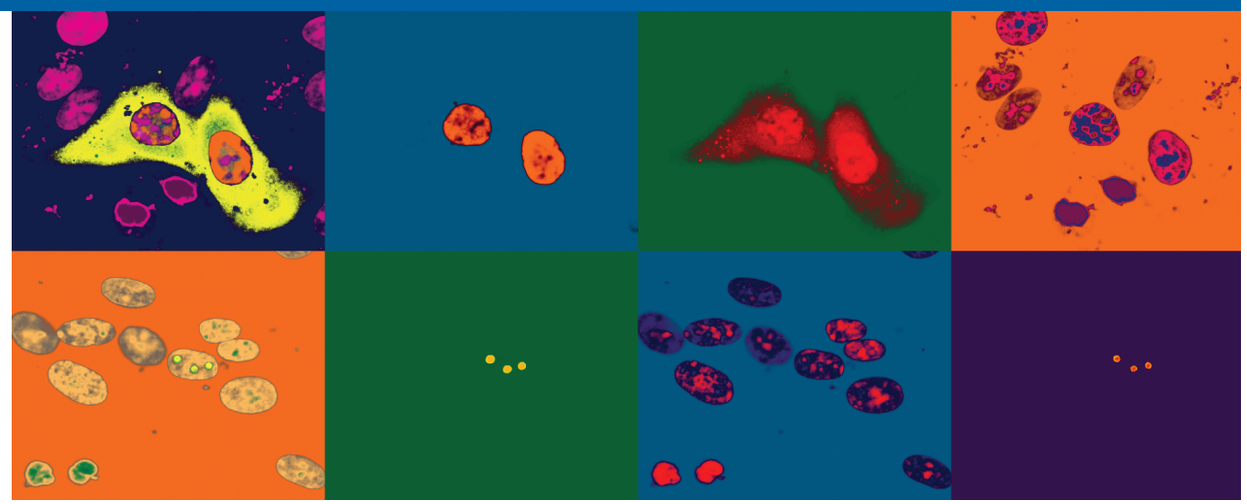
ISBN 978-91-629-0394-7 (PRINT)
ISBN 978-91-629-0395-4 (PDF)

Printed by BrandFactory, Gothenburg

DOCTORAL
THESIS
SAHLGRENSKA
ACADEMY

2018



Molecular aspects on Herpes simplex virus type 1 DNA replication and gene expression | Zhiyuan Zhao

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Cover illustration: Confocal microscope images of mammalian cells transiently expressing HSV-1 replication proteins. Taken and edited by Zhiyuan Zhao.

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“All things are difficult before they are easy”

-Thomas Fuller

Molecular aspects on Herpes simplex virus type 1 DNA replication and gene expression

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ABSTRACT

Herpesviruses infect a variety of animals from molluscs to humans and they have evolved in a close relationship with their hosts. In humans, we find nine herpesviruses, representing all three subfamilies of the *family Herpesviridae* and they can cause a variety of symptoms. The viruses have evolved independently, but they have all kept a conserved molecular machinery for the replication of their genomes. We have been studying the protein interactions within the molecular machinery of herpes simplex virus I (HSV-1), to gain further insight into the molecular mechanism of how the virus replicates and maintains its genome. In addition, we have been investigating the molecular requirements for the expression of the HSV-1 DNA replication-dependent late genes. We expect that detailed knowledge of these molecular events will help the development of new antiviral therapies, and perhaps also promote the understanding of related events in our own cells.

In this thesis, we have shown that the interactions between the seven viral proteins, which are essential for the HSV-1 DNA replication, are species-specific. The proteins cannot be substituted with homologs from a closely related virus, Equine herpesvirus 1. This observation suggests that the seven replication proteins function as a molecular machinery unit, a replisome, which is characterized by numerous protein-protein interactions. Additionally, we have identified important amino acids in an enzymatically inactive protein, UL8, in the HSV-1 helicase-primase complex, which is required for its interaction with the primase component, UL52, in the complex. Mutations of these amino acids in UL8 impaired their interaction and reduced or abolished the DNA replication capacity of the HSV-1 replisome at the non-permissive temperature.

Next, we examined the interactions between UL52 of the HSV-1 helicase-primase complex and other replication proteins. We found that UL52 consisted of different domains, and that the domains had different interaction partners. Stable interactions were detected between the N-terminal domain of UL52 and the helicase component, UL5, while the middle domain showed stable interactions with UL8. We could only detect a relative weak association between UL5 and the C-terminal domain of UL52, which may suggest the existence of a transient interaction. Furthermore, a new group of drugs against HSV infection targets the helicase-primase complex, but their inhibitory action was unknown. We have now demonstrated that these drugs inhibit HSV-1 DNA replication by affecting the interaction between UL5 and UL52. We suggest that the drugs lock these proteins in a certain conformation, preventing them from assisting in viral DNA replication.

In addition to its interaction within the helicase-primase complex, the middle domain of UL52 also exhibited stable interaction with HSV-1 single-strand DNA binding protein, UL29/ICP8. The interaction between these two proteins may indicate how the helicase-primase complex is loaded onto the activated origins of replication in the HSV-1 genome, and synthesizes primers on single-stranded DNA coated with ICP8.

Viral DNA replication is a prerequisite for the expression of HSV-1 true late genes. We have shown that expression of true late genes is also specifically dependent on the activity of CDK9, a cellular protein kinase involved in transcriptional regulation. The inhibition of CDK9 affected the transcription and cytosolic accumulation of viral late mRNAs. Furthermore, a substrate of CDK9, the transcription factor SPT5, was shown to be necessary for late gene expression. The activity of CDK9 was necessary for an interaction between SPT5 and the viral protein ICP27, which is suggested to be involved with the maturation and nuclear export of viral late mRNAs. Our results suggest that the control of HSV-1 true late genes are at least partially regulated by the maturation and nuclear export of viral late mRNAs.

Keywords: Herpes simplex virus type 1, DNA replication, gene expression

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SAMMANFATTNING PÅ SVENSKA

Virus av ordningen *Herpesvirales* infekterar en mängd olika djur, från blötdjur till människor, och har under evolutionen utvecklats i nära relation med sina värdar. I människor har nio herpesvirus identifierats. De återfinns i familjen *Herpesviridae* och kan delas upp i de tre underfamiljerna, alfa-, beta- och gamma-herpesvirus. Infektion med dessa virus kan manifesteras på en mängd olika sätt och i många aspekter har de utvecklat olika egenskaper, men den molekylära mekanismen för att replikera dess genom (DNA-replikation) förefaller vara bibehållen. Under mitt arbete har jag varit intresserad av hur Herpes simplex virus typ 1 (HSV-1) replikerar sitt genom, och i vår grupp har vi studerat hur virusproteiner involverade i DNA-replikationen interagerar med varandra. Dessa studier har ökat förståelsen för DNA replikationens molekylära mekanismer och hur viruset förmår bevara sitt genom intakt. Vi har även undersökt hur uttrycket av en grupp HSV-1 gener som är starkt kopplade till virusets DNA-replikation regleras. En djupare förståelse för dessa molekylära händelser kommer att gynna utvecklingen av nya antivirala läkemedel och därtill främja förståelsen av liknande processer i våra celler.

I denna avhandling har vi visat att interaktionerna mellan de sju virus-proteinerna, som är nödvändiga för HSV-1 DNA-replikationen, samverkar på ett artspecifikt sätt. Varken enskilda proteiner eller proteinkomplex kan bytas ut mot motsvarande komponent från ett närbesläktat herpesvirus, ekvint herpesvirus typ 1 (EHV-1). Detta tyder på att proteinerna fungerar som en molekylär enhet, en replisom, med flera protein-protein interaktioner inom replisomen. I replisomen har vi identifierat aminosyror i det enzymatiskt inaktiva proteinet UL8 som är viktiga för dess interaktion med proteinet UL52. Mutationer som förändrar dessa aminosyror i UL8 försvagade interaktion med UL52 och hämmade replisomens replikationsförmåga vid den icke-permissiva temperaturen.

Vi undersökte också interaktionerna mellan UL52 och övriga proteiner i det helikas-primas komplex som är en del av replisomen. Vi fann att UL52 kunde delas upp i olika domäner, och att dessa domäner interagerade med olika proteiner. En stark interaktion kunde påvisas mellan den N-terminala domänen av UL52 och UL5 som är ett helikas. Mellandomänen av UL52 bildade ett stabilt komplex med UL8. Vi fann också belägg för en betydligt svagare association mellan den C-terminala domänen av UL52 och UL5 tydande på en mer tillfällig interaktion. En ny grupp av läkemedel mot HSV infektioner har utvecklats. De hämmar virusreplikationen genom att påverka

helikas-primaskomplexets funktion, men den molekylära mekanismen har varit okänd. Vi har nu kunnat visa att dessa läkemedel stabiliserar interaktionen mellan UL5 och UL52. Vi föreslår därför att dessa läkemedel låser proteinerna i en ogynnsam konformation vilket hindrar dem från att utöva sina funktioner under pågående DNA syntes.

Utöver interaktioner mellan UL5, UL8 och UL52 inom helikas-primaskomplexet kunde vi för första gången påvisa en stark interaktion mellan virusets enkelsträngbindande protein ICP8 och mellandomänen av UL52. Samspelet och interaktionen mellan dessa två proteiner kan hjälpa till att förklara hur helikas-primaskomplexet rekryteras till aktiverade replikations origin (DNA-replikationens startsekvenser) på HSV-1 genomet samt hur syntesen inleds av primersekvenser på enkelsträngat DNA täckt med ICP8.

För att effektivisera syntesen av nya viruspartiklar under infektionen, måste HSV-1 koordinera uttrycket av sina gener med DNA-replikationen. En grupp av gener, så kallade sena HSV-1 gener, kräver virus DNA-replikation för att uttryckas. Mekanismen bakom detta fenomen är oklar. Vi fann att uttrycket av dessa gener är specifikt beroende av aktiviteten hos det cellulära proteinkinaset CDK9. Inhibition av CDK9 reducerade transkription av sena gener samt minskade ackumuleringen av sena mRNA i cytoplasman. Transkriptionsfaktorn SPT5, vilken är ett substrat av CDK9, visades vara särskilt viktigt för virusets genuttryck. Vi kunde vidare visa att hämning av CDK9 blockerade interaktionen mellan SPT5 och det virala proteinet ICP27, vilket är av betydelse för mognad av viralt mRNA samt dess export ut ur cellkärnan. Våra resultat antyder därför att en CDK9-beroende reglering av mognad och export av mRNA är en av de mekanismer som styr uttrycket av de sena HSV-1 gener.

Sammantaget, genom att kartlägga proteininteraktionerna i HSV-1 replisomen har vi fördjupat vår insikt i den molekylära processen av virusets DNA-replikation, och vi har också ökat förståelsen för hur en ny grupp av läkemedel mot HSV infektioner verkar. Vi har kunnat visa att aktiviteten av ett cellulärt protein, CDK9, är specifikt viktig för uttrycket av virusets sena gener. Kännedom om dessa processer möjliggör till en mer riktad utveckling av antivirala läkemedel framgent.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Muylaert, I, **Zhao, Z**, Andersson, T, Elias P. Identification of Conserved Amino Acids in the Herpes Simplex Virus Type 1 UL8 Protein Required for DNA Synthesis and UL52 Primase Interaction in the Virus Replisome

J Biol Chem. 2009 July; 287(40): 33142-52

II. Muylaert I, **Zhao, Z**, Elias P. UL52 Primase Interactions in the Herpes Simplex Virus 1 Helicase-Primase Are Affected by Antiviral Compounds and Mutations Causing Drug Resistance

J Biol Chem. 2014 October; 289(47): 32583-92

III. **Zhao, Z***, Tang KW*, Muylaert I, Samuelsson T, Elias P. CDK9 and SPT5 proteins are specifically required for expression of herpes simplex virus 1 replication-dependent late genes (*Equal contribution)

J Biol Chem. 2017 July; 292(37): 15489-00

CONTENTS

ABBREVIATIONS

43S – 43 subunit, the translation preinitiation complex
A – Adenosine
α gene – Immediate early gene
ATP – Adenosine triphosphate
β gene – Early gene
bp(s) – Base pair(s)
γ gene – Late gene
γ_2 gene – True late gene
C-domain – C-terminal domain
C-terminal – Carboxy-terminal of polypeptide
ChIP – Chromatin immunoprecipitation
CDK – Cyclin dependent kinase
CTD – Carboxyl-terminus domain of the largest subunit of RNAP II
D – Aspartic acid
DNA – Deoxyribonucleic acid
DRB – 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSIF – DRB sensitive inducing factor
E – Glutamic acid
emerald GFP – Emerald green fluorescent protein
EHV-1 – *Equid alphaherpesvirus 1*
H3 – Histone H3
hpi – Hours post infection
HSV – Herpes simplex virus
HSV-1 – Herpes simplex virus type 1
HSV-2 – Herpes simplex virus type 2
HSE – Herpes simplex encephalitis
gB – Glycoprotein B
gC – Glycoprotein C
gD – Glycoprotein D
gH – Glycoprotein H
gL – Glycoprotein L
HCF-1 – Host cell factor 1
ICP – Infected cell polypeptide
IRF-3 – Interferon regulatory factor-3
kbs – Kilo bases
kbps – Kilo base pairs
kDa – Kilo dalton
LAT – Latency associated transcript
M-domain – Middle domain

MNase – Micrococcal nuclease
MOI – Multiple of infection
MW – Molecular weight
mRNA – Messenger ribonucleic acid
N-domain – N-terminal domain
N-terminal – Amino-terminal of polypeptide
NELF – Negative elongation factor
NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells
OBP – Origin binding protein
PCNA – Proliferating cell nuclear antigen
P-TEFb – Positive elongation factor b
qPCR – Quantitative polymerase chain reaction
rtPCR – Reverse transcription polymerase chain reaction
RING finger – Really interesting new gene finger of ubiquitin ligase
R – Arginine
RNA – Ribonucleic acid
RNAP II – RNA polymerase II
SPT4 – Supressor of Ty homolog 4
SPT5 – Suppressor of Ty homolog 5
STING – Stimulator of interferon genes
T – Thymidine
ts – Temperature sensitive
UL – Unique long segment of HSV-1 genome
US – Unique short segment of HSV-1 genome
VHS – Virion host shutoff
VICE – Virus-induced chaperone-enriched
VP – Virion protein
VZV – Varicella-zoster virus
wt – Wild type

1 INTRODUCTION

Herpes simplex virus type 1 (HSV-1) infection is a common and widespread infection in the human population. Once infected with HSV-1, the virus will remain with its host for life. The infectious cycle of HSV-1 is characterized by a first productive lytic infection, where new virions (virus particles) are produced. Later the infection is spread to the sensory neurons, where HSV-1 establishes a latent infection and remains in a quiescent state [1]. Upon certain stimulation, HSV-1 can reactivate from its state of latency and initiate new productive infections. In the vast majority of cases, the progression of HSV-1 infection is kept under control by the host's immune system. HSV-1 is a successful virus indeed, since it is highly contagious and is efficiently replicated. In addition, it remains without being eliminated by its host and usually does not kill off its host. A combination of these properties explains the large number of individuals infected worldwide and the huge reservoir of infectious virus in the population.

