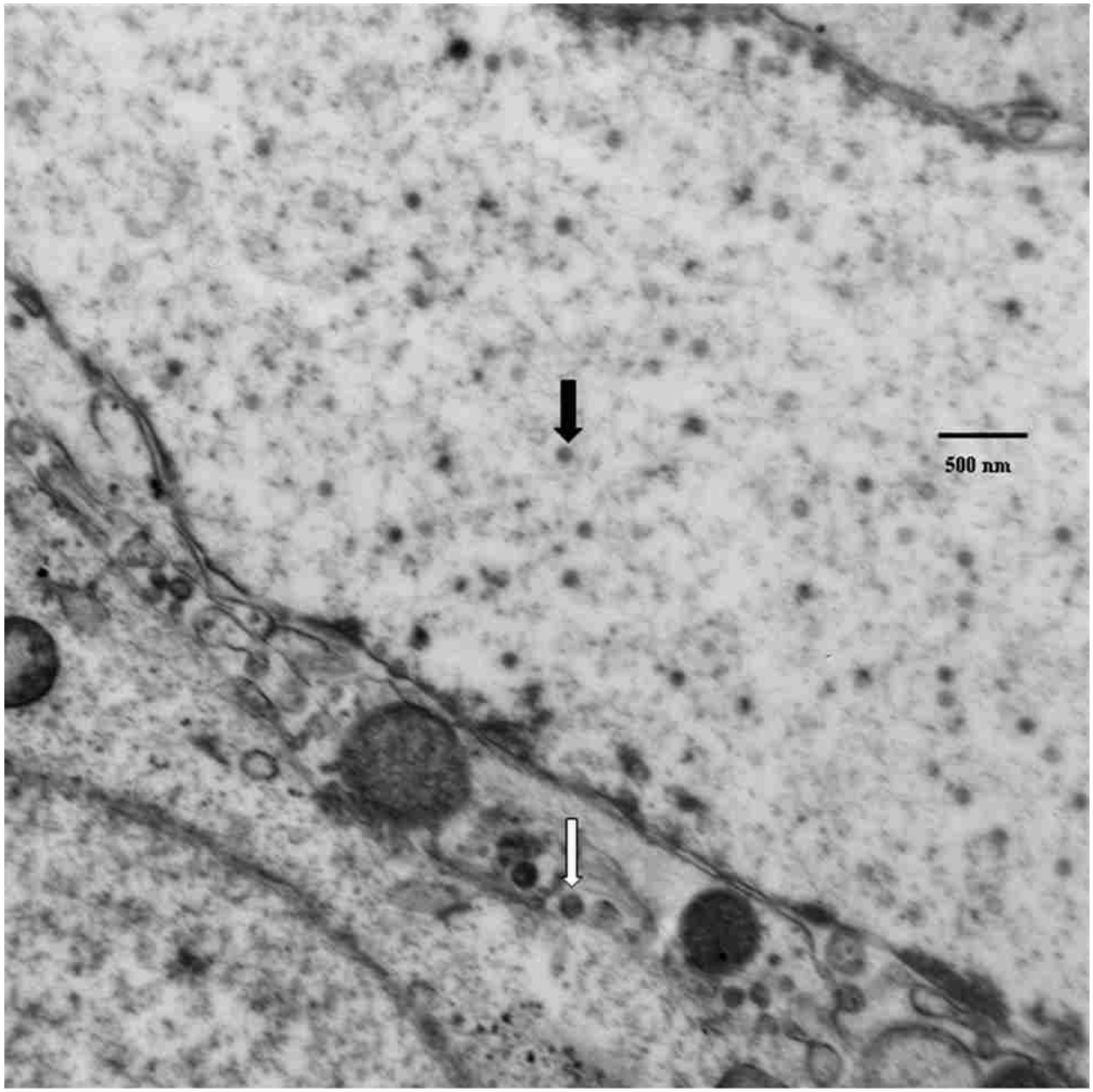


Figure 3.4: Ultrastructural characterization of ΔUL16. Confluent cell monolayers were infected with the YEbac102ΔUL16 virus at an MOI of 2, incubated for 24 hrs at 37°C, and prepared for transmission electron microscopy. Typical intranuclear accumulation of capsids (black arrow). Also indicated are unenveloped cytoplasmic virions (white arrow).

envelopment, presumably because they either directly or indirectly affect proper interactions with the cytoplasmic domains of viral membrane proteins and glycoproteins embedded within TGN-derived vesicles. Of particular interest to this study was the interaction between the membrane associated tegument protein UL11 and the virion associated tegument protein UL16. In this manuscript we created a HSV-1 mutant that contained a single point mutation to silence the UL16 start codon. We compared this virus, ΔUL16, with our previously constructed UL11-null virus, ΔUL11. We found that: 1) ΔUL16 exhibits similar plaque morphology to that of ΔUL11; 2) However, unlike ΔUL11, ΔUL16 exhibited predominantly a nuclear egress defect; 3) UL16 may also have a defect in cytoplasmic envelopment, because examination of multiple electron micrographs suggested the presence of unenveloped capsids in the cytoplasm at time points, where wild-type infections did not contain any unenveloped capsids in the cytoplasm. These results led to the conclusion that UL16 functions primarily in nuclear virion assembly and egress, while secondarily may also function in cytoplasmic virion envelopment. To assess the role of UL16 in virion morphogenesis and egress, the YEbac102ΔUL16 virus was constructed using a recently described RED markerless recombination system (Tischer et al., 2006). This allowed for the construction of ΔUL16 without changing the amino acid coding sequence of the gene found on the complementary strand, UL15. UL15 is involved in HSV-1 viral genome cleavage and packaging into mature virions (Abbotts et al., 2000; Beard, Taus, and Baines, 2002). UL16 is encoded on the complementary strand to UL15, located completely within the single intron of UL15. The role of this intron is not well understood. Previous studies have shown that the UL15 intron did not serve to downregulate UL16 expression by an antisense mechanism, nor is the splicing event itself necessary for proper UL15 function (Baines and Roizman, 1992a). Because the roles of the UL15 splicing event and its intron are not well understood, we sought to create a UL16-null virus that did not alter the amino acid sequence of UL15. This was accomplished by introducing a single point mutation into the UL16 ATG

to change it to ATA. Introduction of this mutation effectively silenced UL16 production, while only altering the UL15 coding sequence from GCC to GCT, both of which code for alanine. ΔUL16 exhibited a similar defect in growth kinetics to that of a previously described UL16-null virus, R710 (Baines and Roizman, 1991).

However, ΔUL16 showed a markedly different ultrastructural phenotype than that of R7210 (Baines and Roizman, 1991)(not shown). Specifically, ΔUL16 virions exhibited predominantly a nuclear defect resulting in accumulation of virions within the nucleus, as well as a slight accumulation of unenveloped virions in the cytoplasm. This observation seems to suggest two roles for UL16, one in nuclear capsid assembly/egress, and a second role in cytoplasmic envelopment. The differences in the ultrastructural phenotypes between ΔUL16 and R7210 may be attributed to a possible role for the UL15 intron sequences deleted in R7210, but remaining in ΔUL16.

The UL11 protein is anchored to TGN membranes through an N-terminal myristylate anchor. In addition, the UL11 protein contains a di-Leucine and an acidic amino acid motif, which are known to be involved in physical interactions with the UL16 protein, as well as recycling from the plasma membranes to TGN. Deletion of either motif abrogates UL16 interaction and plasma membrane TGN recycling (Loomis et al., 2001; Loomis, Courtney, and Wills, 2003). Interaction of this TGN membrane associated UL11 and the capsid associated UL16 provides a potential pathway for interactions between mature cytoplasmic virions and glycoprotein bearing TGN-vesicles for final cytoplasmic envelopment and egress from infected cells. The relatively minor accumulation of cytoplasmic tegumented capsids in ΔUL16 infected cells indicates that cytoplasmic capsids, which escaped the nuclear egress block, may not be able to efficiently envelope by budding into TGN membranes, in agreement with the known interactions between UL11 and UL16. However, the presence of fully enveloped virions suggests that this interaction does not play a major role in cytoplasmic virion envelopment. HSV proteins appear to

be multifunctional contain separable domains that possess different functions in the life cycle of the virus. Therefore, generation of a cadre of UL16 mutations and truncations may be able to segregate domains of UL16 that function in nuclear egress from those that function in cytoplasmic virion envelopment.