In the host, the clinical manifestation of productive HSV-1 infection ranges from the most common symptom of cold scores (sores and blisters around the lip) to severe encephalitis (inflammation in the brain). The main treatment for a HSV-1 infection is acyclovir, a nucleoside analogue, which inhibits the ability of the virus to synthesize new DNA. The drugs inhibit HSV-1 DNA replication, but cannot target the latent state of a HSV-1 infection. Resistance to antiviral drugs is uncommon and is mainly observed in immunocompromised individuals [1]. Such individuals as well as neonates are especially vulnerable groups for HSV-1 infection, since the progression of the viral infection is not limited as in immunocompetent adults.

With the ultimate goal of establishing a rational basis for development of new antiviral compounds against HSV-1 and other herpesviruses, we have studied, in detail, the mechanism of HSV-1 replication. We have also sought to investigate how viral DNA replication is coupled to virus gene expression. These efforts, as presented in this thesis, have given new insights into the molecular machinery responsible for HSV-1 DNA synthesis. We have revealed how a new group of drugs act against HSV-1 infection. Finally, we have identified a cellular enzyme, CDK9, which is required for virus gene expression and for which a variety of inhibitory substances exist that could be further explored as antiviral compounds.

1.1 Brief historical perspective

In ancient Greece, some skin lesions were collectively called herpes [2]. The term herpes originated from the Greek word “to creep” and it was used to describe a group of diseases associated with the spreading of skin lesions. In the Hippocratic Collection, herpes was considered to be a potential severe condition. It was probably used to refer to a variation of skin lesions such as skin cancer, lupus vulgaris, ringworm, eczema and smallpox [2]. It continued to be applied to miscellaneous skin conditions up until the nineteen century [3]. A more modern and familiar description of herpes was proposed by Willan and Bateman in 1814 [4]. They limited the use of herpes to diseases that were characterized by localized vesicles, which were self-limiting and healed within ten to fourteen days. According to their description, despite mild symptoms, the diseases could not be cured or the course be shortened by any medication. Contrary to modern knowledge, they also claimed that the conditions were not contagious [4].

It was not until at the end of nineteenth century, that studies involving human volunteers could demonstrate the infectious nature of Herpes simplex virus (HSV) [5]. The virus was first isolated in the 1920s after the transfer to, and subsequently between, rabbits. At that time, microbiologists were still not certain about the composition and properties of viruses [3]. After it had been shown that herpes could be transferred from humans to rabbits, the cause of recurrent of herpes lesions was again questioned in the 1930s following the discovery that the recurrent lesions only occurred in individuals who carried neutralizing antibodies [5]. This observation clearly differed from what was known about other infectious agents at that time. A theory was put forward that these lesions were caused by the endogenous production of an agent in patients that behaved as a virus following certain stimulation [5]. When the proposed infectious agent was transferred to animals, it then appeared to be converted to an actual virus. This phenomenon was later recognized to be the features of the latent infection by HSV. The new technical advances in the 1950s, such as the introduction of cell culture, electron microscopy and negative staining, allowed the identification of virus particles. Since then, HSV has been one of the most extensively studied viruses, both as a pathogen and as a model organism for DNA replication, recombination and gene expression [5].

1.2 Phylogenetics of herpesviruses

Classification of viruses has been characterized by taking several factors into account, such as virion morphology, as well as physical and biological properties [6]. Virus taxonomy includes the hierarchical levels: order, family, subfamily, genus and species [6]. In 2007, the International Committee on Taxonomy of Virus, raised herpesvirus from the level of family to the level of order, *Herpesvirales* (**figure 1**) [7]. In the past, the phylogeny of herpesviruses have mainly been based on their virion structure [8]. All herpesviruses have a linear double stranded DNA genome, ranging between 124-295 kilobase pairs (kbp). Their genomes are encased in an icosahedral capsid, which in turn are enclosed by a lipid bilayer envelope (**figure 2**). The space between the capsid and the viral envelope is occupied by an amorphous layer called the tegument, which mainly contains viral proteins that prime the host cell for infection.

Three families are included in the order *Herpesvirales*. The family *Herpesviridae* contains herpesviruses that infect mammals, birds and reptiles. Herpesviruses that infect fishes and frogs are included in the family *Alloherpesviridae* and, finally, in the family *Malacoherpesviridae* we can find the herpesviruses that infect bivalves [7]. *Herpesviridae* is in turn divided into the subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. In all three subfamilies of *Herpesviridae* there are viruses that infect humans, and in these subfamilies there are in total nine known herpesviruses that can infect humans. They are herpes simplex virus type 1 and 2, varicella-zoster virus, human cytomegalovirus, human herpes virus 6a, 6b and 7, Epstein-Barr virus and Karposi's sarcoma associated virus [8]. These human herpesviruses have different cell tropism, but they share the common biological properties of lytic infection, which involves high expression of viral genes and the production of new virions, and latent infection, which involves minimal expression of viral genes and no formation of new virions. Human herpesviruses can reactivate from their latent state and induce lytic infection, thereby producing new infectious virions. Infections by herpesviruses crossing species barriers are rare, but may in such instances lead to fatal outcome [8]. For example when humans are infected by *Macacine herpesvirus 1* from monkeys, the mortality rate is about 80 percent, if left untreated [1].

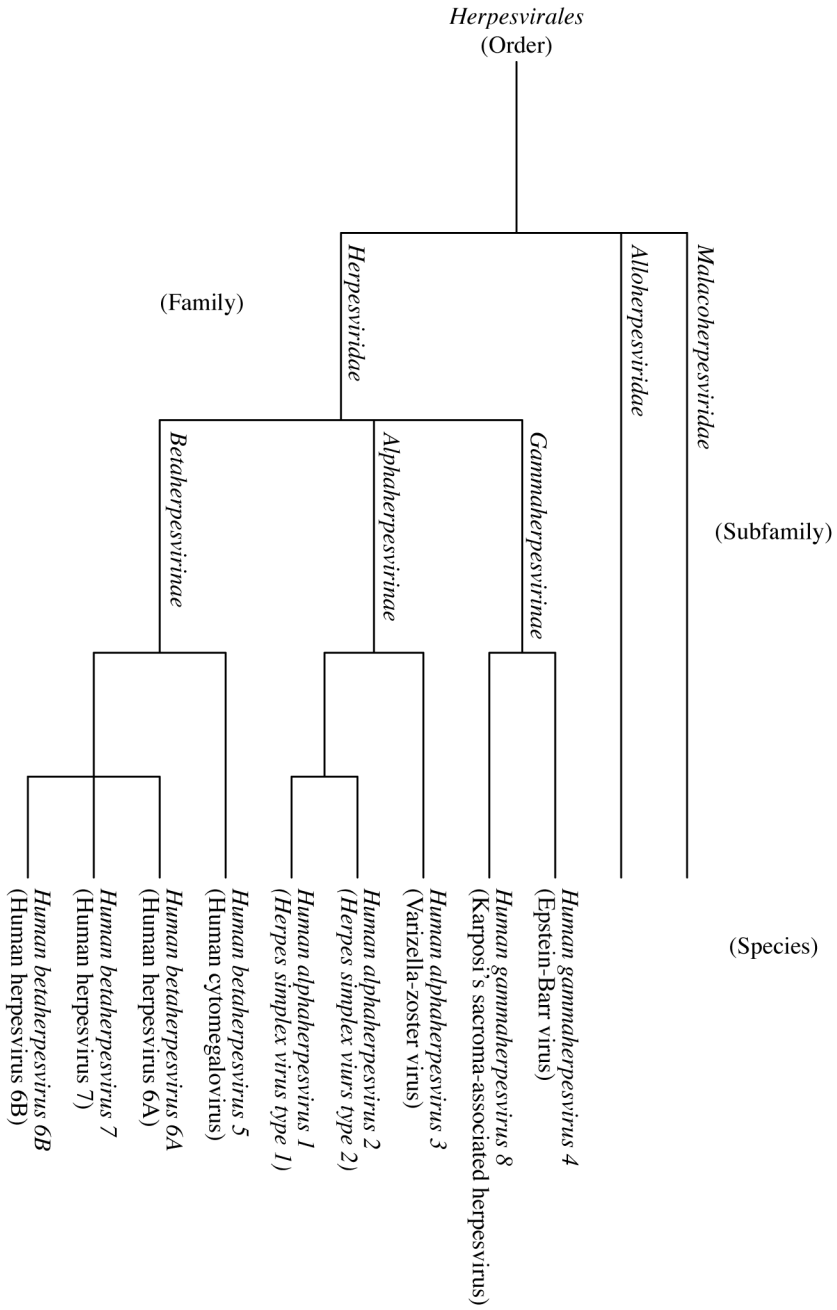

Figure 1. Phylogenic tree of human herpesviruses. Phylogenic relationship between human herpesviruses. The correct virus taxonomy is in italic. The tree was generated in phyloT (http://phylot.biobyte.de/) using NCBI taxonomy and edited in Adobe Illustrator.

1.3 Structure and composition of the virus particle

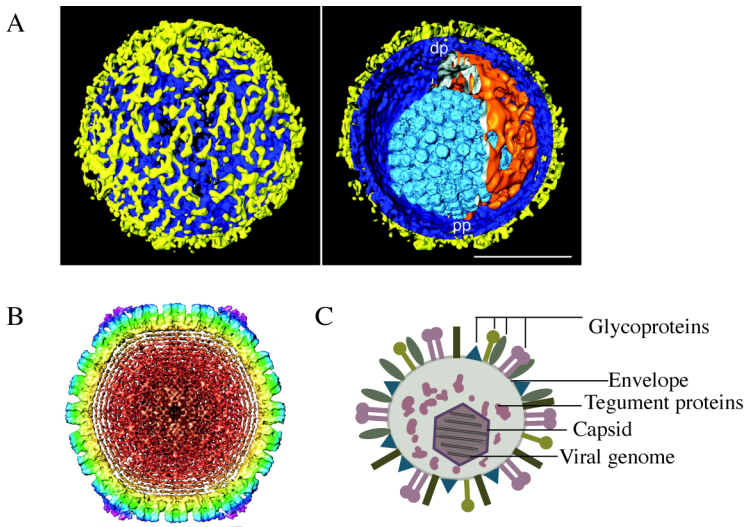

Figure 2. HSV-1 virion. (A) Cryo-electron tomography of HSV-1 virion. The glycoproteins are in yellow, the viral envelope in dark blue, the tegument cap is in orange and the capsid is in light blue. The scale bar indicates 100 nm, pp and dp short for proximal pole and distal pole. Reprinted with permission [9]. (B) A reconstruction of cryo-electron microscopy showing a cross-section of the HSV-1 capsid and the viral genome inside. Reprinted with permission [10]. (C) Schematic picture of the HSV-1 virion.

As for all herpesviruses, the HSV-1 virion is surrounded by a lipid bilayer, called an envelope, as its outer layer (**figure 2**). Viral proteins are embedded in the envelope and the majority of these proteins are glycosylated. The glycoproteins protrude out from the envelope like spikes. The average diameter of the virion is 225 nm including the glycoprotein spikes and 186 nm without [9]. The viral glycoproteins are essential for the attachment of the virion to susceptible cells and subsequently to promote the fusion of the viral envelope with the cell membrane [1]. Inside the envelope resides the capsid, with one side closer to the envelope than the other (**figure 2**) [9]. The capsid has an icosahedral structure and the linear double stranded DNA genome of HSV-1 is packaged in a condensed manner like a spool (**figure 2b**) [10]. The space between the capsid and envelope is referred to as tegument and contains viral proteins that are important for the viral life cycle [11]. Tegument proteins may prime the host cell for HSV-1 infection by shutting down cellular gene expression, or directing it towards expression of viral

genes. For example, the viral transcription activator VP16 is synthesized late in the infection and becomes incorporated in the virion as a tegument protein [1]. Upon infection VP16 is released into the cell and strongly stimulates HSV-1 immediate early gene expression [1]. The tegument also contains viral proteins that are necessary for the transport of the capsid to the cellular nucleus from the place of viral entry. In total the HSV-1 virion consist of 30 to 44 viral proteins, and in addition there are traces of 49 cellular proteins in the virion [1, 11]. The specific role of these cellular proteins in the virion are still under investigation [12].