REFERENCES

Abbotts, A. P., Preston, V. G., Hughes, M., Patel, A. H., and Stow, N. D. (2000). Interaction of the herpes simplex virus type 1 packaging protein UL15 with full-length and deleted forms of the UL28 protein. *J Gen Virol* 81(Pt 12), 2999-3009.

Baines, J. D., Jacob, R. J., Simmerman, L., and Roizman, B. (1995). The herpes simplex virus 1 UL11 proteins are associated with cytoplasmic and nuclear membranes and with nuclear bodies of infected cells. *Journal of Virology* 69(2), 825-33.

Baines, J. D., and Roizman, B. (1991). The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. *Journal of Virology* 65(2), 938-44.

Baines, J. D., and Roizman, B. (1992a). The cDNA of UL15, a highly conserved herpes simplex virus 1 gene, effectively replaces the two exons of the wild-type virus. *J Virol* 66(9), 5621-6.

Baines, J. D., and Roizman, B. (1992b). The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. *Journal of Virology* 66(8), 5168-74.

Beard, P. M., Taus, N. S., and Baines, J. D. (2002). DNA cleavage and packaging proteins encoded by genes U(L)28, U(L)15, and U(L)33 of herpes simplex virus type 1 form a complex in infected cells. *J Virol* 76(10), 4785-91.

Bowzard, J. B., Visalli, R. J., Wilson, C. B., Loomis, J. S., Callahan, E. M., Courtney, R. J., and Wills, J. W. (2000). Membrane targeting properties of a herpesvirus tegument protein-retrovirus Gag chimera. *J Virol* 74(18), 8692-9.

Browne, H., Bell, S., Minson, T., and Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* 70(7), 4311-6.

Foster, T. P., Alvarez, X., and Kousoulas, K. G. (2003). Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion. *J Virol* 77(1), 499-510.

Foster, T. P., Melancon, J. M., Baines, J. D., and Kousoulas, K. G. (2004). The Herpes Simplex Virus Type 1 UL20 Protein Modulates Membrane Fusion Events during Cytoplasmic Virion Morphogenesis and Virus-Induced Cell Fusion. *J Virol* 78(10), 5347-57.

Foster, T. P., Rybachuk, G. V., and Kousoulas, K. G. (2001). Glycoprotein K specified by herpes simplex virus type 1 is expressed on virions as a Golgi complex-dependent glycosylated species and functions in virion entry. *J Virol* 75(24), 12431-8.

Fulmer, P. A., Melancon, J. M., Baines, J. D., and Kousoulas, K. G. (2007). UL20 protein functions precede and are required for the UL11 functions of herpes simplex virus type 1 cytoplasmic virion envelopment. *J Virol* 81(7), 3097-108.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N., and Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *J Virol* 75(8), 3675-84.

Harley, C. A., Dasgupta, A., and Wilson, D. W. (2001). Characterization of herpes simplex virus-containing organelles by subcellular fractionation: role for organelle acidification in assembly of infectious particles. *J Virol* 75(3), 1236-51.

Kopp, M., Granzow, H., Fuchs, W., Klupp, B. G., Mundt, E., Karger, A., and Mettenleiter, T. C. (2003). The pseudorabies virus UL11 protein is a virion component involved in secondary envelopment in the cytoplasm. *J Virol* 77(9), 5339-51.

Lee, E. C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D. A., Court, D. L., Jenkins, N. A., and Copeland, N. G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73(1), 56-65.

Loomis, J. S., Bowzard, J. B., Courtney, R. J., and Wills, J. W. (2001). Intracellular trafficking of the UL11 tegument protein of herpes simplex virus type 1. *J Virol* 75(24), 12209-19.

Loomis, J. S., Courtney, R. J., and Wills, J. W. (2003). Binding partners for the UL11 tegument protein of herpes simplex virus type 1. *J Virol* 77(21), 11417-24.

MacLean, C. A., Clark, B., and McGeoch, D. J. (1989). Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristylated. *Journal of General Virology* 70(Pt 12), 3147-57.

Melancon, J. M., Foster, T. P., and Kousoulas, K. G. (2004). Genetic analysis of the herpes simplex virus type 1 UL20 protein domains involved in cytoplasmic virion envelopment and virus-induced cell fusion. *J Virol* 78(14), 7329-43.

Melancon, J. M., Luna, R. E., Foster, T. P., and Kousoulas, K. G. (2005). Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment. *J Virol* 79(1), 299-313.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J Virol* 76(4), 1537-47.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* 106(2), 167-80.

Mettenleiter, T. C. (2006). Intriguing interplay between viral proteins during herpesvirus assembly or: the herpesvirus assembly puzzle. *Vet Microbiol* 113(3-4), 163-9.

Nalwanga, D., Rempel, S., Roizman, B., and Baines, J. D. (1996). The UL 16 gene product of herpes simplex virus 1 is a virion protein that colocalizes with intranuclear capsid proteins. *Virology* 226(2), 236-42.

Oshima, S., Daikoku, T., Shibata, S., Yamada, H., Goshima, F., and Nishiyama, Y. (1998). Characterization of the UL16 gene product of herpes simplex virus type 2. *Arch Virol* 143(5), 863-80.

Roizman, B., and Sears, A. E. (2001). Herpes Simplex Viruses and Their Replication. Third edition ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2399-2459. 2 vols. Lippincott-Williams & Wilkins, Philadelphia, PA.

Skepper, J. N., Whiteley, A., Browne, H., and Minson, A. (2001). Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment --> deenvelopment --> reenvelopment pathway. *J Virol* 75(12), 5697-702.

Tanaka, M., Kagawa, H., Yamanashi, Y., Sata, T., and Kawaguchi, Y. (2003). Construction of an excisable bacterial artificial chromosome containing a full-length infectious clone of herpes simplex virus type 1: viruses reconstituted from the clone exhibit wild-type properties in vitro and in vivo. *J Virol* 77(2), 1382-91.

Tischer, B. K., von Einem, J., Kaufer, B., and Osterrieder, N. (2006). Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. *Biotechniques* 40(2), 191-7.

Vittone, V., Diefenbach, E., Triffett, D., Douglas, M. W., Cunningham, A. L., and Diefenbach, R. J. (2005). Determination of interactions between tegument proteins of herpes simplex virus type 1. *J Virol* 79(15), 9566-71.

Ward, P. L., Barker, D. E., and Roizman, B. (1996). A novel herpes simplex virus 1 gene, UL43.5, maps antisense to the UL43 gene and encodes a protein which colocalizes in nuclear structures with capsid proteins. *J Virol* 70(5), 2684-90.

Ward, P. L., Ogle, W. O., and Roizman, B. (1996). Assemblons: nuclear structures defined by aggregation of immature capsids and some tegument proteins of herpes simplex virus 1. *J Virol* 70(7), 4623-31.

Wing, B. A., Lee, G. C., and Huang, E. S. (1996). The human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. *J Virol* 70(6), 3339-45.

Zhu, Z., Gershon, M. D., Hao, Y., Ambron, R. T., Gabel, C. A., and Gershon, A. A. (1995). Envelopment of varicella-zoster virus: targeting of viral glycoproteins to the trans-Golgi network. *J Virol* 69(12), 7951-9.

CHAPTER 4
CONCLUDING REMARKS

Summary

Herpes simplex virus type-1 (HSV-1) morphogenesis occurs in multiple stages within infected cells. Initially, the virion capsid assembles within the nucleus and the virion acquires an initial envelope by budding into the perinuclear spaces (Roizman and Sears, 2001). Subsequently, these enveloped virions fuse with the outer nuclear lamellae leading to the accumulation of unenveloped capsids into the cytoplasm. Within the cytoplasm, a number of additional tegument proteins attach to the capsid and the fully tegumented capsids bud into cytoplasmic vesicles, which mostly likely originate from the *trans*-Golgi network (TGN). Enveloped virions are ultimately secreted to extracellular spaces through the utilization of cellular vesicular trafficking systems (Browne et al., 1996; Granzow et al., 2001; Harley, Dasgupta, and Wilson, 2001; Mettenleiter, 2002; Skepper et al., 2001; Zhu et al., 1995). The process by which the tegumented cytoplasmic capsids bud into TGN-derived vesicles is not well-understood. The prevalent model calls for specific interactions among viral tegument proteins and membrane proteins and glycoproteins embedded within TGN membranes as key factors that drive cytoplasmic virion envelopment. This model is supported by evidence that specific mutations within tegument proteins and multiple membrane proteins and glycoproteins inhibit cytoplasmic envelopment (Mettenleiter, 2004; Mettenleiter, 2006).