1.4 The different terminology of HSV-1 proteins

The proteins of HSV-1 have different names depending on how they were initially characterized [13]. Initially, purified HSV-1 virions were isolated from infected cells and the proteins in the virion were numbered according to decreasing molecular weight (MW) on an acrylamide gel and the prefix ,virion protein (VP), was added to their number [14]. Additionally, in similar fashion, HSV-1 proteins that accumulated in virally infected cells were numbered with decreasing MW and the prefix infected cell polypeptide (ICP), was added to the numbers [15]. Also, by the use of antiserum against HSV-1 virion, viral glycoproteins on the envelope were isolated and were assigned alphabetically, e. g. glycoprotein B (gB), glycoprotein C (gC) and so forth [16]. More recently, HSV-1 proteins have been given the name of the gene by which they are encoded in the HSV-1 genome [17, 18]. For example, the viral single-stranded DNA binding protein ICP8 can also be referred to as the UL29 protein. The overlapping nomenclatures are in some extent still in use today and may cause some confusion. Although, their names are usually used in different context. Many of the proteins in the virion, especially the capsid proteins, are referred as VP, while the glycoproteins are designated alphabetically [13]. HSV-1 proteins that are expressed early in the infection cycle are often called by ICP, and some viral proteins are simply referred by their function.

1.5 Clinical manifestations of HSV-1

The prevalence of HSV-1 increases with age [19]. In a report from the World Health Organization, it was estimated that the prevalence of HSV-1 in 2012 was 67% between the ages of 0 to 49 among the world population [19]. It also showed that the prevalence was higher in socio-economically vulnerable regions. Africa, for example, had the highest prevalence (87%) and lowest prevalence was seen in North and South America (40 to 50%). In Europe the prevalence ranged between 60 to 70%.

HSV-1 is spread between individuals through close contact [1]. The lytic infection of HSV-1 can either be primary, initial or recurrent [1]. Primary infection is characterized by the infection of individuals that have never been in contact with neither HSV-1 nor HSV-2 before and therefore have no antibodies against HSV. Once infected with HSV, the virus will remain with its host for life and cannot be cleared. A new infection of individuals that have been previously infected with a different type of HSV at a different site is defined as initial infection. As an example, a genital HSV-2 infection can been seen in individuals which earlier in their life have become infected with orolabial HSV-1. Finally, infection caused by reactivation of latent HSV is called recurrent infection.

Clinical symptoms in primary infection are rare but, if they occur, gingivostomatitis is more commonly observed [1, 20]. However, the most common clinical manifestation of HSV-1 infection is recurrent orolabial infection [1, 21]. Usually recurrent infections are less severe. External factors, such as emotional and physical stress, illness, UV exposure and trauma have been reported to trigger recurrent orolabial HSV [1, 22-24]. Although the primary infection of HSV-1 in the ocular region is normally asymptomatic or mild , the recurrent infection, HSV-1 keratitis, is the leading cause of non-traumatic cornea blindness in the United States [1, 21, 25].

Human alpha herpesviruses are neuroinvasive and the most feared manifestation of HSV-1 is encephalitis [22]. This can either be caused by primary or recurrent infection. Although herpes simplex encephalitis (HSE) is rare, it is the leading cause of sporadic encephalitis. Untreated patients have mortality rate of 70% and only 2 to 3% recover to a normal neurological function [1].

Neonatal HSV infections are usually caused by HSV-2, since in the majority of cases transmission to the newborn occurs by the mother at partum, but HSV-1 infections do occur [1, 21, 23, 26]. Neonatal HSV infection is almost

always symptomatic. It can be manifested as a localized disease to the skin, eye and mouth, but in about 60% of cases it either presents as HSE or in combination with disseminated disease, which affects multiple organs. In the case of neonatal HSE and disseminated infection, it is associated with high mortality and morbidity when left untreated.

Immunocompromised individuals, such as patients which receive immunosuppressive therapy and HIV positive patients, constitute a particular vulnerable group for HSV-1 infections. Progression of infections, which would have normally been limited in immunocompetent individuals, are common and infections in the respiratory tract, esophagus, gastrointestinal tract and as well as disseminated disease can occur in these individuals following reactivation [1]. In general, resistance to HSV-1 treatment is rare, but in immunocompromised individuals the risk for developing resistance increases ten-fold [27, 28].

1.6 Lytic infection

The productive infection starts with the cellular entry of HSV-1 virion. Depending on the condition and cell type, the uptake of the virion is either mediated through the fusion of the viral envelope with the cell plasma membrane, or through endocytosis [1, 29]. The same viral glycoproteins are involved in both processes. The first step is the attachment HSV-1 gB and gC to glycoaminoglycans on the cell membrane. The initial attachment then enables gD to interact with its cellular receptor; such as nectins, herpesvirus entry mediator or 3-O-sulfated heparan sulfate. The interaction between gD and its receptor will induce a conformational change in gD, which in turn is transmitted to gB and the gH/gL heterodimer and these 3 glycoproteins are responsible for the fusion of the HSV-1 envelope either with the plasma membrane or with the membranes of endocytic vesicles [29].

Following the fusion of the viral envelope, the tegument proteins and the naked HSV-1 capsid are released in the cytosol (**figure 3**). The tegument protein, virion host shutoff (VHS) protein, an endoribonuclease encoded by the UL41 gene, inhibits the expression of the cellular genes by degrading messenger RNA (mRNA) at the ribosomes [30, 31]. This will halt the cellular machinery and also down regulate the cellular defense against HSV-1 infection, by targeting the mRNA responsible for the interferon response, such as cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) [32]. Other tegument proteins assist in the nuclear translocation of the capsid and injection of HSV-1 DNA into the nucleus through nuclear pores [1]. Inside the nucleus, the viral DNA is circularized by DNA ligase IV/XRCC4 [33]. Subsequently, the first set of HSV-1 genes, referred to as immediate early genes or α genes, are expressed [34, 35]. The immediate early (or α) proteins, encoded by the α genes, are in turn involved in the regulation of the expression of the early and late HSV-1 genes. Their function is to ascertain vigorous viral protein synthesis during the infectious cycle [1, 26].

The α proteins are essential for the expression of early (or β) genes. These gene products are needed for HSV-1 DNA synthesis. Both α proteins and HSV-1 DNA replication are necessary for the full expression of HSV-1 late (or γ) genes (**figure 4**) [34, 36]. Protein products of γ genes are either components of the HSV-1 virion or involved in the formation of the virion. The viral transcription, DNA replication and capsid assembly takes place in the host nucleus, while the translation of HSV-1 mRNA and consequently the viral protein synthesis occurs in the cytosol.

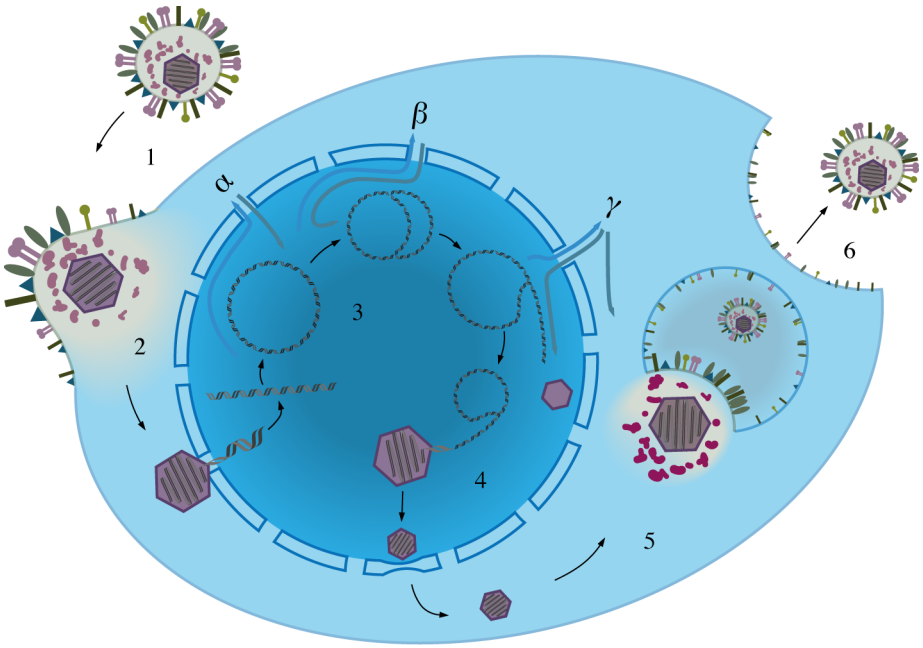

Figure 3. The lytic infectious cycle of HSV-1. (1) HSV-1 virion attaches to the cellular membrane and fuses with it. (2) Capsid is transported to nuclear pore and injects its DNA genome into the nucleus. (3) Viral gene expression is initiated and HSV-1 genome is circularized. The genes are expressed in the order of α, β and γ. Viral DNA replication is initiated after the expression β genes. (4) HSV-1 genome is packaged into capsids and cut into single unit length. Nuclear capsids exits the nucleus by being enveloped and de-enveloped through the nuclear membranes (5) In the cytosol, virion acquires its tegument protein and viral envelope through a second envelopment into endosomes. (6) Finally, newly synthesized HSV-1 is released.

HSV-1 capsids are formed in the nucleus from viral procapsid (immature capsid) proteins [37]. In the nucleus the procapsid is first assembled around viral scaffold proteins. The replicated HSV-1 DNA genome is then injected into the procapsids. With the entrance of the viral DNA, the scaffold proteins are cleaved and dissociated from the capsid. Nucleocapsids then exit the nucleus through the envelopment and de-envelopment of the inner and outer nuclear membrane, respectively (**figure 3**) [38]. In the cytosol, the capsids acquire the viral tegument proteins and also acquire an envelope by undergoing a secondary envelopment into endosomes. Finally, the virions are released through the fusion of the endosomes with the cell membrane.

Inevitably, a productive lytic infection of HSV-1 leads to the death of the infected cell. Lytic infection is ultimately limited by the adaptive immune system in immunocompetent individuals [1].

There is a constant struggle between HSV-1 and their host. For the viruses it is a matter of survival and reproduction. While for their host it is a matter of preventing, eliminating or, if the previous two are not possible, suppressing the viral infection. These struggles have forced the host and virus to continuously adapt and evolve antiviral defenses and ways to overcome these defense, respectively. The cellular lines of defenses against HSV infection consist of the intrinsic, innate and adaptive immunity, in which the viral molecules are recognized, and counter measurements are induced that limit viral replication, signal and protect nearby cells and, as a last resort, to commit suicide in order to limit the spread of virus [1]. Although these cellular responses have been known for a few decades, some of the crucial components have just been identified in recent years. One such component in the innate immunity, is the pathway of the stimulator of interferon genes/interferon regulatory factor-3/nuclear factor kappa-light-chain-enhancer of activated B cells (STING/IRF-3/NF-κB) [39-41]. The activation of this pathway leads to the production of type I interferon and induces the autocrine and paracrine inflammatory response. HSV-1 activates the STING/IRF-3/NF-κB through gamma-interferon-inducible protein 16 (IFI 16) and cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), which senses foreign nuclear double DNA in the nucleus and the cytosol, respectively [42]. Investigating how HSV-1 has adapted and evades the cellular defenses is an intriguing and active field of research.

1.7 Latent infection

After the productive infection cycle of HSV-1, the virions are spread to nearby epithelial cells and sensory nerve endings. In the epithelial cells, the HSV-1 virion undergoes additional rounds of lytic infection as discussed previously. In contrast, in sensory neuron cells HSV-1 establishes latent infection instead [1, 43, 44]. After fusion of the viral envelope and release of the HSV-1 capsid into the axon terminal, the capsid is transported in a retrograde manner to the cell body of the neuron in sensory nerve ganglion. The viral DNA is injected into the nucleus, but instead of initiating the productive viral replication, the circularized HSV-1 genome will adopt an episomal conformation and remain quiescent. The viral genome will, in this case, exist in a state that is characterized by a regular nucleosome structure [45]. The latent infection will persist in the sensory neurons and in the host for the rest of the host's life. Upon certain stimulation, e. g. physical-, emotional stress and immunosuppression of the host, HSV-1 can reactivate from its silent mode and produce new virion in the neurons. These newly produced HSV-1 virions are transported anterograde to the nerve terminals where they are released and can cause recurrent lytic infections. It should be noted that HSV-1 can also induce lytic productive infection in neuronal cells both in animal models and in humans, as demonstrated by encephalitis [1].

The state of latency was first proven in mouse models, by infecting the mouse and harvesting the neurons from affected ganglia [46]. No infectious virion or viral product could be detected in these neurons, but, when the neurons were cultured together with susceptible cells, productive lytic infection could be detected. It still holds true that minimal viral protein synthesis is detected in latent infected neurons, although with more sensitive methods, α, β and γ gene transcripts (mRNA) are detected 24 to 72 hours post infection (hpi) [1]. Also it is suggested that the virus undergoes DNA replication before it is silenced, since several HSV-1 genomes are found in an individual latent infected cell [1].

A characteristic feature of latent infection is the presence of latency associated-transcripts (LATs) [47]. LATs are a group of transcripts regulated by a common promoter on the HSV-1 genome. The whole transcript is about 8 kbs and it is only found in limited amounts in latent infected cells. However, processed shorter transcripts, 1,5 kbs and 2,0 kbs, can be readily detected. The LATs appear 24 hours post infection, but in contrast to the lytic transcripts that peak and decline, the LAT transcripts accumulate during

latency [1]. The detailed molecular mechanisms of how HSV-1 establishes and maintains latency followed by reactivation is still largely unknown.

It is proposed that the silencing of α gene expression is the key step to initiate latency, and that the LATs are important for maintaining HSV-1 in a latent state [1, 43]. During latency, it has been shown that the region coding for the LATs on the HSV-1 genome is associated with euchromatin nucleosome structure, while the regions of lytic genes are in heterochromatin structure [43]. This relationship is reversed in lytic infection. Knock-down and mutant viruses of LATs yield fewer latent infected neurons and a higher rate of apoptosis among sensory neurons in animal models [1].

The kinetics of the lytic gene expression during the reactivation process is still under debate. It is, however, agreed upon that the frequency by which spontaneous reactivation leads to a symptomatic infection is modulated by the host immune system [1, 43, 48].

1.8 HSV-1 gene expression

The HSV-1 genome, which is about 152 kbps in size, encodes for more than 80 viral genes [1]. It is divided in an unique long and an unique short region (**figure 6**) [17, 18]. They are separated by an internal repeat sequence and, in both ends, flanked with terminal repeat sequences (**figure 6**). During productive viral infection, gene expression initiates promptly after the entry of the HSV-1 genome into the cell nucleus. Gene expression occurs sequentially in a cascade manner as discussed previously in the order of α, β and γ genes (**figure 4**). Viral genes are ordered accordingly to their localization on the HSV-1 genome, e. g. unique long (UL) 1, UL2, UL3 and unique short (US) 1, US2, US3 and so forth.

The first molecular event of gene expression is transcription. All of the HSV-1 genes are transcribed by the cellular transcription machinery, specifically by the RNA polymerase II (RNAP II) holoenzyme [49]. Viral proteins act to modify the cellular transcription machinery, either directly or through cellular factors, to facilitate the transcription of HSV-1 genes [1]. A complete picture of the regulatory elements of gene transcription including promoters is still not available. However, the virus must clearly be able to distinguish its own genes from the cellular genes, and it must also employ strategies to regulate the transcription pattern of its genes in order to accomplish effective virion production. The cellular transcription machinery is regulated at the steps of initiation, elongation and termination. Many aspects of HSV-1 transcriptional processes overlap with its host transcriptional control. In fact, for many years, HSV-1 transcriptional control has been a good model to study cellular transcription machinery [50]. All HSV-1 transcripts (mRNA) are 5' capped and polyadenylated at 3' [51]. A clear difference is that, with only a few exceptions, the majority of HSV-1 transcripts lack introns [1]. In the following sections, some of the well established transcriptional control mechanisms are summarized.

Since, the eukaryotic cells have developed several strategies to regulate gene expression during different stages of development and in response to changes in the surrounding environment, viruses, such as HSV-1, have to adapt and make use of these strategies [52]. This includes getting access to the coding and regulatory domains of genes. In the cell, the histone proteins are bound to DNA and form nucleosomes. Modification of histones regulates DNA accessibility for transcription and loading of the transcription complex on to the upstream regions of the transcription start site (promoters) [43]. When foreign double stranded DNA is presented in the cell nucleus, the cell tries to

silence it by assembling histones to form nucleosome structures, as seen during transfection with DNA plasmids and during virus infections [43, 48, 53]. Initial investigations of parental and replicated HSV-1 DNA indicated that only a small amount of, if any, viral HSV-1 is associated with the nucleosome structure in cell nucleus during lytic infection [54]. This conclusion was drawn based on the digestion of viral DNA with micrococcal nuclease digestion (MNase), which randomly digests naked double-stranded DNA but leaves DNA associated with histones uncut. This issue has been readdressed, since accumulating evidence has shown that cellular factors involved in chromatin remodeling machinery are recruited to and are necessary for HSV-1 gene expression [43, 55-57]. In these studies, the authors found that up to 43 percent of HSV-1 DNA was protected against MNase digestion. In contrast to the chromosomes of the cell, which produce regular patterns of bands, the HSV-1 DNA produced a smear when it was incompletely digested. However, vigorous MNase treatment of both cellular and viral DNA produced a single band corresponding to the size of a mononucleosome [55]. These findings have been interpreted to mean that HSV-1 DNA is associated with histone in a mononucleosome structure during productive viral replication, but not in a regular pattern as in the cellular chromosomes. Indeed, it was found that HSV-1 DNA is associated with histone H3 (H3) by chromatin immunoprecipitation (ChIP) assays [55-57]. It has now been accepted that HSV-1 DNA is associated with histones in a nucleosome manner, and that the initiation of viral gene expression is accompanied by the recruitment of H3 modifying and chromatin remodeling factors to their promoter regions [1, 43, 58]. However, many aspects of how chromatin remodeling regulates HSV-1 gene expression is still not yet clear.

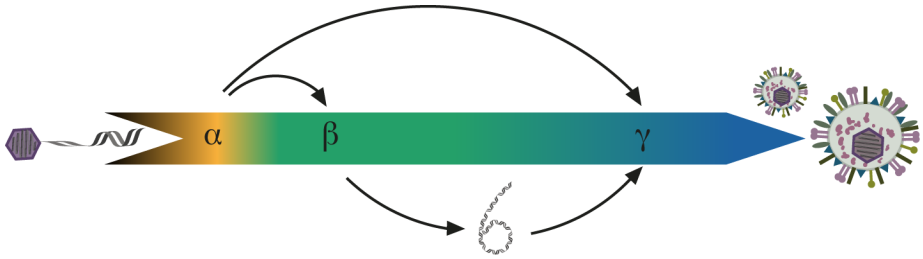

Figure 4. The HSV-1 gene expression program. α gene products are required for the expression of β and γ genes. β are required for viral DNA replication, which in turn stimulates the expression of γ genes.

HSV-1 α genes are originally defined as genes in which gene expression is independent of prior viral protein synthesis [34]. The function of these gene products of this group of viral genes is to promote and regulate the continued viral gene expression and to subdue the cell to the need of the virus [1]. In the promoter region of α genes, there are several binding sequences for cellular transcription factors, including a TATA box and SP1 binding sites (**figure 5**). Also in these regions there are binding sites for VP16, a tegument protein, in complex with two cellular partners, host cell factor 1 (HCF-1) and octamer-binding protein 1 (Oct-1). Together, the complex of VP16, HCF-1 and Oct-1 recruit histone modifying factors and general transcription factors to the promoters of α genes and enable the expression of these genes [56, 58, 59].

Following after α genes, the β genes are expressed. The protein products of these genes are involved with the metabolism of nucleotides, and they are essential for HSV-1 DNA replication [1]. One of the α genes, ICP4, is specifically required for the expression of all β genes and γ genes as well, while ICP0 is necessary for their proper expression, especially at low multiple of infection (MOI), and for successful infection *in vivo* [50]. For specific β and γ genes, ICP27 is also needed.

TATA box and cellular enhancer regions are also found in the promoter region of β genes, upstream of the transcription start site (**figure 5**) [1, 50]. Except for the TATA box, the cellular enhancers in the promoter vary among of these genes. No viral enhancer sequences have been identified in the promoters in the β genes.

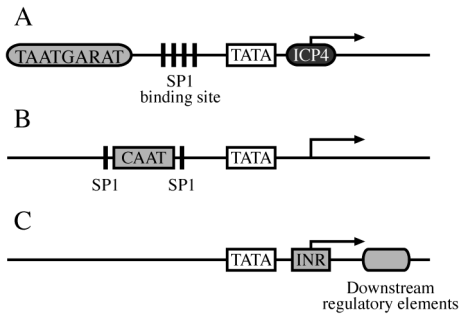

Figure 5. Schematic representation of the promoter regions of HSV-1. (A) The viral element TAATGARAT can be found in the promoter region of α genes which is recognized by the VP16/HCF-1/Oct-1. (B) Only cellular elements can be found in the promoter region of β genes. (C) No additional elements can be found upstream of TATA in true late gene promoters, although downstream activation sites have been identified for some late genes. Picture adapted from [50].

At the promoters of β genes, ICP4 is bound and interacts with the general transcription factors. ICP4 stimulates the formation of preinitiation complex and subsequent initiation of transcription of all β and γ genes [1, 50].

The multifunctional protein, ICP0 which was originally identified by its ability to activate the expression of foreign genes and to down regulate cellular proteins, has recently been shown to modify the histones at β genes to make these genes more accessible for transcription [13, 57]. ICP0 does not have any DNA binding activity, but has a zinc-binding really interesting new gene (RING) finger motif, which is shared among ubiquitin ligase E3 family [60]. How it achieves histone modification and transactivation is still not clear [50]. Acetylation of H3 in nucleosomes is generally associated with derepression of gene expression. Treatment with inhibitors of histone deacetylation could partially compensate for ICP0 deficient HSV-1, which strengthens the argument that ICP0 acts through histone modifications [61]. It has also been shown that ICP0 has a down-regulating effect on antiviral pathways [62]

ICP27 exhibits mRNA binding properties and it has been reported to interact with RNAP II and nuclear pore proteins [63-66]. It is generally considered to stimulate gene expression of certain β and γ genes through post-transcriptional events, such as mRNA maturation and nuclear export [1, 67].

γ genes are subdivided in γ_1 (leaky-late) genes and γ_2 (true late) genes [1, 34, 36, 50]. The requirements for the expression of γ_1 genes are the same as for β genes, but they are expressed later and their expression is enhanced by HSV-1 DNA replication. The γ_2 gene expression, in addition to requiring β genes, is strictly dependent on HSV-1 DNA replication. *In vivo* and in some cells, γ_2 gene expression may also require the α gene, ICP22. No enhancer elements, neither viral nor cellular, have been found upstream of the TATA-box in the promoters of γ_2 genes. Instead regulatory elements can been found down-stream of the transcription start site (**figure 5**) [1, 50]. The promoter region of γ_1 genes resembles a hybrid between β and γ_2 promoters.

Viral DNA replication, which is a prerequisite for γ_2 genes expression, cannot be substituted by any trans-acting factors produced during the DNA replication process [36]. Mutant HSV-1 virus with a fusion gene that consists of a 5' region of a γ_2 gene, which contained both transcribed and non-transcribed regions, with a gene body of a β gene, resulted in a γ_2 expression kinetics of the fusion gene [68]. However, when the same construct is inserted into the host genome, the chimeric, γ_2/β gene, is expressed as an β gene upon HSV-1 infection. A similar construct in a DNA plasmid, in which

the 5' end of a γ_2 gene is coupled to a reporter gene, expresses the reporter gene only as a HSV-1 γ_2 gene upon transfection and subsequently infected, when an HSV-1 origin of replication was inserted into the plasmid [69]. The insertion of the origin stimulated the replication of the plasmid construct upon viral infection, but the authors argued that the expression of the reporter gene cannot solely be explained by an increase of gene dosage, since equal amounts of non-replicated plasmid fail to express the gene in similar fashion. Taken together, these observations suggest that the context in which the γ_2 gene is in is important for how it is regulated.

ICP22 affects γ_2 gene expression independently of viral DNA replication and mutant viruses do not affect HSV-1 DNA replication in neither restrictive nor non-restrictive cell cultures [70-72]. ICP22 has been suggested to contribute to several functions in the viral life cycle. First, ICP22 affects the modification of RNAP II in several ways; it appears to regulate phosphorylation and dephosporylation of amino acid residues in the carboxyl-terminus domain of the largest subunit of RNAP II (CTD) [71, 73, 74]. Modification of CTD is associated with the transition of RNAP II holoenzyme from a transcription pre-initiation complex to functional states involved in initiation, elongation and termination transcription. One hypothesis is that ICP22 is necessary for the transcriptional elongation of γ_2 genes through modifications of CTD [72]. Secondly, ICP22 has been found to be important for the formation of nuclear bodies called virus-induced chaperone-enriched (VICE) domains [75]. These domains contain molecular chaperones, the proteasome, ubiquitin ligases and viral proteins, and they are thought to function as protein quality control centers in the nucleus during HSV-1 infection [76]. The amino-terminal (N-terminal) domain of ICP22 is important for the formation of VICE domains, while the carboxy-terminal (C-terminal) domain is responsible for the phosphorylation effect on CTD. Both domains of ICP22 are important for the expression of γ_2 genes in restrictive cell lines. Third, reports have shown that ICP22 stimulates the modification and stabilization of a certain cellular cyclin dependent kinase (CDK), cdc2, and, at the same time, promotes the degradation of its cyclin subunit [77]. cdc2 together with a viral protein, UL42, involved in HSV-1 DNA replication, appears to recruit cellular topoisomerase II after viral DNA synthesis. Possibly, topoisomerase II untangles the newly synthesized viral DNA and enables the expression of γ_2 genes [78]. How γ_2 gene expression occurs in the absence of ICP22 in non-restrictive cells is not clear.

The temporal viral gene expression program cannot solely rely on positive regulating mechanisms such as the activation of transcription. Negative regulation must also play a role. For example, some viral proteins may act to

inhibit expression of others [1]. Temporal patterns of HSV-1 gene expression at an MOI of 10, start with α protein synthesis at 2 to 4 hours post infection (hpi), and the production of β proteins reaches its peak between 6 to 12 hpi. Finally, γ proteins appear later and accumulate until the end of the infection cycle. Many of the inhibitory elements of HSV-1 gene expression program are still not well understood. However, it has been shown that ICP4 can bind to a consensus sequence in its own promoter region and thereby inhibit transcription of its gene (**figure 5**) [79]. It is also thought that ICP8, the HSV-1 single strand DNA binding protein, has an general inhibitory effect on the HSV-1 gene transcription by an as yet unknown mechanism [80, 81]. Finally, the late gene product VHS/UL41 has been shown to control HSV-1 gene expression by destabilizing certain viral mRNAs and facilitates the transition between α and β gene expression [1].