To further investigate the roles of tegument proteins in cytoplasmic virion envelopment, we constructed deletion mutants of UL11 (ΔUL11), UL20 (ΔUL20), both UL11 and UL20 (ΔUL11ΔUL20), and UL16 (ΔUL16). The UL11 ORF encodes a 96 amino acid tegument protein, which is N-terminally myristylated (MacLean, Clark, and McGeoch, 1989) and palmitylated (Loomis et al., 2001). The UL11

protein localizes to nuclear and TGN-derived apparatus derived membranes in infected cells (Baines et al., 1995), but only to the TGN-membranes in non-infected cells (Bowzard et al., 2000). UL11 is believed to play a significant role in cytoplasmic envelopment as UL11 deletion mutants display a severe defect at this step in both HSV-1 (Baines and Roizman, 1992b; Fulmer et al., 2007) and PRV (Kopp et al., 2003). The interaction of UL11 with UL16 is dependant upon the presence of an acidic amino acid cluster and a di-leucine motif located within the first 50 amino acids of UL11 (Loomis, Courtney, and Wills, 2003). The interaction between UL11 on TGN-membranes and the capsid associated UL16 presents a possible pathway for the recruitment of cytoplasmic capsids to TGN-derived membranes for final envelopment. The UL20 ORF encodes a multipass transmembrane proteins of 222 amino acids and is conserved in all alphaherpesviruses (Debroy, Pederson, and Person, 1985; MacLean et al., 1991; Ramaswamy and Holland, 1992). UL20p localizes to TGN membranes after endocytosis from cell surfaces (Foster et al., 2004b). UL20p is essential for cytoplasmic virion morphogenesis, since mutant viruses lacking UL20p accumulate capsids within the cytoplasm that are unable to acquire envelopes by budding into TGN-associated membranes (Dietz et al., 2000; Foster, Alvarez, and Kousoulas, 2003; Foster et al., 2004b; Fuchs et al., 1997; Melancon, Foster, and Kousoulas, 2004b). Furthermore, UL20p is essential for virus-induced cell fusion caused by either gB or gK syncytial mutations, and it is necessary for gK cell-surface expression. Recently, our laboratory has shown that gK and UL20 interact and that this interaction is essential for their co-transport and membrane fusion and virion morphogenesis functions (Foster et al., 2004d; Melancon et al., 2005). The UL16 ORF encodes a 373 amino acid protein (Nalwanga et al., 1996) that is associated with intra-nuclear assemblons early in the HSV-1 life cycle and is later found associated with peri-nuclear virions and is a component of purified virions in HSV-1 and HCMV(Nalwanga et al., 1996; Ward, Barker, and Roizman, 1996; Wing, Lee, and Huang, 1996), but not HSV-2 (Oshima et al., 1998). Assemblons are believed to be sites at

which capsid assembly and/or DNA cleavage and packaging may occur (Ward, Ogle, and Roizman, 1996), suggesting a possible role for UL16 in these processes.

Chapter 2 presents evidence that UL11 is involved in cytoplasmic virion envelopment. The ΔUL11 virus exhibits large amounts of unenveloped capsids in the cytoplasm of infected cells. To examine if UL11 worked synergistically with UL20 in cytoplasmic envelopment, the ΔUL11ΔUL20 virus containing deletions of both UL11 and UL20 was examined. The phenotype of the double null virus most closely resembled that of the UL20 single null virus (ΔUL20) in all areas: plaque phenotype, growth kinetics, and ultrastructural characteristics. Similar to the UL20/gK heterodimer, UL11 localizes to TGN-derived vesicles during virus infection (Foster et al., 2004b; Loomis, Courtney, and Wills, 2003; Vittone et al., 2005). To asses whether UL11 has any affect on UL20/gK localization, confocal experiments to determine the localization of UL11, UL20 and gK, revealing that UL11 transport was completely independent of UL20/gK. Taken together these results indicate that UL11 acts at a step in cytoplasmic envelopment downstream of UL20, and UL20 is required for proper UL11 function. However, UL11 is not dependent upon the UL20/gK heterodimer for its transport.

Chapter 3 presents evidence that UL16 serves a two-fold purpose in HSV-1 virion morphogenesis and egress from infected cells. To assess the role of UL16 in virion morphogenesis and egress, the YEbac102ΔUL16 virus was constructed using a recently described RED markerless recombination system (Tischer et al., 2006). This allowed for the construction of ΔUL16 without changing the amino acid coding sequence of the gene found on the complementary strand, UL15. UL15 is involved in HSV-1 viral genome cleavage and packaging into mature virions (Abbotts et al., 2000; Beard, Taus, and Baines, 2002). UL16 is encoded on the complementary strand to UL15, located completely within the single intron of UL15. The role of this intron is not well understood, past studies have shown that it does not serve to downregulate UL16 production by an antisense mechanism, nor is

the splicing event itself necessary for proper UL15 function (Baines and Roizman, 1992a). Because the role of the UL15 splicing event and its intron are not well understood, we sought to create a UL16-null virus that did not alter the amino acid sequence of UL15. This was accomplished by introducing a single point mutation into the UL16 ATG to change it to ATA. This effectively silenced UL16 production while altering the UL15 coding sequence from GCC to GCT, both of which code for alanine(Baines and Roizman, 1991). ΔUL16 exhibited a plaque phenotype and growth kinetics similar to that of the ΔUL11 virus. However, the viruses differed slightly with respect to their ultrastructural characteristics. While both ΔUL16 and ΔUL11 exhibited a cytoplasmic envelopment defect, the defect seen in ΔUL16 was much less severe than that of ΔUL11. Instead, ΔUL16 showed a large accumulation of intranuclear capsids not seen in the ΔUL11 virus. This result indicates a two-fold role for UL16 in virion morphogenesis and egress: 1) The nuclear accumulation of capsids seems to suggest that the first and most important role of UL16 is in intranuclear capsid assembly/egress. 2) The cytoplasmic accumulation of capsids suggests that UL16 also plays a role in cytoplasmic envelopment. These results indicate a possible pathway for the juxtaposition of cytoplasmic capsids with TGN-derived vesicles for final cytoplasmic envelopment.

In conclusion, this dissertation has capitalized on the recent availability of herpes simplex virus type-1 BAC mutagenesis protocols to quickly and precisely make targeted mutations to any gene of HSV-1. This work has led to a greater understanding of how HSV-1 undergoes cytoplasmic envelopment and egress from infected cells.

Current and future research

The work presented in Chapter 3 leaves open the question of whether or not UL11 and UL16 act synergistically in HSV-1 cytoplasmic envelopment. Our original hypothesis was that UL16 functioned

in cytoplasmic events of the lifecycle based on a previous publication with a partially deleted U16 virus, which was reported to exhibit replication defects due to the accumulation of unenveloped capsids in the cytoplasm (ref). Based on our previous work comparing the relative contributions of UL20 and UL11 genes to cytoplasmic virion envelopment (ref), we constructed a double-null UL16/UL11 virus and begun to characterize its replication and phenotypic properties. This work is currently in progress. The finding that the DUL16 virus constructed with the pYEBac102 plasmid accumulated capsids within the nucleus, apparently, unable to properly egress from the nucleus to the cytoplasm, does not allow an ordering of the UL11 and U16 functions with respect to the potential roles of these proteins in cytoplasmic virion envelopment, by a simple comparison of the replication and other characteristics of the single mutants versus that of the double-null mutant virus. It is anticipated that the double-null virus will possess the defects of the individual null mutations exhibiting approximately two logs of inhibition of virus replication versus one log inhibition of infectious virus production by each of the null mutants.