After transcription, mRNA are required to be translated by the ribosomes into polypeptide chains, which later mature to functional proteins. As in all viruses, HSV-1 utilizes the cellular system for protein translation, and must therefore transport the newly synthesized viral transcript to the sites of translation in the cytosol. In uninfected cells, the transportation of mRNA to the cytosol is coupled to mRNA splicing. However, since most of the HSV-1 genes do not contain introns, it has been argued that HSV-1 inhibits splicing to redirect the cellular machinery to the expression viral genes [1, 51]. The viral transcripts must then have adapted other mechanisms for their translocation to the cytosol. ICP27 has been shown to both interfere with splicing and to promote the translocation of viral transcript to the cytosol [82]. Some other mechanism for nuclear export must also exist, since several viral transcripts are efficiently exported in ICP27 deficient HSV-1. There is evidence that the reorganization and the movements of the HSV-1 replication compartment in the nucleus are important for the export of viral transcripts [83].

In the cytosol, cellular translation occurs in 3 phases, initiation, elongation and termination. Regulation of the cellular translation process is mainly at the level of initiation [51]. For most cellular mRNAs, translation is initiated by a 5' cap-dependent manner, and this is also the case for HSV-1 translation [51]. Interestingly, cellular responses to viral infection have been developed to limit the components necessary for translation and thereby preventing viral protein production [84]. HSV-1 in return, has developed strategies to overcome these effects, e.g. the viral proteins ICP34.5 and US11 promote the formation of the translation pre-initiation complex (43 subunit), and ICP6 seems to promote the formation of the translation initiation complex on the 5'cap of mRNA [51, 84].

HSV-1 re-directs the cellular translation machinery by first destabilizing cellular mRNAs, in a VHS and ICP0 dependent manner at early times, and by ICP27 and VHS later in the infection, and then acts to maintain a general cap-dependent translation [1]. Recently it has been suggested in a reporter system that ICP27 may induce mRNA specific translation initiation down stream of 5'cap- dependent recognition, relying on the interaction with the poly(A) binding protein [85]. However the role of this mechanism during HSV-1 infection needs to be further explored.

1.9 HSV-1 DNA replication

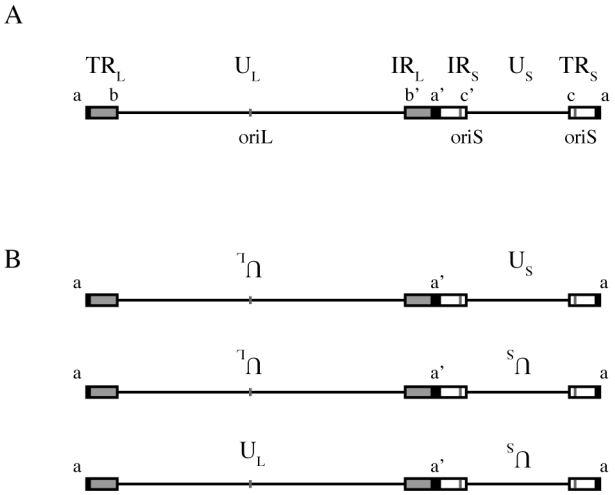

Figure 6. HSV-1 genome. (A) Schematic representation of HSV-1 genome unique long (U_L), unique short (U_S), and the terminal (TR) and internal repeats (IR). The three origins of replication can be found in the middle domain of U_L (oriL) and in the repeat sequences flanking U_S (oriS). (B) Four different isomers of the inverted unique sequences exist in equal molar amounts in HSV-1 infection.

In order to produce new infectious HSV-1 virion particles, the virus needs to properly copy its genome before packaging it into capsids. The process of copying the viral genome is accomplished by DNA replication. HSV-1 encodes seven proteins required for its DNA replication. In addition, a number of cellular and viral proteins contribute to efficiency and accuracy during the production of new genomes [86]. The seven replication core enzymes are: UL9, the origin binding protein (OBP); UL29 (ICP8), single-strand DNA binding protein; UL5, UL8 and UL52 make up the trimeric helicase-primase complex; the HSV-1 DNA polymerase, UL30, and its processivity factor, UL42 (**table 1**) [86]. Together these proteins form the HSV-1 replisome and they have been well studied since they pose as good potential targets for antiviral therapy, but they can also serve as model for studying the molecular mechanism of DNA replication. The initiation of the viral DNA replication starts with the assembling of HSV-1 replisome on circularized virus DNA at unique sites referred to as origins of replication (**figure 6a**). At these sites, the replisome then synthesizes new DNA in a continuous leading strand and lagging strand mode to ultimately generate concatemers of viral DNA, which can be cleaved into monomers during

encapsidation of the genomes (**figure 3**) [1, 86, 87]. Below, these steps will be reviewed in more detail.

The generally accepted model of HSV-1 DNA states that after its nuclear entry, the HSV-1 genome adapts a circular conformation (**figure 3**) [1, 86]. This step is dependent on the cellular DNA ligase IV/XRCC4 and therefore it suggests that non-homologous end joining facilitates the formation of endless HSV-1 genomes after its nuclear entry [33, 88]. On the HSV-1 genome there are three origins of replication (**figure 6a**), one in the middle UL, referred as oriL, and two in the "c" sequences flanking US, referred as oriS [86]. These sites contain cis-elements which are recognized and bound by the HSV-1 replication initiator protein, OBP. OBP is encoded by the UL9 gene and has a predicted molecular mass of 94 kilodalton (kDa) [89, 90]. The N-terminal domain has a DNA helicase activity and belongs to the superfamily II of DNA helicases [86]. The C-terminal domain of OBP is responsible for its sequence-specific recognition and binding to the oriL and oriS. In addition, the last 13 amino acids of OBP display an evolutionarily conserved motif, WPXXXGAXXFXXL, which binds to ICP8 [91]. At oriS and oriL, the binding sites for OBP, box I and box II, are separated by repeats of adenosine (A) and thymidine (T). Two dimers of OBP bind to oriS, one dimer to each binding site flanking the AT-rich sequence. Interaction beteween ICP8 and the OBP dimers will promote a conformational change in OBP and lead to the distortion of the AT-rich sequence, and expose a single-stranded DNA which will attract ICP8 (**figure 7B**) [86, 92]. The activation of HSV-1 oriS can be achieved *in vitro* by a cooperation between OBP and ICP8 in the presence of ATP. This will generate a single-strand hairpin structure bound by the C-terminal domain of OBP, while the N-terminal domain of OBP will see single-stranded DNA and hydrolyse ATP. Ultimately, ICP8 will be released from OBP and become cooperatively bound to single-stranded DNA [86, 92-95]

After the activation of origins, the heterotrimeric (UL5, UL8 and UL52) helicase-primase complex is recruited to the ICP8 coated single stranded DNA **(figure 7C)** [86, 96, 97]. Analysis of the sequence motifs of UL5 have shown that it is a part of the superfamily I DNA helicases and while analysis of UL52, which has primase activity, has recently shown that it is related to the human PrimPol [98-100]. Both helicase and primase activities are retained in the UL5 and UL52 (UL5/UL52) subcomplex, and their enzymatic activities have been shown to be interdependent [86]. For example, mutations in the C-terminal part of UL52 will not only affect the primase activity but also the DNA dependent ATPase and helicase activities of UL5/UL52 subcomplex [101]. In contrast, UL8 is devoid of any enzymatic activity, but

it is necessary for the proper function of the helicase-primase complex in HSV-1 viral infections. It has been shown that UL8 is essential for the nuclear localization of helicase-primase complex, and it may mediate the interaction with the other components of the HSV-1 DNA replication machinery [102-106]. *In vitro*, the addition of ICP8 stimulates the helicase activity of the full helicase-primase complex (UL5/UL52/UL8), but not in the absence of UL8 [104]

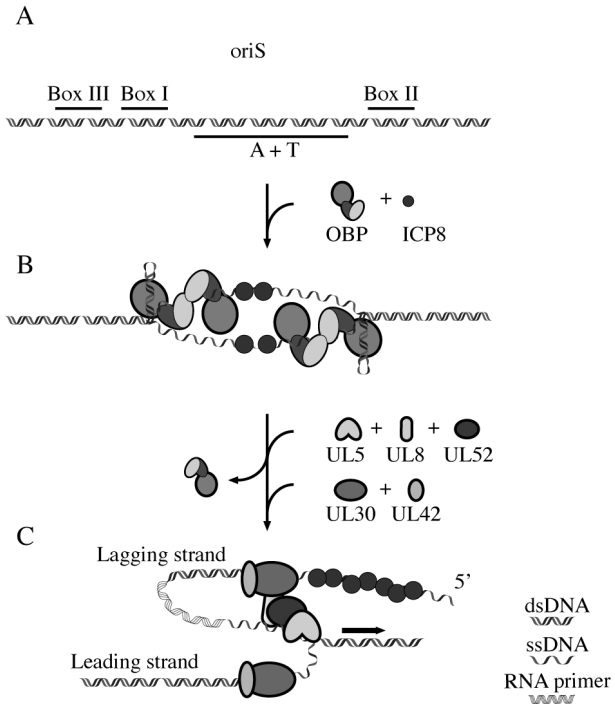

Figure 7. HSV-1 DNA replication. (A) Schematic representation of oriS. (B) Activation of HSV-1 origin. (A) and (B) adapted from [107]. (C) HSV-1 replication fork.

The helicase-primase complex will continuously unwind DNA duplex at the HSV-1 replication fork and synthesize short primers for the lagging strand synthesis [103]. Primer synthesis seems to be a prerequisite for the recruitment of HSV-1 DNA polymerase and its processivity factor [108]. However, a direct and specific interaction between HSV-1 DNA polymerase and the helicase-primase complex has yet not been identified. The HSV-1 DNA polymerase, UL30, has a molecular mass of approximately 134 kDa and its crystal structure has been solved [109, 110]. In addition to its

polymerase activity, UL30 also exhibits a 3' to 5' exonuclease activity [111, 112], which can act as proof-reading, and contributes to its replication fidelity [109]. It has also been shown that UL30 has a 5' deoxyribose phosphate lyase activity [113]. The extreme C-terminal domain of UL30 interacts with UL42, in which in return tethers UL30 to double stranded DNA, and thereby enhances processivity of UL30 [114, 115]. The crystal structure of UL42 has also been solved and its structure resembles one subunit of the homotrimer complex of proliferating cell nuclear antigen (PCNA), which is the processivity factor of the eukaryotic DNA polymerase δ [116]. Unlike PCNA, which forms a ring around the duplex DNA, UL42 binds DNA and UL30 as monomer. Also different from PCNA, instead of sliding along double stranded DNA , UL42 appears to diffuse along duplexed DNA through tiny jumps [117]. After the initiation, viral DNA replication is believed to occur bi-directionally from the origins, with two replication forks travelling in opposite direction away from the origins (**figure 8**). *In vitro* evidence support that leading and lagging strand synthesis are coupled at HSV-1 replication forks (**figure 7c**) [87, 118]. Initially, DNA replication will proceed by a theta (θ) mode of replication generating a circular DNA as product [86, 109, 119]. However, at later times replication will switch to a rolling circle mode of replication by an unknown mechanism (**figure 3** and **8**) [87].

At this later stage, during rolling circle replication, OBP is not needed. It may, in fact, have an inhibitory effect on viral DNA replication during later times of infection [120, 121]. The product of the origin independent rolling circle DNA replication are multiple unit repeats of the HSV-1 genome coupled to each other in a head to tail conformation [1, 86, 109]. The majority of replicated viral DNA is found in the form of concatemers and in complexes of branched networks, as result of the high frequency of recombination [122-124]

The complex network of branched viral genomes needs to be further processed and resolved before it can be cut into single genome units and packaged in to capsids. The viral alkaline nuclease, UL12, has been suggested to play a role in resolving these branches [125, 126]. Mutant HSV-1, with defective UL12, produces more branched replication products in the cell, and it also produces capsids containing defective viral DNA. While DNA replication *per se* is only minimally affected for this mutant [125, 126].

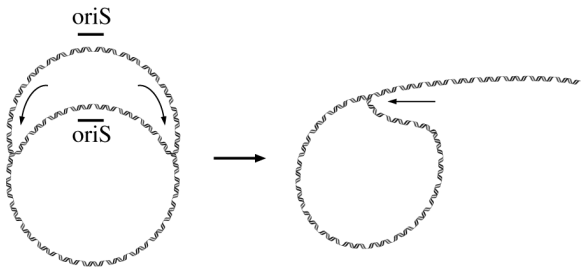

Figure 8. The different modes of HSV-1 DNA replication. DNA replication initiates as theta mode but later switches to rolling circle replication. (Small arrow indicates the direction of replication forks)

The UL sequence in the HSV-1 genome is flanked by repeated sequences termed "ab" at the terminal end, while in the internal end it is flanked by inverted repeated sequence, "b'a'", and US in turn, is flanked in the terminal end by the sequence "ca", while in the internal end by the inverted sequence, "a'c'" (**figure 6a**) [17, 18]. At the "a" sequences the recombination frequency is enhanced [1, 86, 103, 124]. As a consequence of this, the number of repeats of a sequences varies in the terminal end of UL, and at the junction between UL and US. Also, the orientation of the UL and US compared to the terminal and internal repeats exist in four different isomers at equal molar concentration during productive infections (**figure 6b**) [86].

The processing of the head-to-tail concatemers into single units of HSV-1 genomes occurs in a sequence-specific manner also at the terminal "a" sequences, and this event is tightly associated with the packaging of the genome into capsids [127]. This is demonstrated by the depletion of cut genomes in the absence of structural capsid proteins [128]. The proteins UL15, UL28 and UL33 make up the terminase which cleaves the replication products into a single unit of one HSV-1 genome and this complex is highly conserved among all herpesviruses [7, 127].

Table 1. HSV-1 replisome.

Protein name	Gene	Protein functions
OBP	UL9	Origin binding protein, 3'-5' helicase
DNA helicase-primase complex	UL5	5'-3' helicase
	/UL52	Primase
	/UL8	Suggested to have an integrative role
DNA polymerase	UL30	DNA polymerase, 3'-5' exonuclease
	/UL42	DNA polymerase processivity factor

1.10 Treatment of HSV-1 infections

The most efficient way to reduce the burden of infectious disease in a population is to identify a good vaccine. Despite an active search, there is currently no available vaccine against HSV-1 on the market [129]. While, for the related varicella-zoster virus (VZV), another alpha herpesvirus that infects humans and causes commonly chicken pox, there has been a live attenuated vaccine for the last twenty years, and this gives hope for the development of a successful vaccine against HSV-1 in the future [130]. Other strategies to limit the spread of HSV infections have been employed, such as the use of condoms, and specific hospital routines aimed at reducing the risk of transmission to immunocompromised individuals and to neonates. However, the worldwide incidence and prevalence of HSV infections have not declined [19].

All currently used clinically antiviral therapies against HSV-1 infections are targeting viral DNA replication [1]. Acyclovir, an antiviral nucleoside analog against HSV, has pioneered the development of nucleoside analogs against virus infections. It efficiently inhibits the productive HSV replication and has a low toxicity for uninfected cells [131]. In fact, acyclovir and its derivatives are still the most widely used therapies against HSV and VZV infections [1]. Its effectiveness depends on the conversion of acyclovir to acyclovir monophosphate by herpes virus thymidine kinase. Acyclovir is additionally phosphorylated by cellular kinases after its phosphorylation by the virus kinase and used by the viral DNA polymerase to be incorporated into the replicated viral DNA. Since acyclovir lacks the 3' carbon atom, additional nucleotides cannot be added to the acyclovir incorporated viral DNA. As a consequence of this, viral DNA replication is terminated [132].

The use of acyclovir and its related drugs has decreased the mortality rate of adult HSV encephalitis and disseminated neonatal infection, from 75% and 80% respectively to 25% [1]. Although, only one third of the survivors with encephalitis recover to normal neurological function and 60% of the survivors from disseminated neonatal disease suffer from neurological impairment even after treatment. Treatment of orolabial and genital HSV infections with acyclovir related drugs eases the symptoms and shortens the healing time [133, 134]. Also if used as suppressive therapy, acyclovir related drugs diminish the rate of recurrence and viral shedding. However, they do not completely inhibit the shedding of virus, and HSV can still be transmitted to seronegative individuals upon contact with seropositive individuals, even if the seropositive individual is on suppressive therapy. Although uncommon,

resistant viruses to acyclovir do appear and occur mainly in individuals who are immunocompromised [27, 28].

Non-nucleoside drugs have also been developed against HSV infection. Foscarnet, a pyrophosphate analogue, targets HSV DNA polymerase enzymatic activity and is used for treating acyclovir resistant infections [1]. A new group of drugs which target HSV helicase-primase complex have shown great potential and are currently undergoing clinical trials [135].

There are still no current treatment against latent HSV-1 infection and, as mentioned earlier, once infected with HSV-1, the host will retain the virus for life.

2 AIMS

Despite at least three decades of intense research, a number of issues concerning the molecular mechanism for HSV-1 DNA replication and its coordination with other events in the viral life cycle remain. In this thesis we focus on the protein interactions within the HSV-1 replisome that are important for their functions. Also, we have been interested in the regulation of HSV-1 late gene expression, which is strictly dependent on viral DNA replication. Specifically, we have looked at:

I. Species-specific and functionally significant interactions within the HSV-1 replisome using transiently expressed HSV-1 and EHV-1 proteins **(paper I)**

II. An in depth analysis of the interactions between HSV-1 UL8 protein and other viral replisome components **(paper I and II)**

III. Mapping of the protein domains of HSV-1 primase UL52 and the interactions with other replication proteins **(paper II)**

IV. Analysis of helicase-primase inhibitors on interactions within the helicase-primase complex **(paper II)**

V. Identification of the cellular and viral factors involved in late gene expression **(paper III)**

3 RESULTS AND DISCUSSION

3.1 Paper I

HSV-1 encodes for its own core proteins for DNA replication. The equivalent proteins can be found in all herpesviruses in the family of *Herpesviridae* [136], which indicates that the viruses replicate their genomes in a conserved manner. Since origin independent DNA replication has been reconstituted *in vitro* using a rolling circle model for DNA replication, it is evident that the replisome proteins interact with each other. Furthermore, it has been shown that these interactions are functionally important for the proper replication of HSV-1 DNA [104, 137, 138]. UL8, a protein of HSV-1 replisome, is a part of heterotrimeric helicase-primase complex, but it has no detectable enzymatic activity. Earlier works have indicated that it interacts with other proteins in the viral replisome, suggesting an integrative role of UL8 in the replisome. During viral replication *in vivo* UL8 is indispensable, and it is continuously required for HSV-1 DNA replication *in vitro* in the presence of the remaining core proteins [86].

In this work, we have tried to identify species-specific protein-protein interactions within the HSV-1 DNA replisome, and to examine the relevance of these interactions with regard to DNA replication.

To assess the function of HSV-1 replisome in cells, we transiently expressed viral replication proteins in a mammalian cell line together with an indicator plasmid containing HSV-1 oriS [139, 140]. We also established a homologous system in a close relative, *Equid alphaherpesvirus 1* (EHV-1). Using expression plasmids for UL9, UL5 UL8, UL52, UL30, UL42 and UL29, we were able to detect oriS-dependent plasmid replication (**figure 1** in paper I). The two replisomes had a preference for their cognate origin of replication. Importantly, neither subcomplexes nor individual proteins of the replisomes could be exchanged between HSV-1 and EHV-1 to achieve comparable amounts of replicated DNA (**figure 1a**, **b** and **figure 2** in paper I).

These observations indicate that the functional interactions between the proteins within the herpes replisome are species-specific, despite the fact that all herpesviruses replicate their genomes in a similar manner. As noted, the two OBPs, HSV-1 and EHV-1, seem to prefer their own origin sequences, even if the recognition sequences at their origins are identical [141]. A

notable difference between the origins of these two different species, is that the EHV-1 oriS contains four additional AT-base pairs in the AT-rich spacer sequence compared to HSV-1 oriS [141]. It has previously been demonstrated that the length of the spacer sequence affects the ability of HSV-1 OBP to activate oriS [93, 142]. This discrepancy of DNA replication observed during different oriS, might be explained by the fact that HSV-1 and EHV-1 OBP prefer different lengths of AT-rich spacer sequences to modulate the effects of the cooperative protein-protein interaction at oriS [86].

We next turned our attention to UL8, whose protein sequence is one of the least conserved among the replisome proteins within the family *Herpesviridae* (Paper I). Its low conservation was in line with a proposed integrator role in herpes replisomes. Besides being a part of the helicase-primase complex, it had also been reported previously that UL8 interacts with OBP, ICP8 and UL30 [104-106]. To map the amino acids that were responsible for these functional interactions and to elucidate other unknown roles of UL8, we employed a systematic mutational analysis of HSV-1 UL8. Based on the fact that charged amino acids are usually exposed on the outer surface of proteins [143], charged amino acids situated within 4 residues of the peptide chain of UL8 were replaced with alanines. In total, 52 mutants were generated (**figure 3** in paper I). The ability of the mutants to support origin dependent replication was examined in the transient replication assay described earlier. Only four out of the 52 mutants exhibited a significant decrease in DNA replication efficiency (**supplement table 1** in paper I). Mutant A23, where the arginine at position 254 and aspartic acids at positions 255 and 257 were mutated to alanines (R254A, D255A and D257A respectively), did not support DNA replication. While three mutants in the far C-terminal of UL8, A49 (R640A, D642A and E644A), A52 (D671A and E673A) and A53 (R677A and R678A), exhibited a temperature sensitive (ts) phenotype. At the non-permissive temperature, 39 ºC; A49, A52 and A53 did not support DNA replication, but at the permissive temperature, 32 ºC, the ts mutants could support replication up to 80 to 90 % of wild type (wt) UL8 (**table 1** in paper I).

UL8 has been attributed two functions: nuclear localization of the viral helicase-primase complex, and an integrative role in the HSV-1 replisome. We therefore examined the cellular localization and protein-protein interactions of the defective, A23, mutant and the ts, A49, A52 and A53, mutants, using confocal microscopy and co-immunoprecipitation experiments.

In transient expression assays, the proteins of the HSV-1 replisome and the plasmid containing oriS are translocated to the nucleus, forming replication compartments. It is in these replication compartments where active DNA replication occurs [144, 145]. A similar process is believed to occur during viral infection. When analyzed with confocal microscopy, mutant A23 and the ts mutants failed to form replication compartments as seen with wt UL8 at the non-permissive temperature (**figure 7** in paper I). Furthermore, accumulation in the nucleus of UL8 for mutant A23 was less pronounced when compared with wt and the other mutant proteins (results not shown).

The only stable interaction detected by co-immunoprecipitation between UL8 and other proteins of the HSV-1 replisome using the transient expression assay was with UL52 (results not shown). Further experiments revealed that the ts mutants, A49 and A53, could not pull down UL52 (**figure 6** in paper I). Mutant A52 also pulled down lower amounts of UL8. These observations indicated that the interaction with UL52 was affected. Specifically, the arginine at residue 640 seemed to be specifically important for the UL52 interaction in the mutant A49 (**figure 8b** in paper I). The pull-down efficiency of the ts mutants was not affected by temperature, and suggested that the reason for their inability to support DNA replication was not due to gross misfolding. Instead, our results suggest a deficient interaction with UL52 as an explanation. The amount of UL52 pull-down by the defective mutant A23 was not significantly affected (**figure 6** in paper I).

Sequence alignment of proteins corresponding to UL8 in the family of *Herpesviridae*, showed that the mutated amino acids of A23, A49, A52 and A53 mutants were conserved (**figure 3** in paper I). To relate the importance of these conserved amino acid residues, corresponding mutants of EHV-1 UL8 (EA23, EA49, EA52 and EA53) were generated, and their ability to support DNA replication was examined in the transient replication assays as described previously. Interestingly, EA49 and EA52, for which corresponding mutations in HSV-1 showed defective interaction with UL52, significantly affected the DNA replication efficiency also in the EHV-1 replisome at the non-permissive temperature, although not as severely as in HSV-1 (**table 1** and **figure 9** in paper 1). The mutant EA23 exhibited a temperature sensitive phenotype and the mutant EA52 seemed to behave in similar manner as A52. The mutational analysis of EHV-1 UL8 demonstrated that similar regions in EHV-1 UL8 and HSV-1 UL8 were important for supporting DNA replication and that the function of EHV-1 UL8 function is similar to HSV-1 UL8.

Taken together the results in paper I demonstrate that protein-protein interactions within the herpes replisomes have evolved to become species-specific, even between the closely related viruses HSV-1 and EHV-1. However, our results confirm that the two different replisomes function in similar manner, since corresponding mutations in UL8 had similar effects on the HSV-1 and EHV-1 replisome. We also demonstrated, for the first time, specific amino acid residues that are important for the interaction between HSV-1 UL8 and UL52. These mutations were all located in the C-terminal domain of the UL8 protein. It cannot be ruled out that other domains of UL8 also interact with UL52, since mutants A49, A52 and A53 exhibited temperature sensitive phenotypes. In contrast to the other UL8 mutants which affected DNA replication, the mutated amino acids of A23 were located in the middle part of UL8, and they did not affect the interaction with UL52. This mutant failed to support DNA replication and immunofluorescence analysis using confocal microscope showed that it was deficient in its nuclear localization. We reasoned that A23 was either deficient in its nuclear localization sequence or was structurally impaired. Finally, even if UL8 did not interact with other proteins of HSV-1 replisome in co-immunoprecipitation experiments, we cannot dismiss other interactions within the viral replisome or, for that matter, other functions of UL8.

3.2 Paper II

The trimeric helicase-primase complex in human herpesviruses has been suggested to be a good target for antiviral compounds, since a similar complex does not exist in the host cells [135]. The helicase-primase complex UL5, UL52 and UL8 of HSV-1 is essential for HSV-1 replication, but a detailed understanding of its biochemical properties and biological function is lacking. The helicase component, UL5, has been suggested to be related to the evolutionary conserved cellular Pif 1 helicase, and it travels along the lagging strand template in 5' to 3' direction at the HSV-1 replication fork [86, 146]. The primase component, UL52, has recently been demonstrated to be related to the cellular primpol, and it synthesizes short RNA primers from 5' to 3' direction [100]. As discussed previously, the UL8 component of the trimeric helicase-primase complex has been shown to lack any enzymatic activity [147]. The detailed interactions within the helicase-primase complex have not been characterized in detail yet, and the mechanism which allows the helicase to unwind duplex DNA (5' to 3') in the opposite direction to the primer synthesis (3' to 5' direction on the lagging strand template) is puzzling. To address these issues, we have looked at the detailed interactions within the helicase-primase complex.