Based on the observation that a number of HSV proteins and glycoproteins are multifunctional, it can be predicted that potential roles of UL16 in virion assembly within the nucleus and in cytoplasmic tegumentation and concomitant cytoplasmic virion assembly may be discerned through the investigation of UL16 mutants that can affect one, but not necessarily the other function. An example of this experimental approach is the delineation of functional domains of the UL20 protein (UL20p) by alanine-scanning mutations, single amino acid mutations and truncations (refs), which allowed for the segregation of UL20p domains that functioned in virion assembly from those that functioned in intracellular transport of UL20p and its interacting partner gK (ref). In this regard, a collection of UL16 mutants with different mutations can be quickly tested in conjunction with the U16-null virus using complementation analysis to segregate functional domains of UL16 protein.

The ultimate goal of these studies was to begin dissecting the order of molecular events that are responsible for intracellular virion envelopment. The most current and prevalent hypothesis is that cytoplasmic nucleocapsids mature by the addition of a limited number of tegument proteins in the cytoplasm, while another set of tegument proteins bind independently of the virion capsid to the cytoplasmic portions of the membrane bound proteins and glycoproteins (ref Metenleiter). Subsequently, tegument-tegument and tegument-membrane protein interactions drive the budding of cytoplasmic capsids into the TGN derived vesicles. The molecular genetics approach used in these investigations revealed valuable information about the sequence of cytoplasmic virion maturation events that require the UL11 and UL20 functions suggesting that UL20 functions preceded those of UL11. Construction of the UL16-null mutant virus revealed an unexpected role of UL16 in nuclear egress of virions. In this regard, the genetic approach followed here provides one pathway for mining the necessary information required for understanding intracellular virion assembly. Additional experimental approaches need to be pursued including proteomic characterization of the components of fully and partially assembled virions. Furthermore, reconstitution of intracellular cytoplasmic envelopment using cellular extracts may become possible in the future in which individual mutant virion particles can be tested for their ability to acquire envelopes by budding into TGN-derived vesicles. Ultimately, complete knowledge of virion assembly may enable future researchers to build "custom" viruses as tailored "nanomachines" that could deliver specific payloads and/or perform specific tasks.

REFERENCES

Abbotts, A. P., Preston, V. G., Hughes, M., Patel, A. H., and Stow, N. D. (2000). Interaction of the herpes simplex virus type 1 packaging protein UL15 with full-length and deleted forms of the UL28 protein. *J Gen Virol* 81(Pt 12), 2999-3009.

Baines, J. D., Jacob, R. J., Simmerman, L., and Roizman, B. (1995). The herpes simplex virus 1 UL11 proteins are associated with cytoplasmic and nuclear membranes and with nuclear bodies of infected cells. *Journal of Virology* 69(2), 825-33.

Baines, J. D., and Roizman, B. (1991). The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. *Journal of Virology* 65(2), 938-44.

Baines, J. D., and Roizman, B. (1992a). The cDNA of UL15, a highly conserved herpes simplex virus 1 gene, effectively replaces the two exons of the wild-type virus. *J Virol* 66(9), 5621-6.

Baines, J. D., and Roizman, B. (1992b). The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. *Journal of Virology* 66(8), 5168-74.

Beard, P. M., Taus, N. S., and Baines, J. D. (2002). DNA cleavage and packaging proteins encoded by genes U(L)28, U(L)15, and U(L)33 of herpes simplex virus type 1 form a complex in infected cells. *J Virol* 76(10), 4785-91.

Bowzard, J. B., Visalli, R. J., Wilson, C. B., Loomis, J. S., Callahan, E. M., Courtney, R. J., and Wills, J. W. (2000). Membrane targeting properties of a herpesvirus tegument protein-retrovirus Gag chimera. *J Virol* 74(18), 8692-9.

Browne, H., Bell, S., Minson, T., and Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* 70(7), 4311-6.

Debroy, C., Pederson, N., and Person, S. (1985). Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. *Virology* 145(1), 36-48.

Dietz, P., Klupp, B. G., Fuchs, W., Kollner, B., Weiland, E., and Mettenleiter, T. C. (2000). Pseudorabies virus glycoprotein K requires the UL20 gene product for processing. *J Virol* 74(11), 5083-90.

Foster, T. P., Alvarez, X., and Kousoulas, K. G. (2003). Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion. *J Virol* 77(1), 499-510.

Foster, T. P., Melancon, J. M., Baines, J. D., and Kousoulas, K. G. (2004a). The Herpes Simplex Virus Type 1 UL20 Protein Modulates Membrane Fusion Events during Cytoplasmic Virion Morphogenesis and Virus-Induced Cell Fusion. *J Virol* 78(10), 5347-57.

Foster, T. P., Melancon, J. M., Olivier, T. L., and Kousoulas, K. G. (2004b). Herpes simplex virus type 1 glycoprotein K and the UL20 protein are interdependent for intracellular trafficking and trans-Golgi network localization. *J Virol* 78(23), 13262-77.

Fuchs, W., Klupp, B. G., Granzow, H., and Mettenleiter, T. C. (1997). The UL20 gene product of pseudorabies virus functions in virus egress. *J Virol* 71(7), 5639-46.

Fulmer, P. A., Melancon, J. M., Baines, J. D., and Kousoulas, K. G. (2007). UL20 protein functions precede and are required for the UL11 functions of herpes simplex virus type 1 cytoplasmic virion envelopment. *J Virol* 81(7), 3097-108.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N., and Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *J Virol* 75(8), 3675-84.

Harley, C. A., Dasgupta, A., and Wilson, D. W. (2001). Characterization of herpes simplex virus-containing organelles by subcellular fractionation: role for organelle acidification in assembly of infectious particles. *J Virol* 75(3), 1236-51.

Kamen, D. E., Gross, S. T., Girvin, M. E., and Wilson, D. W. (2005). Structural basis for the physiological temperature dependence of the association of VP16 with the cytoplasmic tail of herpes simplex virus glycoprotein H. *J Virol* 79(10), 6134-41.

Klupp, B. G., Granzow, H., Fuchs, W., Keil, G. M., Finke, S., and Mettenleiter, T. C. (2007). Vesicle formation from the nuclear membrane is induced by coexpression of two conserved herpesvirus proteins. *Proc Natl Acad Sci U S A* 104(17), 7241-6.

Kopp, M., Granzow, H., Fuchs, W., Klupp, B. G., Mundt, E., Karger, A., and Mettenleiter, T. C. (2003). The pseudorabies virus UL11 protein is a virion component involved in secondary envelopment in the cytoplasm. *J Virol* 77(9), 5339-51.

Loomis, J. S., Bowzard, J. B., Courtney, R. J., and Wills, J. W. (2001). Intracellular trafficking of the UL11 tegument protein of herpes simplex virus type 1. *J Virol* 75(24), 12209-19.

Loomis, J. S., Courtney, R. J., and Wills, J. W. (2003). Binding partners for the UL11 tegument protein of herpes simplex virus type 1. *J Virol* 77(21), 11417-24.

MacLean, C. A., Clark, B., and McGeoch, D. J. (1989). Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristylated. *Journal of General Virology* 70(Pt 12), 3147-57.

MacLean, C. A., Efstathiou, S., Elliott, M. L., Jamieson, F. E., and McGeoch, D. J. (1991). Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. *Journal of General Virology* 72(Pt 4), 897-906.

Melancon, J. M., Foster, T. P., and Kousoulas, K. G. (2004). Genetic analysis of the herpes simplex virus type 1 UL20 protein domains involved in cytoplasmic virion envelopment and virus-induced cell fusion. *J Virol* 78(14), 7329-43.

Melancon, J. M., Luna, R. E., Foster, T. P., and Kousoulas, K. G. (2005). Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment. *J Virol* 79(1), 299-313.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J Virol* 76(4), 1537-47.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* 106(2), 167-80.

Mettenleiter, T. C. (2006). Intriguing interplay between viral proteins during herpesvirus assembly or: the herpesvirus assembly puzzle. *Vet Microbiol* 113(3-4), 163-9.

Nalwanga, D., Rempel, S., Roizman, B., and Baines, J. D. (1996). The UL 16 gene product of herpes simplex virus 1 is a virion protein that colocalizes with intranuclear capsid proteins. *Virology* 226(2), 236-42.

O'Regan, K. J., Bucks, M. A., Murphy, M. A., Wills, J. W., and Courtney, R. J. (2007). A conserved region of the herpes simplex virus type 1 tegument protein VP22 facilitates interaction with the cytoplasmic tail of glycoprotein E (gE). *Virology* 358(1), 192-200.