In paper I, we could not precipitate UL5 using UL8 in co-immunoprecipitation experiments, but we could successfully pull-down UL52. We thus focused on UL52 interaction domains in the helicase-primase complex. With the guidance of a previous work [148], truncation experiments of UL52 showed that the middle (M-) domain (amino acids 422 to 914) was sufficient to pull-down UL8, and that the N-terminal (N-) domain of UL52 (amino acids 1 to 421) was sufficient to pull-down UL5 in co-immunoprecipitation experiments (**figure 3** and **table 1** in paper II). These interactions were confirmed by reverse pull-down experiments with UL8 and UL5 (**figure 2a** and **4a** in paper II). Interestingly, the N-domain, as well as the full length UL52, were found in lower amounts in the lysate compared to the M-domain and C-terminal (C-) domain as detected by immunoblot experiments, but the low abundance of the N-domain was reversed in the presence of UL5 (**figure 2a** compared to **figure 2b** in paper II). The corresponding effect could also be observed for UL5; simultaneous expression of full length UL52 and the N-domain increased the level of UL5 (**figure 2b**, **3** and **4** in paper II). Although not to the same extent, the level of UL5 was also somewhat increased in the presence of the C-domain, which could possibly indicate a weaker transient interaction.

Also, whenever UL5 was co-expressed with full length UL52, a smaller fragment of UL52 could also be detected in the cell lysates (**figure 2b**, **4a** and **c** in paper II). This fragment had a similar size to the N-domain. Since UL52 and its domains were detected with an antibody raised against the emerald green fluorescent protein (emarld GFP) tag at their N-terminal, the small fragment might be a N-terminal fragment of UL52 produced by produced by proteolytic cleavage. In addition, since we have shown earlier that UL5 interacts with the N-domain, this observation indicates that the interaction with UL5 either stabilizes the fragment or/and increases cleavage at a possible putative hinge region.

With the help of high-throughput screening, an efficient anti-HSV compound, BAY 54-6322, has been identified [149]. This drug has been shown to be superior to valacyclovir, in that it promotes less genital shedding of HSV-2 and reduced frequency of genital lesions in clinical trials [150]. The inhibitory action of this drug has been identified to be on the helicase-primase complex, based on the appearance of a resistant mutant virus [149, 151]. The mechanism of inhibition is, however, not known. Previously in paper I, we showed that the interactions within the HSV-1 helicase-primase complex were important for its function, and we hypothesized that BAY 54-6322 might affect these interactions. To investigate if this was the case, co-immunoprecipitation experiments against UL5 were performed in the presence and absence of the drug (**figure 5b** and **d** in paper II). A dose dependent increase of UL52 in pull-down experiments was observed in the presence of the drug. At the highest concentrations, levels of UL5 and UL52 in the lysate were reduced, but even then the relative amount of UL52 pulled-down by UL5 still increased. These observations suggest that the inhibitory effects of the drug were mediated through the action of stabilizing the UL5 and UL52 interaction and preventing a conformational change required for their function. To investigate the effect of resistant mutations against BAY 54-6322, we made expression plasmids harboring mutations in the UL5 (K356T) and the UL52 (A899T) gene. Co-immunoprecipitation experiments with these mutants revealed that the interaction between the resistant mutants were not affected by the presence of the drug (**figure 5b**, **c** and **d** in paper II). The effects seen on the interactions within the helicase-primase complex in the co-immunoprecipitation experiments was related with the function of the replisome by a quantitative polymerase chain reaction (qPCR) assay (**figure 5e** in paper II). Consistent with the co-immunoprecipitation experiments, the resistant mutations made the replisome more resistant to the inhibitory effect of the drug. The fifty percent inhibitor concentration (IC_{50}) of the drug on DNA replication was at least 10-fold higher for BAY 54-6322 with the resistant mutant proteins than for the wt proteins (~30 μM vs. ~3 μM). The

resistant UL5 and UL52 mutant protein levels were also seen to be affected by the high concentration of the drug in the cell lysates, and this could, at least partially, explain the reduced DNA replication efficiency observed for the resistant mutant proteins.

It had also been reported for another drug against HSV-1 infections, APS2152, that resistant viruses harbored mutations mapped to the UL5 and UL52 genes (UL5; G352V and M355I, and UL52; S364G and R367H) [152]. Compared to their parental strain, these resistant HSV-1 viruses also showed deficient infectivity, but the reason for this was unknown. We noted that these resistance mutations in the UL52 protein were found in the N-domain. To see if these mutations could affect the interaction between UL5 and UL52 in the helicase-primase complex, they were transferred to UL5 and UL52 genes in expression plasmids. The ability of the mutant proteins to interact was assessed in co-immunoprecipitation experiments (**figure 5a** in paper II). As suspected, mutations in UL52 affected its ability to be pulled down by UL5 and this property could reflect the effect of the mutations on viral infectivity. Unfortunately, we did not have access to ASP2151 and could not perform similar experiments as we did for BAY 54-6322.

The domain structure of UL52 may serve different roles in the HSV-1 replisome. To see if it was possible to separate these roles, the N-, M- and C-domains were transiently expressed together with the rest of the HSV-1 replisome proteins, as well as an oriS-containing plasmid. The ability to support DNA replication and the effect on cellular localization were examined with qPCR and confocal microscopy, respectively. The combination of N-, M- and C-domains expressed simultaneously did not support DNA replication (result not shown), but strikingly they did form prominent nuclear dots co-localized with ICP8 (**figure 6** in paper II). Although bigger in size, these dots resembled the pre-replicative sites seen during HSV-1 infection [153]. The minimum requirement for the formation of HSV-1 pre-replicative sites has been shown to be dependent on UL5, UL52, UL8 and UL29 expression [153]. To compare the requirement for the formation of the nuclear dots with the pre-replicative sites, we performed a series of experiments in which the viral replication proteins as well as the origin-containing plasmid were omitted one by one (results not shown). Finally, it was found that the M-domains of UL52, UL8 and ICP8 were sufficient for their formation (**figure 7a** in paper II). This was in agreement with our earlier observation that UL8 and the M-domain of UL52 interacted in co-immunoprecipitation experiments. The co-localization with, and the requirement for, ICP8 for the formation of the nuclear dots, indicated that there might exist a ternary complex between ICP8, M-domain of UL52 and

UL8. The interaction between ICP8 and the M-domain of UL52 was confirmed by co-immunoprecipitation experiments and was independent of UL8 (**figure 7b** and **c** in paper II). This interaction between ICP8 and the M-domain of UL52 may reveal how the helicase-primase complex is recruited to ICP8-coated single-stranded DNA at the activated origins on the HSV-1 genome. The role of UL5 for the formation of the nuclear dots and the pre-replicative sites, could possibly be explained by that interaction between UL5 and UL52 affects the accessibility of the M-domain of UL52. When only the M-domain is present with UL8 and ICP8, their interactions become independent of UL5. An implication of this idea could be, that in the presence of UL5, UL8 could pull down larger amounts of full length UL52 (**figure 2a** vs **b** in paper II).

To summarize, we have demonstrated, for the first time, the specific functional domains of UL52 using deletion mutants of the protein. With these mutants, we could show that the helicase component, UL5, bound stably to the N-domain of UL52. We found that the N-terminal domain of UL52 was stabilized and could be proteolytically cleaved from UL52 in the presence of UL5 in cells. Proteolytic cleavage is often observed in hinge or linker peptide sequences and this may also be the case for the UL52 N-domain. We speculate that a hinge domain may be necessary in order to handle the forces created by the opposite directions of movement of the helicase and the primase at the replication fork. Only small amounts of the UL52 C-domain could be detected in co-immunoprecipitation experiments with UL5, and this may indicate that they interact only transiently. The functional significance of the interactions between UL5 and UL52 was highlighted by the observation that inhibitors of the HSV-1 helicase-primase complex affected the interaction between the UL5 and UL52. Finally, binding of the UL52 M-domain to ICP8 was demonstrated by co-immunoprecipitation experiments and may demonstrate a mechanism of how the helicase-primase complex is loaded on single-stranded DNA coated with ICP8. The M-domain also bound to UL8 in co-immunoprecipitation experiments and evidence of a ternary complex of M-domain of UL52, UL8 and ICP8 were observed by confocal microscopy.

3.3 Paper III

The aim of a virus is basically to produce new infectious virion particles by infecting cells. Two processes are fundamental for productive virus infections: expression of viral genes and replication of the viral genome. As discussed previously, HSV-1 encodes its own DNA replication proteins and in order to effectively produce new infectious virion particles it has an strictly regulated program for gene expression. The HSV-1 genes are grouped as immediate early (α), early (β) and late (γ) genes, and they are expressed sequentially (discussed in further details in section 1.8). It has been shown that the expression of a subgroup of γ genes, true late (γ_2) genes, strictly requires HSV-1 DNA replication and that no transacting factor produced during HSV-1 infection can overcome this requirement [36]. However, the molecular mechanism underlying this phenomenon is poorly understood and we have therefore re-explored the requirements for HSV-1 γ_2 gene expression with modern analytical techniques.

First, the requirements for loading of RNA polymerase II to the three different classes of gene promoters was investigated using chromatin immunoprecipitation (ChIP). In addition to wt HSV-1, two temperature sensitive HSV-1 viruses were used. Temperature sensitive mutant tsK had an alanine to valine substitution at position 475 of the ICP4 peptide sequence and at the non-permissive temperature, 39 ºC, the function of ICP4 was impaired for this ts mutant [154]. The other temperature sensitive mutant virus, tsS, had alanine to threonine substitution at position 90 of the peptide sequence of UL9 and did not support the initiation of DNA replication at the non-permissive temperature 39 ºC [120]. ChIP and subsequently qPCR were used to assess the loading of RNAP II to the promoter of the ICP4 coding gene (an α gene), UL23 (a β gene) and UL38 (a γ_2 gene) on the wt HSV-1, as well as on tsK and tsS genomes at the non-permissive temperature of the mutants (**figure 1** in paper III). For wt HSV-1, RNAP II was enriched on the promoters of the ICP4 gene, UL23 and UL38 at 4 hours post infection (hpi). While later, at 7 hpi, the RNAP II occupancy decreased for the promoters of UL23 and UL38 on the genome of wt virus. For tsK virus, RNAP II accumulated at the promoter of ICP4, but failed to bind to the UL23 and UL38 promoters. In the absence of DNA replication, RNAP II enrichment increased with time at the promoters of all three classes, ICP4, UL23 and UL38. These observations were consistent with previous findings stating that loading of RNAP II on γ_2 gene promoters is independent on DNA replication and that ICP4 is required for loading of RNAP II to both β and γ gene promoters [155].

The difference in regulation of β and γ_2 gene expression thus seemed to occur in downstream of the loading of RNAP II to the promoter. We next examined binding of RNAP II to the neighboring genes, UL38 (γ_2) and UL39 (β), using ChIP (**figure 2a** in paper III). Clearly, an increase in RNAP II binding to the UL38 promoter could be observed between 2 to 4 hpi. For the UL39 promoter, RNAP II was highly enriched already 2 hpi and changed only little over time. This observation suggests that RNAP II was loaded on the UL39 gene before the UL38 gene, and that the transcription elongation of RNAP II was regulated differently for these two genes.

An important elongation factor for cellular transcription elongation of RNAP II is the positive elongation factor b (P-TEFb) [52]. It consists of the cyclin dependent kinase 9 (CDK9) and a cyclin, usually cyclin T1 or T2. It had been shown previously that inhibition of CDK9 with the drug 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) could affect the expression of certain HSV-1 γ_2 genes [156]. The primary action exerted by CDK9 is phosphorylation of the second residues of the heptapeptide repeat sequence of RNAP II CTD, phosphorylation of suppressor of Ty homolog 5 (SPT5) and of the negative elongation factor (NELF) complex. In eukaryotic cells, SPT5 and suppressor of Ty homolog 4 (SPT4) forms the DRB inducing sensitivity factor (DSIF). Together with NELF, DSIF will associate with initiated transcription complexes and induce stalling at 50 bp downstream of the transcription start site [52]. This phenomenon is called promoter proximal stalling of RNAP II and can be released by CDK9. Specifically, phosphorylation of the NELF complex leads to its disassociation from the transcription complex and phosphorylation of SPT5 turns it into a positive elongation factor [52]. While the phosphorylation of two serine residues on the CTD is coupled to the recruitment of factors involved with mRNA maturation. Promoter proximal stalling of RNAP II transcription complexes have been shown to be a part of the general regulatory mechanism of cellular gene expression [52]. The human immunodeficiency virus (HIV) uses the viral Tat protein to overcome promoter proximal stalling [157, 158]. Tat protein recognizes and binds to the TAR elements in the newly transcribed viral transcript and the TAR associated Tat will recruit P-TEFb to the stalled complex. In this manner HIV selectively stimulates transcription elongation of its own genes during infection.

HSV-1 may have utilized similar transcriptional control for γ_2 gene expression and therefore the distribution of RNAP II on UL38 and UL39 genes was re-investigated in the presence of DRB (**figure 2b** in paper III). Similar patterns were observed for RNAP II distribution in the presence as in the absence of DRB. However, the increase of RNAP II enrichment on the

UL38 gene was delayed in the presence of the drug. In fact, at 2 hpi, the RNAP II could not be detected above background levels at some regions of the genes (results not shown). These observations suggest that inhibition of CDK9 affected RNAP II differently on UL38 and UL39 genes and that transcription elongation could be involved in the regulation of β and γ_2 gene expression.