Oshima, S., Daikoku, T., Shibata, S., Yamada, H., Goshima, F., and Nishiyama, Y. (1998). Characterization of the UL16 gene product of herpes simplex virus type 2. *Arch Virol* 143(5), 863-80.

Ramaswamy, R., and Holland, T. C. (1992). In vitro characterization of the HSV-1 UL53 gene product. *Virology* 186(2), 579-87.

Roizman, B., and Sears, A. E. (2001). Herpes Simplex Viruses and Their Replication. Third edition ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2399-2459. 2 vols. Lippincott-Williams & Wilkins, Philadelphia, PA.

Skepper, J. N., Whiteley, A., Browne, H., and Minson, A. (2001). Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment --> deenvelopment --> reenvelopment pathway. *J Virol* 75(12), 5697-702.

Tischer, B. K., von Einem, J., Kaufer, B., and Osterrieder, N. (2006). Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. *Biotechniques* 40(2), 191-7.

Vittone, V., Diefenbach, E., Triffett, D., Douglas, M. W., Cunningham, A. L., and Diefenbach, R. J. (2005). Determination of interactions between tegument proteins of herpes simplex virus type 1. *J Virol* 79(15), 9566-71.

Ward, P. L., Barker, D. E., and Roizman, B. (1996). A novel herpes simplex virus 1 gene, UL43.5, maps antisense to the UL43 gene and encodes a protein which colocalizes in nuclear structures with capsid proteins. *J Virol* 70(5), 2684-90.

Ward, P. L., Ogle, W. O., and Roizman, B. (1996). Assemblons: nuclear structures defined by aggregation of immature capsids and some tegument proteins of herpes simplex virus 1. *J Virol* 70(7), 4623-31.

Wing, B. A., Lee, G. C., and Huang, E. S. (1996). The human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. *J Virol* 70(6), 3339-45.

Zhu, Z., Gershon, M. D., Hao, Y., Ambron, R. T., Gabel, C. A., and Gershon, A. A. (1995). Envelopment of varicella-zoster virus: targeting of viral glycoproteins to the trans-Golgi network. *J Virol* 69(12), 7951-9.

APPENDIX
ADDITIONAL WORK

Disclaimer

The work presented in this appendix was begun in our lab by a previous student, Jeff Melancon. Jeff left the lab before this work could be completed, so I finished the project. This work has not been presented before, either in Jeff's dissertation, or in a peer-reviewed publication. However, as I was not the sole party responsible for the body of the work, I did not feel it warranted a separate chapter and instead it is presented here. The work presented in this appendix highlights my contributions to the project.

Introduction

Herpes simplex viruses (HSV) specify at least eleven virus-specified glycoproteins, as well as several non-glycosylated membrane associated proteins, most of which play important roles in multiple membrane fusion events during virus entry and intracellular virion morphogenesis and egress (Roizman and Sears, 1996; Spear, 1993a; Spear, 1993b; Spear, Eisenberg, and Cohen, 2000). Spread of infectious virus occurs either by release of virions to extracellular spaces or through virus-induced cell-to-cell fusion. In vivo, the latter mechanism allows for virus spread without exposing virions to extracellular spaces containing neutralizing antibodies. Mutations that cause extensive virus-induced cell fusion predominantly arise in four genes of the HSV genome: the UL20 gene (Baines et al., 1991; MacLean et al., 1991), the UL24 gene (Jacobson et al., 1998; Sanders, Wilkie, and Davison, 1982), the UL27 gene encoding glycoprotein B (gB) (Bzik et al., 1984; Pellett et al., 1985), and the UL53 gene coding for glycoprotein K (gK) (Bond and Person, 1984; Debroy, Pederson, and Person, 1985; Hutchinson

et al., 1992b; Pogue-Geile et al., 1984; Ryechan et al., 1979). Of these four membrane associated proteins, only UL20 and gK are absolutely essential for the intracellular transport of virions to extracellular spaces in all cell types (Baines et al., 1991; Foster and Kousoulas, 1999; Fuchs et al., 1997; Hutchinson and Johnson, 1995; Jayachandra, Baghian, and Kousoulas, 1997). Virus-induced cell fusion requires the coordinate action of multiple viral glycoproteins including gD, gH, gL, gB, gM, gK, as well as UL20p. This conclusion is supported by the fact that gB syncytial mutations do not cause virus-induced cell fusion in the absence of either gH, gM, gK, or UL20. Similarly, syncytial mutations in gK do not cause virus-induced cell fusion in the absence of gB, or gH.

The most prevalent model of morphogenesis and egress of infectious herpes virions is thought to involve sequential de-envelopment and re-envelopment steps in transit to extracellular spaces: a) primary envelopment by budding of capsids assembled in the nuclei through the inner nuclear leaflet leading to the production of enveloped virions within perinuclear spaces; b) de-envelopment by fusion of viral envelopes with the outer nuclear leaflet leading to the accumulation of unenveloped capsids in the cytoplasm; c) assembly of sets of tegument proteins on the cytoplasmic capsids, as well as potentially on vesicle sites to be used for cytoplasmic envelopment; d) re-envelopment of cytoplasmic tegumented capsids into TGN-derived vesicles. This final event in cytoplasmic virion envelopment is thought to be largely mediated by interactions between tegument proteins and cytoplasmic portions of viral glycoproteins embedded within the TGN-derived membranes. Cytoplasmically enveloped viruses are thought to be transported to extracellular spaces within Golgi or TGN-derived vesicles (reviewed in: (Johnson and Huber, 2002; Mettenleiter, 2002; Tomishima, Smith, and Enquist, 2001).

The UL20 gene encodes a 222 amino acid non-glycosylated transmembrane protein that is conserved by all alphaherpesviruses. The UL20p is a structural component of extracellular enveloped virions and it is expressed in infected cells assuming a predominantly perinuclear and cytoplasmic distribution (Ward et al., 1994). An initial report indicated that partial deletion of the UL20 gene resulted in perinuclear accumulation of capsids indicating that the UL20 gene functioned, most likely, in the de-envelopment of enveloped virions found within perinuclear spaces (Baines et al., 1991). However, we showed previously that a precise deletion of the UL20 gene revealed that the UL20 gene strictly functioned in cytoplasmic envelopment of capsids (Foster et al., 2004a). In addition, syncytial mutations in either gB or gK failed to cause fusion in the absence of the UL20 gene, suggesting that the UL20 protein was essential for virus-induced cell fusion (Foster et al., 2004a). Furthermore, we showed that UL20 is required for cell-surface expression of gK and TGN localization, suggesting a functional interdependence between gK and UL20 for virus egress and cell-to-cell fusion (Dietz et al., 2000; Foster, Alvarez, and Kousoulas, 2003). Recently, we delineated via site-directed mutagenesis the functional domains of UL20p involved in infectious virus production and virus-induced cell fusion. Importantly, we showed that both amino and carboxyl terminal portions of UL20p, which are predicted to lie within the cytoplasmic side of cellular membranes, function both in cytoplasmic virion envelopment and virus-induced cell fusion.

In this manuscript, we extend our previous findings by examining the effect previous as well as new mutations within UL20p in intracellular transport, cell surface expression and TGN localization of UL20 and gK. Our findings show that the amino and carboxyl termini of UL20p contain distinct domains that function in infectious virion production and intracellular transport and suggest that putative phosphorylation sites in the amino terminus of UL20p play important

roles in infectious virus production and virus-induced cell fusion, but not intracellular transport of UL20p and gK.

Materials and Methods

Cells and viruses

African green monkey kidney (Vero) cells were obtained from ATCC (Rockville, MD). The Vero-based UL20 complementing cell line, G5, was a gift of Dr. P. Desai, (John Hopkins Medical Center) (Desai et al., 1993). Cells were maintained as previously described (Desai et al., 1993; Foster, Alvarez, and Kousoulas, 2003; Foster and Kousoulas, 1999). The parental wild-type strain used in this study HSV-1 (KOS) was originally obtained from P. A. Schaffer (Harvard Medical School). Δ20DIV5, Δ20gBsyn3 and Δ20gKsyn1DIV5 viruses were as described previously (Foster et al., 2004a). Virus stocks were grown on the UL20 complementing cell line Fd20-1, the construction of which was described previously (Melancon, Foster, and Kousoulas, 2004a). In this paper, for simplification purposes, the Δ20DIV5 virus is referred to as Δ20 virus and the Δ20syngK1DIV5 virus is referred to as Δ20gKsyn1 virus (Melancon, Foster, and Kousoulas, 2004a).

Plasmids

pCR2.1-UL20, which was used as the parental vector for UL20 mutagenesis, was generated by cloning a 773bp DNA fragment containing the UL20 gene, obtained by PCR amplification of HSV-1(KOS) viral DNA, into pCR2.1/TOPO (Invitrogen) as described in detail previously (Melancon, Foster, and Kousoulas, 2004a). The generation of UL20 cluster to alanine mutants CL38, Cl49, CL153, and CL209, the single point mutant Y49A, and truncation mutants, 204t,

211t, 216t were reported previously (Melancon, Foster, and Kousoulas, 2004a). A set of new UL20 mutants generated for this study included, a UL20 mutant containing both the CL38 and CL49 mutations (CL38—CL49), the alanine cluster UL20 mutant CL61, and the single point mutants Y38A, and the UL20 mutant Y-Y containing both the Y38A and Y49A mutations. The additional single point UL20 mutants as well as the double mutants were generated by splice-overlap extension (SOE) PCR (Aiyar, Xiang, and Leis, 1996) as described previously (Melancon, Foster, and Kousoulas, 2004a) . CL2 and CL61 mutants were generated using the GeneTailor™ Site-Directed Mutagenesis Kit as directed by the manufacturer (Invitrogen), and as described previously for the other CL mutants (Melancon, Foster, and Kousoulas, 2004a).

UL20 complementation assay for infectious virion production

Confluent Vero monolayers in six well plates were transfected with 2 μg of wild-type or mutant UL20 plasmid with Lipofectamine 2000 as described by the manufacturer (Invitrogen). Six hours post-transfection, the monolayers were infected with a UL20-null virus at an MOI of 1. Infections were placed on a rocker for 1 hour at 4°C, and then transferred to 37°C for 2 hours. Residual virus was inactivated using an acid wash (PBS containing .5M glycine, pH3) for 2 min, and monolayers were subsequently washed 3 times with DMEM to restore the pH to a normal level. Infections were incubated at 37°C for 24 hours. After repeated freeze/thaw cycles, virus stocks were titered in triplicate on Fd20-1 cells, which effectively complement the UL20-null defect (Melancon, Foster, and Kousoulas, 2004a). The complementation ratio for each mutant was calculated with the formula (virus titer of mutant / virus titer of positive control).

UL20 complementation assay for virus-induced cell-to-cell fusion

Confluent Vero monolayers in six-well plates were transfected with 2 μg of wild-type or mutant UL20 plasmid with Lipofectamine 2000 as described by the manufacturer (Invitrogen). 18 hours post transfection, the monolayers were infected at an MOI of 0.1 with either Δ20gKsyn1 or Δ20gBsyn3 viruses. Infections were placed on a rocker at room temperature for 1 hour, then transferred to 37°C for 30 minutes. Cells were overlaid with DMEM containing 1% methylcellulose. 24 hours post-infection, cell fusion was determined by visualization of syncitia formation by light microscopy. Cells were stained with a polyclonal HRP conjugated HSV-1 antibody as directed by the manufacturer (DakoCytomation). Briefly, cells were washed with PBS to remove methylcellulose media, and fixed with 4°C methanol for 15 minutes. TBS containing a 1:750 dilution of the polyclonal HSV-1 antibody was added to the cells and placed on a rocker at 4°C for 1 h. Cells were washed with TBS and developed using the VECTOR NovaRED peroxidase substrate kit as directed by the manufacturer (VECTOR , Inc).

Results

Mutagenesis of HSV-1 UL20

Previously, we reported on the construction and characterization of a panel of 31 mutations within the UL20 gene (Melancon, Foster, and Kousoulas, 2004a). These mutations included: 1) cluster-to-alanine mutants in which a cluster of proximal amino acids were changed to alanine residues; 2) single amino acid replacement mutants within alanine cluster regions; 3) carboxyl terminal truncations of UL20p. Two additional double mutants where constructed for the present study. UL20 mutant CL38—CL49 combined the two cluster mutations targeting the two putative phosphorylation sites in the amino terminus of UL20p. Similarly, the Y38A—Y49A double

mutant combined the two specific tyrosine modifications without altering adjacent amino acids. In addition, UL20 mutants CL2, CL61, Y38A, and Y117A, which were not reported previously, were included in these investigations. All UL20 mutants were tested for their ability to complement UL20-null infectious virus production as well as either gB or gK-mediated virus-induced cell fusion. The mutated amino acids for each type of mutation included in this study are shown in Table 1. The carboxyl terminal truncations are identified with the number of the last remaining amino acid (i.e. 204t retains UL20p amino acids 1-204). The location of each mutation with respect to the predicted and experimentally confirmed topology of UL20p (Melancon, Foster, and Kousoulas, 2004a) is shown in Figure A.1.

Complementation assay for infectious virus production.

It was previously shown that deletion of the HSV-1 UL20 and the PRV UL20 genes resulted in up to two logs reduction in infectious virus production relative to their parental wild type strains (Baines et al., 1991; Foster et al., 2004a; Fuchs et al., 1997). The cadre of single or double UL20 mutants and UL20p truncations were tested for their ability to complement the HSV-1(KOS) UL20-null virus. Complementation experiments involved transfection of Vero cells with plasmids encoding wild-type or mutant UL20 genes, followed by infection with the UL20-null virus as reported previously (Foster et al., 2004a) and described in Materials and Methods. A complementation ratio was calculated for each mutant UL20 plasmid as a percent ratio to complementation levels provided by the wild-type UL20 gene. The UL20 wild-type gene effectively complemented UL20-null virus infectious virus production, while most of the UL20 mutants failed to complement the UL20-null virus with the exception of the CL2 and Y117A mutants (Figure A.2).

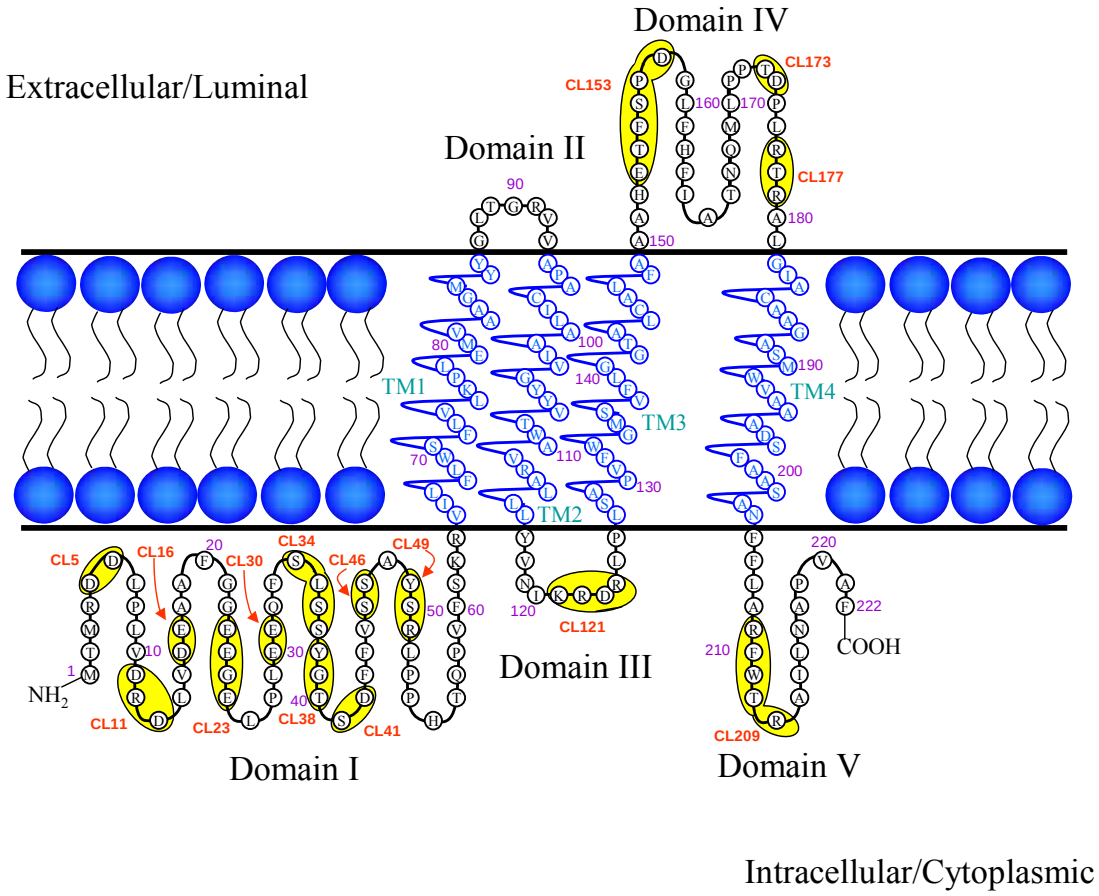


Figure A.1: Predicted membrane topology of UL20p and location of the 15 cluster-to-alanine mutations. Membrane topology was predicted using the TMPred and SOSUI algorithms (19, 20). UL20p domains where cluster-to-alanine mutations are located are indicated by a shaded oval. Naming of cluster mutations is based on the first amino acid mutated in each cluster. Transmembrane region (TM), Cluster mutant (CL).