In order to investigate the specific effect on viral protein levels of CDK9 inhibition during HSV-1 infection, infected cell lysates were analyzed using immunoblotting for a subset of α, β and γ proteins. In these experiments, only γ_2 proteins were found to be sensitive to DRB at concentrations which selectively inhibit CKD9 (between 2,5 to 25 μM [159, 160]) (**figure 3a**, **4a**, **b,** and **7b** in paper III). To exclude that the effect on γ_2 protein, was a consequence of either direct or indirect effects on HSV-1 DNA replication, qPCR experiments were performed on infected cell lysates to investigate the efficiency of HSV-1 DNA replication in the presence of DRB (**figure 3c** and **supplement figure 2** in paper III). At the concentration of 25 μM DRB, the onset of viral DNA replication was delayed, but once it had started, the rate of DNA replication was not affected by the drug. Also, the small decrease in the amount of viral DNA synthesized in the presence of DRB could not explain the effect on γ_2 protein levels. To determine in which phase of the productive infection cycle DRB acts in order to inhibit γ_2 gene expression, DRB was added at different times of an HSV-1 infection and it was observed that the drug could be added as late as 7 hpi and still fully inhibit the expressionof a γ_2 protein, glycoprotein C (gC) (**figure 6b** in paper III). These experiments also showed that the amount of gC in infected cells was reduced even if DRB was added as late as 13 hpi. At this time point, the viral DNA replication had already initiated and was on-going. These results indicate that the inhibition of γ_2 gene expression by DRB cannot be explained by effects on DNA replication.

The specificity of DRB inhibition on CDK9 activity was further addressed with the use of another CDK9 inhibitor, flavopiridol [161]. Similar effects on the expression of gC could be observed in immunblots of cell lysates infected with HSV-1 in the presence of flavopiridol at low concentrations (**figure 4c** in paper III). Finally, the importance of CDK9 activity for gC expression was validated by a siRNA knock-down experiments directed against CDK9 (**figure 5a** in paper III).

After being convinced that the inhibitory effect of DRB on γ_2 gene expression was mediated specifically through CDK9 inhibition, we went on to investigate how CDK9 might specifically stimulate expression γ_2 late

genes. Since the known activities of CDK9 are coupled to transcription elongation, we used reverse transcription polymerase chain reaction (rtPCR) to measure the accumulation of γ_2 transcripts in the presence of DRB during the progression of HSV-1 infection. In these experiments, the effect of the drug was assessed by measuring the ratio of the γ_2 gene transcription product of UL38 over the accumulation of the transcription product of the β gene, UL23 (**figure 6a** in paper III). We found that even though the accumulation of UL38 transcripts was significantly inhibited at later time points, the difference between the ratio of UL38 and UL23 in the presence and absence of DRB did not correspond to the difference in protein levels detected by immune blots in the total cell extracts. Specifically, at 17 hpi the ratio in the presence of DRB was two-fold lower than in the absence (0,3, versus 0,6 respectively) and at 21 hpi, the ratio was three-fold lower (0,35 versus 1,0 respectively). It suggested that DRB also acted at other regulatory steps important for gene expression.

We went on to further examine effects of DRB on β and γ_2 gene transcript accumulation and performed a transcriptome analysis on HSV-1 infected cell lysates in the presence and absence of DRB. The transcriptome analysis showed that reads mapped to the HSV-1 genome decreased from 11 to 6,9 percent in the presence of DRB (**table 1** in paper III). Generally, the γ_2 gene transcription was inhibited slightly more than the other classes of viral genes (**supplemental table 2** in paper III). The most severely affected gene was UL44 coding for gC. However, the ratio between number of reads mapped to UL44 gene in presence and absence of DRB was not lower than 0,64 indicating a less than two-fold decrease following DRB treatment. Thus, the transcriptome analysis confirmed the results of rtPCR demonstrating that the pronounced inhibition of γ_2 gene expression caused by DRB cannot solely depend on its effect on transcription. We also analyzed if the distribution of reads was altered with DRB treatment, as might be expected if promoter proximal stalling occurred (**figure 6c** in paper III). Here the number of reads at each position was normalized to the total number of reads mapped to the whole gene. We could not detect a significant effect caused by the addition of DRB, suggesting that promoter proximal stalling might not be a dominant feature of HSV-1 gene expression control.

It is be possible that DRB could affect nuclear export of transcripts, the step between transcription and translation. To address this matter, we used HSV-1 infected cells obtained in the presence and absence of DRB and examined mRNA in nuclear and cytosolic fractions by rtPCR. Specifically, we compared the ratio of UL38 and UL23 mRNA in the nucleus and cytoplasm in HSV-1 infected cells (**figure 7a** in paper III). These measurements showed

that in the absence of DRB the cytosolic and nuclear quotient of UL38 and UL23 was twice as high as the quotient obtained in the presence of DRB. These observed differences could not distinguish between effects directly on viral mRNA nuclear export or on the maturation process of viral, but if combined with the effect of DRB on transcription it might account for the pronounced inhibition of γ_2 gene expression.

The mechanism by which the inhibition of CDK9 activity could lead to a decreased level of γ_2 transcripts in the cytosol was explored by siRNA knock-down experiments of the phosphorylation substrates of CDK9, SPT5 and NELF-E, an essential subunit of NELF [162] (**figure 5b** and **c** in paper III). These experiments revealed that gC gene expression (γ_2) was strongly dependent on the levels of SPT5. The expression levels of ICP8 (β) were affected to a lesser extent and the synthesis of viral DNA was reduced to 30% when siRNA against SPT5 was used. In contrast, NELF-E seemed to play little role in HSV-1 gene expression. The result might imply that during an HSV-1 infection, CDK9 controls γ_2 gene expression by regulating the activity of SPT5. A significant role for SPT5 is supported by the observation that it is found on HSV-1 DNA, close to the replication fork during productive infection [163, 164]. Together, these observations support that SPT5 has a central role in viral gene expression.

It has recently been shown that in the transcription elongation complex, SPT5 is positioned over the DNA containing cleft of RNAP II, close both to upstream DNA and newly synthesized mRNA [165]. The positioning of SPT5 is in line with several reports that, in addition to promoter proximal stalling, SPT5 is also involved in the maturation processes of mRNA [166-169]. This would match our observations on the inhibitory effects of DRB seen on γ_2 gene expression. We therefore sought after possible interactions between SPT5 and viral factors that had previously been associated with transcriptional control or processing and found interactions between ICP27 and SPT5 using co-immunoprecipitation experiment with an antibody directed against SPT5 (**figure 7b** in paper III). The α gene product ICP27 has been reported to be involved in viral mRNA processing and nuclear export of a group of γ_2 genes [63-66]. In the presence of DRB, the levels of ICP27 co-immunopreciptated with SPT5 decreased by 80% (**figure 7c** in paper III). An interaction between ICP27 and CTD has been reported before, but in that case the interaction was found to be independent of phosphorylation [64]. Decreased association of ICP27 with the viral transcription machinery caused by inhibition of CDK9 by DRB could explain decreased levels of γ_2 gene transcripts ready for nuclear export and subsequent translation.

In summary, our results indicate that the reduced expression of HSV-1 γ_2 genes observed during CDK9 inhibition by DRB cannot be easily explained by effects on viral DNA replication, promoter proximal stalling or solely by reduced transcription activity. It rather suggests that transcript maturation and/or the nuclear export of γ_2 mRNA has an important regulatory role in the expression of these viral genes. We have also shown that DRB decreased either direct or indirect interactions between SPT5 and ICP27, which is known to be involved with the posttranscriptional expression of γ_2 genes. Knock-down experiments of SPT5 demonstrated that it has an central role in HSV-1 gene expression and it is tempting to speculate that the interaction between SPT5 and ICP27, which is dependent on the phosphorylation activity of CDK9, is important for the proper maturation and nuclear export of γ_2 transcripts.

4 CONCLUSION

In this thesis we have provided novel and detailed structural insights into the organization of the HSV-1 helicase-primase complex. This complex is responsible for continued unwinding of duplex DNA at the replication fork, while , at the same time, synthesizing primers for lagging strand synthesis. In addition, it is likely to be responsible also for synthesizing the primer for leading strand synthesis at the viral origins of DNA replication. These functions require a number of protein-DNA and protein-protein contacts as well as an ability to accommodate conformational strain acquired during fork movement.

In short, we have elucidated a domain-structure of the UL52 helicase. Our results show that the N-terminal domain binds the UL5 helicase; the middle domain, the M domain, can bind to the UL8 and UL29, also referred to as ICP8, proteins. Our results are compatible with formation of a ternary complex composed of the M-domain, UL8 and ICP8. In addition, we identified a protease sensitive site between the N- and M domains, which might serve as a hinge domain allowing conformational changes taking place during unwinding and primer synthesis. The UL8 protein interacts with UL52 by a defined surface in its C-terminal part. This interaction seems to be preserved also in a related herpesvirus indicating a high degree of evolutionary conservation of the helicase-primase complex. Interestingly, co-expression in cells of the UL52 M-domain, UL8 and ICP8 lead to formation of prominent intranuclear dots which may resemble pre-replicative foci formed during an HSV-1 infection.

We also were able to demonstrate that a novel antiviral compound, BAY 54-6322, acts to stabilize the UL5-UL52 interaction and possibly prevent conformational changes required for function of helicase-primase.

In our last publication we investigated the role of CDK9 for expression of HSV-1 genes. Using the drug DRB at carefully titrated concentrations we found evidence for a role very early during a virus infections causing a delay in the onset of virus replication and delayed loading of RNA polymerase II on early and late promoters. The phenomenon might be caused by delayed expression of immediate early genes. However, once DNA replicated started it progressed at normal rates and near normal levels of DNA, immediate early and early proteins were synthesized. However, DRB strongly suppressed expression of late genes primarily the γ_2 genes. Our results indicate that DRB cause a modest reduction in transcription of late genes as well as reduced

accumulation of late mRNAs in the cytoplasm. Together our results indicate that CDK9 regulates co- and post-transcriptional processes required for late gene expression. We could furthermore demonstrate that SPT5, a direct target of CDK9, was required for γ_2 gene expression. In addition, DRB inhibited a direct or in-direct association of between SPT5 and HSV-1 ICP27, suggesting a CDK9 regulated pathway for transcription, maturation and export of late viral mRNAs ready for efficient translation.

5 FUTURE PERSPECTIVES

There are some ideas for future research which present themselves immediately. We would like to reconstitute *in vitro*, with purified proteins, the various complexes described in paper II and investigate their biochemical properties. For example, can a complex between UL5 and the UL52 N-domain be isolated and analyzed for enzymatic activities? What enzymatic properties does the various variants of the UL52 primase domains exhibit? How does single-stranded DNA affect the interactions between ICP8 and the complex between the UL52 M-domain and UL8?

Our recent results, together with previous studies, greatly facilitate structural studies of herpes virus replication. We are trying to crystallize individual protein domains and protein complexes for structural studies. We would also like to get involved in attempts to reveal the mechanisms for origin activation and fork movement using cryo-electron microscopy.

Our studies can also be extended to include other herpesviruses, such as Epstein-Barr virus and cytomegalovirus, which infect man and for which efficient antiviral treatments are wanted. Information from structural analysis is likely to facilitate a search for compounds inhibit replication of these herpesviruses.

We would also like to pursue our analysis of regulation of HSV-1 late gene expression by clarifying the role of SPT5 and its putative interaction with ICP27. Are there structural elements in late genes that facilitate SPT5 mediated loading of ICP27 on late mRNAs? CDK9 appears to be recruited to replicating HSV-1 DNA in order to stimulate gene expression. What are the factors need for such recruitment?

The vast number of herpesviruses co-evolving with their hosts raises interesting questions about virus evolution. After paper I was published, a bioinformatic investigation confirmed our result of which amino acids on UL8 were important for its interaction with UL52 [170]. It also showed that UL8 protein sequence is related to family B DNA polymerase. The thought of an inactivated DNA polymerase; UL8, interacting with a PrimPol related primer; UL52, is indeed intriguing and further analysis to elude the functional implication of this is needed.

Also, inasmuch as origin activation as well as replisome composition varies between herpesviruses from different families and there appears to be a

number of different stages during evolution when cellular replication proteins have been recruited for virus replication. When and why these events took place is a fascinating topic.

ACKNOWLEDGEMENTS

I will be forever grateful towards my supervisor, **Per Elias**, for giving me the opportunity to do research. Thank you for all your guidance and for providing me with enough freedom to develop as a researcher. I will forever treasure your lessons in both life and in science.

Thank you, **Isabella Muylaert**, for all your help and support. Basically, what would have become of me without you?

Monica Olsson, I am glad that you decided to retire from your retirement. The combination of your tenacity and kind heart is unique.

Koviljka Hofenskjöld, utan dina hembakade kakor hade jag haft mindre energi under mitt labbande (without your home baked cookies, I would have had less energy to work in the lab).

It was fun when you were in the lab, **Ka-Wei Tang**.

I would also like to thank my co-supervisor, **Maria Falkenberg**. With her encouragement and generosity, it has been a pleasure to work on the third floor of medkem.

Thanks, **Kerryn Elliott**, for your proofreading and comments on my thesis.

I hope to meet you soon again in China, **Tore Samuelsson**!

My **lunch buddies** on the second floor, I really enjoyed our time at Lyktan!

To all the people at the department of **Medical Biochemistry and Cell biology** over the years, thanks for making my graduate student period fun and stimulating.

Mom, **Zhi**, and Dad, **Xiaohe**, I am eternally thankful for your unconditional love and support. You have always believed in me. You have given me the courage and inspiration to pursue the things in life that makes me happy and have always pushed me to become a better person.

Finally, **Jenny**, my partner in life, I never knew that there could be so much color and pleasure in life before I met you. Thank you for your patience during these past three months. By setting a daily reminder on your phone to be more supportive towards me, you truly have been. I love you and I look forward to all the adventures we have ahead of us.

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APPENDIX