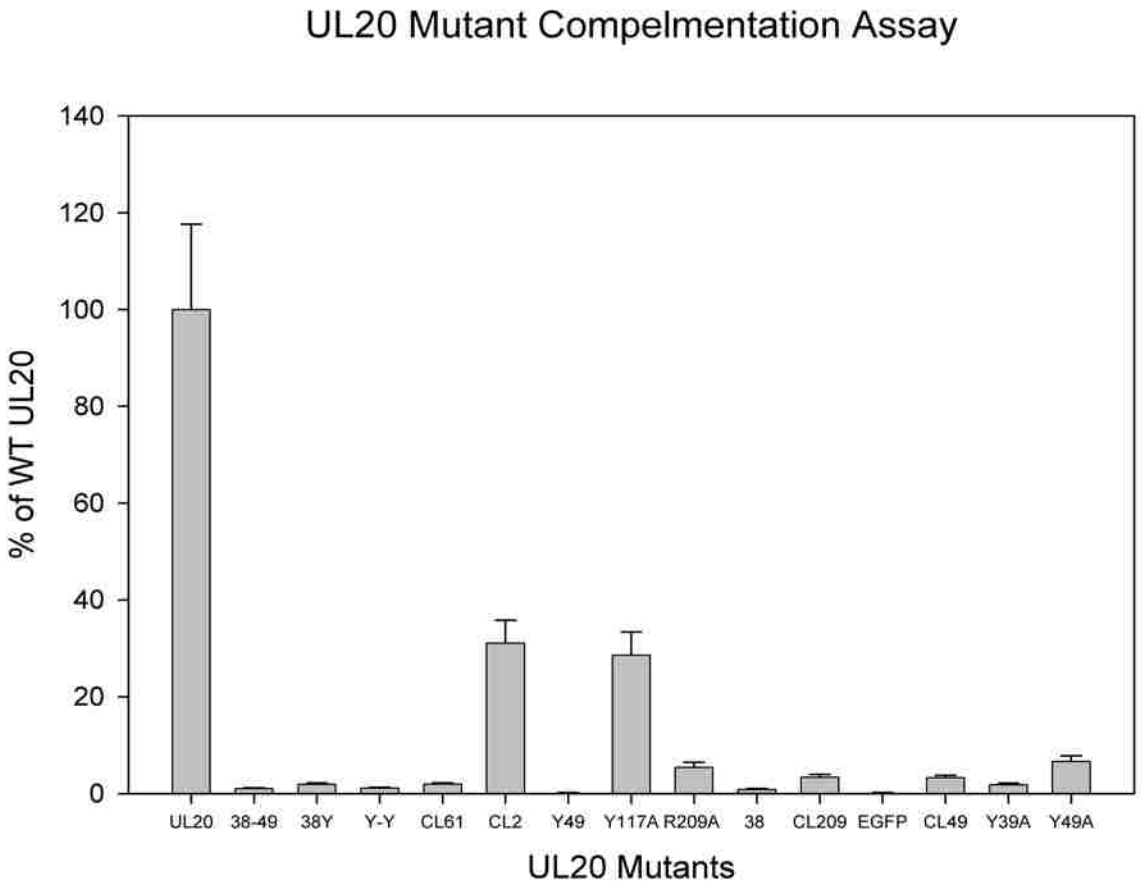


Figure A.2: Complementation by UL20 mutants. Ratios of complementation for various UL20 mutants. Vero cells were transfected with the indicated mutant then infected with the ΔUL20 virus.

Complementation for virus-induced cell-to-cell fusion

We previously showed that syncytial mutations in either gB or gK failed to cause virus-induced cell fusion in the absence of the UL20 gene (Foster et al., 2004a). Furthermore, a panel of 31 different UL20 mutants revealed that UL20 domains that functioned in infectious virus production segregated from those that functioned in virus-induced cell fusion (Melancon, Foster, and Kousoulas, 2004a). The panel of UL20 mutants shown in Table 1 containing additional UL20 mutants was tested for the ability to complement UL20-null viruses containing syncytial mutations in either gB (syn3) or gK (syn1) for virus-induced cell fusion as described previously (Melancon, Foster, and Kousoulas, 2004a). Briefly, confluent Vero monolayers were transfected with plasmids encoding either wild type or mutant UL20p, and subsequently infected with either Δ20gKsyn1 or Δ20gBsyn3 viruses. Viral plaques appearing as larger plaques in a background of uniformly small UL20-null viral plaques were stained with anti-HSV-1 polyclonal antibody as described in Materials and Methods (Figure A.3). In this complementation assay, 20-40% of all viral plaques appeared considerably larger than the uniformly small UL20-null plaques (not shown). The CL2 UL20 mutant (Figure A. 3) and Y117A (not shown) complemented effectively both gB and gK-mediated virus-induced cell fusion producing rescued viral plaques similar in size to those produced by the wild-type UL20 gene. The CL49 and Y49A mutations partially complemented virus-induced cell fusion caused by syncytial mutations in either gB or gK, as evidenced by the production of visibly larger than the UL20-null viral plaques; however, these plaques were substantially smaller than those produced by complementation with the wild-type UL20 gene. For comparative purposes, previously, we showed that the 204t and 211t UL20

truncations failed to complement virus induced cell fusion, while the 216t truncation efficiently complemented virus-induced cell fusion (Melancon, Foster, and Kousoulas, 2004a).

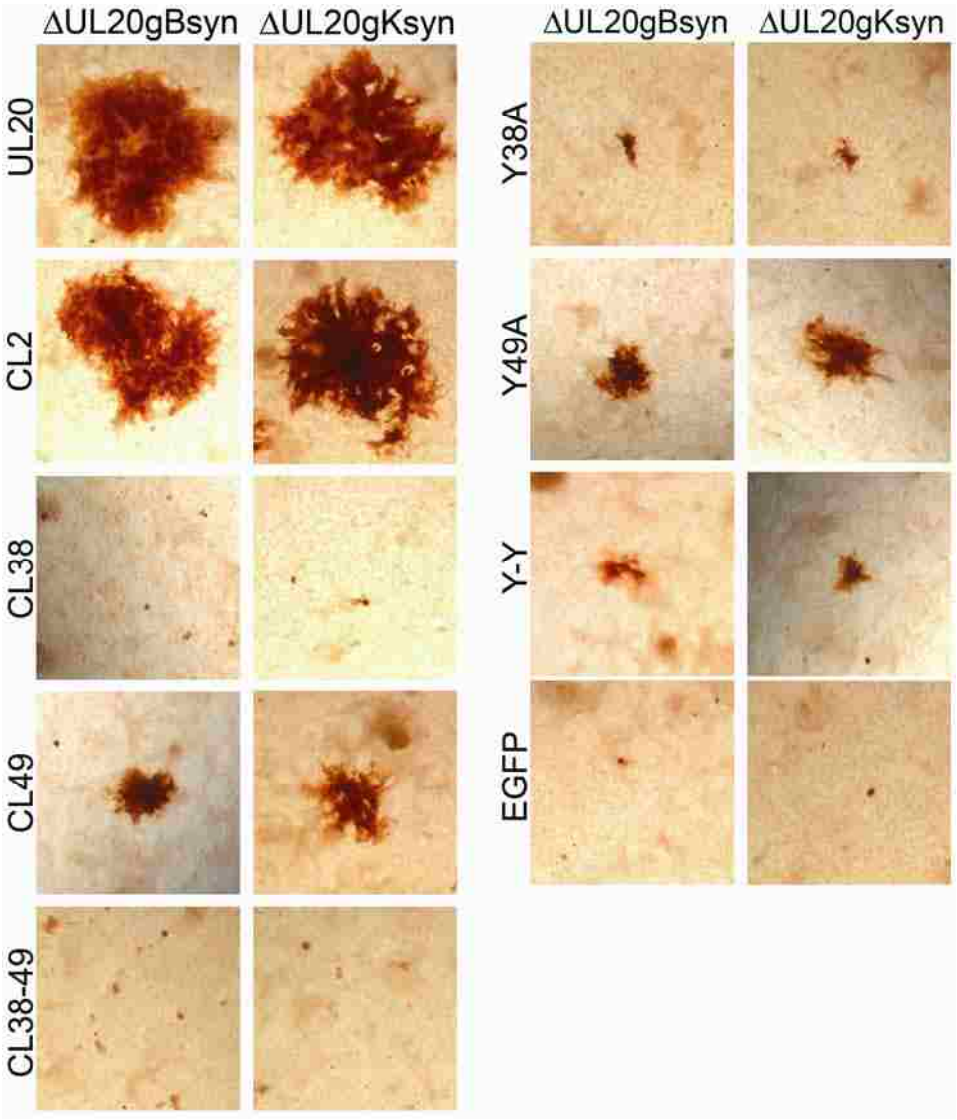


Figure A.3: UL20 mutant complementation for fusion. Vero cells were transfected with the indicated mutant and then infected with either ΔUL20gBsyn or ΔUL20gKsyn virus.

REFERENCES

Aiyar, A., Xiang, Y., and Leis, J. (1996). Site-directed mutagenesis using overlap extension PCR. *Methods Mol Biol* **57,** 177-91.

Baines, J. D., Ward, P. L., Campadelli-Fiume, G., and Roizman, B. (1991). The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. *Journal of Virology* **65**(12)**,** 6414-24.

Bond, V. C., and Person, S. (1984). Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. *Virology* **132**(2)**,** 368-76.

Bzik, D. J., Fox, B. A., DeLuca, N. A., and Person, S. (1984). Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. *Virology* **137**(1)**,** 185-90.

Debroy, C., Pederson, N., and Person, S. (1985). Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. *Virology* **145**(1)**,** 36-48.

Desai, P., DeLuca, N. A., Glorioso, J. C., and Person, S. (1993). Mutations in herpes simplex virus type 1 genes encoding VP5 and VP23 abrogate capsid formation and cleavage of replicated DNA. *Journal of Virology* **67**(3)**,** 1357-64.

Dietz, P., Klupp, B. G., Fuchs, W., Kollner, B., Weiland, E., and Mettenleiter, T. C. (2000). Pseudorabies virus glycoprotein K requires the UL20 gene product for processing. *J Virol* **74**(11)**,** 5083-90.

Foster, T. P., Alvarez, X., and Kousoulas, K. G. (2003). Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion. *J Virol* **77**(1)**,** 499-510.

Foster, T. P., and Kousoulas, K. G. (1999). Genetic analysis of the role of herpes simplex virus type 1 glycoprotein K in infectious virus production and egress. *J Virol* **73**(10)**,** 8457-68.

Foster, T. P., Melancon, J. M., Baines, J. D., and Kousoulas, K. G. (2004). Herpes simples virus type 1 (HSV-1) UL20 protein modulates membrane fusion events during cytoplasmic virion morphogenesis and virus-induced cell fusion. *J Virol* **(Submitted)**.

Fuchs, W., Klupp, B. G., Granzow, H., and Mettenleiter, T. C. (1997). The UL20 gene product of pseudorabies virus functions in virus egress. *J Virol* **71**(7)**,** 5639-46.

Hutchinson, L., Goldsmith, K., Snoddy, D., Ghosh, H., Graham, F. L., and Johnson, D. C. (1992). Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. *J Virol* **66**(9)**,** 5603-9.

Hutchinson, L., and Johnson, D. C. (1995). Herpes simplex virus glycoprotein K promotes egress of virus particles. *Journal of Virology* **69**(9)**,** 5401-13.

Jacobson, J. G., Chen, S. H., Cook, W. J., Kramer, M. F., and Coen, D. M. (1998). Importance of the herpes simplex virus UL24 gene for productive ganglionic infection in mice. *Virology* **242**(1)**,** 161-9.

Jayachandra, S., Baghian, A., and Kousoulas, K. G. (1997). Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space. *Journal of Virology* **71**(7)**,** 5012-24.

Johnson, D. C., and Huber, M. T. (2002). Directed egress of animal viruses promotes cell-to-cell spread. *J Virol* **76**(1)**,** 1-8.

MacLean, C. A., Efstathiou, S., Elliott, M. L., Jamieson, F. E., and McGeoch, D. J. (1991). Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. *Journal of General Virology* **72**(Pt 4)**,** 897-906.

Melancon, J. M., Foster, T. P., and Kousoulas, K. G. (2004). Genetic analysis of the herpes simplex virus type 1 (HSV-1) UL20 protein domains involved in cytoplasmic virion envelopment and virus-induced cell fusion. *J Virol* **78**(14)**,** 7329-43.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J Virol* **76**(4)**,** 1537-47.

Pellett, P. E., Kousoulas, K. G., Pereira, L., and Roizman, B. (1985). Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. *Journal of Virology* **53**(1)**,** 243-53.

Pogue-Geile, K. L., Lee, G. T., Shapira, S. K., and Spear, P. G. (1984). Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. *Virology* **136**(1)**,** 100-9.

Roizman, B., and Sears, A. E. (1996). Herpes Simplex Viruses and Their Replication. Third edition ed. *In* "Feilds Virology" (B. N. Feilds, D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2231-2295. 1 vols. Lippincott-Raven Publishers, Philadelphia, PA.

Ryechan, W. T., Morse, L. S., Knipe, D. M., and Roizman, B. (1979). Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. *J Virol.* **29,** 677-697.

Sanders, P. G., Wilkie, N. M., and Davison, A. J. (1982). Thymidine kinase deletion mutants of herpes simplex virus type 1. *Journal of General Virology* **63**(2)**,** 277-95.

Spear, P. G. (1993a). Entry of alphaherpesviruses into cells. *Seminars in Virology* **4,** 167-180.

Spear, P. G. (1993b). Membrane fusion induced by herpes simplex virus. *In* "Viral fusion mechanisms" (J. Bentz, Ed.), pp. 201-232. CRC Press., Boca Raton, Fla.

Spear, P. G., Eisenberg, R. J., and Cohen, G. H. (2000). Three classes of cell surface receptors for alphaherpesvirus entry. *Virology* **275**(1)**,** 1-8.

Tomishima, M. J., Smith, G. A., and Enquist, L. W. (2001). Sorting and transport of alpha herpesviruses in axons. *Traffic* **2**(7)**,** 429-36.

Ward, P. L., Campadelli-Fiume, G., Avitabile, E., and Roizman, B. (1994). Localization and putative function of the UL20 membrane protein in cells infected with herpes simplex virus 1. *Journal of Virology* **68**(11)**,** 7406-17.

VITA

Preston was born on June 27, 1979 to Wayne and Paula Fulmer in Little Rock, Arkansas. He spent the next 18 years of his life growing up in Des Arc, Arkansas spending time with his younger brother Paul and playing sports for Des Arc High School. In May 1997 Preston graduated Valedictorian of his high school class. He was awarded a scholarship to attend the University of Arkansas, and in December 2000 graduated with a Bachelor of Science degree in microbiology. Later that month he married Amanda Beck of Hope, Arkansas and moved to Baton Rouge, Louisiana where she was attending veterinary school. Preston was accepted into the Louisiana State University graduate school and began work on a Master of Sciences degree in August of 2001. This degree was completed in May 2003 and Preston moved to Raleigh, North Carolina where he worked for Duke University Medical Center for one year. In August 2004, Preston returned to Baton Rouge and began work on a Doctor of Philosophy degree under Dr. K.G. Kousoulas. After graduation in August 2007, Preston intends to further his education by taking a post-doctoral position with the United States Federal Government in a bio-defense lab in Washington, D.C.