

Herpes Simplex Virus 1 DNA replication and its role in recombination and transcription

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Being a scientist is like being a boxer,
you need to take punches to your stomach over and over again.

– Per Elias 2003

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ABSTRACT

Herpes simplex virus 1 (HSV-1) is one of nine different herpesvirus infecting man. They are all capable of establishing a life-long latent state following the primary infection. HSV-1 as well as other herpesviruses may reactivate from the latent state and give rise to a productive infection with clinical symptoms or asymptomatic shedding. HSV-1 infections are primarily treated by targeting the viral DNA replication carried out by a molecular machinery, a replisome, encoded by the virus. Here we examine the mechanism of initiation of viral DNA replication and also how viral DNA replication interacts with DNA recombination and gene expression.

In our first study we examined the initiation-step of HSV-1 replication. The origin binding protein (OBP) initiates replication by binding to the origin of replication (oriS and/or oriL). We showed, using phylogenetics and biochemical experiments, that there was a step-wise evolutionary development of herpesvirus DNA replication initiation. The initial divergence was seen in herpesviruses acquiring an amino-acid motif RVKNL in OBP which binds the sequence TTCGCAC in the oriS. The next step was the development of an ICP8 binding motif at the C-terminus of OBP and finally the arrangement of the binding-sites for OBP in the oriS-sequence and the ability to form a stable hairpin. We presented molecular in vitro data to support the phylogenetic analysis and thereby defining essential motifs in OBP for protein-protein and protein-DNA interactions.

The next study was focused on genetic recombination between different HSV-1 strains. We followed the propagation of HSV-1 in cells infected with one to three genotypes of HSV-1 and calculated the number of recombination

events. We found evidence for Rad51 and Rad52 involvement in recombination of the unique long and unique short gene segments of HSV-1. We also observed an increased recombination rate in cells with retarded ligation of Okazaki-fragments. The fidelity of recombination in virus propagated through mixed infections appears to be high since expansion or shortening of repeated sequences in the US7 gene was not detected.

In the third study we examined the replication-coupled transcription of HSV-1 late genes, which are known to depend on DNA replication for efficient expression. Using chromatin immuno-precipitation we could determine that recruitment of RNA polymerase II to late gene promoters occur even in the absence of replication. Recruitment was dependent on ICP4, but delayed in comparison with early gene promoters. These observations suggested the involvement of transcription elongation and/or maturation in the expression of gamma genes. By using the drug DRB, which inhibits the kinase CDK9, a component of the positive transcription elongation factor B, and siRNA against Spt5, a transcription processivity factor, we could show a specific impairment of late gene expression, with only minimal effect on early gene expression and DNA synthesis. We suggest that CDK9 and Spt5 are specifically recruited to replicated late genes and mediate a maturation of transcribed mRNA for nuclear exit.

In summary, we have studied essential molecular processes in the HSV-1 life cycle and identified molecular interactions as well as mechanistic pathways, which may serve as future drug targets.

Keywords: Herpes Simplex Virus 1, DNA replication, DNA recombination, Rad51, Rad52, Transcription, CDK9, Spt5

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SAMMANFATTNING PÅ SVENSKA

Herpes Simplex Virus 1 (HSV-1) är ett av nio herpesvirus som infekterar människor. De kan alla etablera ett livslångt latent stadium efter primär infektion. Herpesvirus kan därefter reaktiveras från det latent stadium och ge kliniska symptom eller utsöndras asymptomatiskt. HSV-1 infektioner behandlas med läkemedel som angriper maskineriet som replikerar viralt DNA. Vi har studerat mekanismer för initiering av viral DNA replikation samt hur viral DNA replikation interagerar med DNA rekombination och genexpression.

I det första arbetet studerade vi det första steget i replikationen, initiering via interaktion mellan det virala proteinet OBP och sekvensen oriS. Vi kunde med hjälp av fylogenetiska studier och in vitro-försök visa hur denna process stegvis har utvecklats under evolutionen. Både aminosyra-motiv, interaktion med andra proteiner i replikationsmaskineriet samt sekvensarrangemanget i oriS har varit essentiella i utvecklandet av denna initierings-process.

I det andra arbetet analyserade vi DNA rekombination vid infektioner med olika typer av HSV-1. Genom att använda oss av markörer räknade vi fram hur ofta rekombination sker. Resultaten visade att Rad51 och Rad52 är delaktiga i rekombination av virussegmenten U_L och U_S . Vi visade även att rekombination sker oftare i celler med förlångsammad ligering av Okazaki-fragment. Noggrannheten vid rekombination med olika typer av virus visade sig vara hög då ett repetitivt område i genen US7 förblev oförändrat.

I det tredje arbetet studerade vi replikationskopplad transkription av HSV-1 sena gener som kräver DNA replikation för effektivt uttryck. Vi visade att rekrytering av RNA polymeras II till sena genes promotor sker trots avsaknad av DNA replikation. Rekryteringen kräver dock ICP4 och är förlångsammad i förhållande till tidiga gener. Detta ledde oss till att studera mRNA-mognad med hjälp av en inhibitor, DRB som blockerar funktionen av kinaset CDK9, en positiv elongeringsfaktor, samt med siRNA mot Spt5, en processivitetsfaktor. Vi kunde med dessa två metoder förhindra sent genuttryck med minimal påverkan på tidigt genuttryck och DNA replikation. Vi föreslår därför en modell där CDK9 och Spt5 rekryteras till sena gener som genomgått DNA replikation.

Sammantaget har vi studerat essentiella mekanismer under HSV-1 proliferativa stadium som kan vara viktiga mål för framtida läkemedelsutveckling.

LIST OF PAPERS

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

- I. Olsson M, Tang KW, Persson C, Wilhelmsson LM, Billeter M, Elias P. Stepwise evolution of the herpes simplex virus origin binding protein and origin of replication.
J Biol Chem. 2009 Jun 12;284(24):16246-55.
- II. Tang KW, Norberg P, Holmudden M, Elias P, Liljeqvist JÅ. Rad51 and Rad52 are involved in homologous recombination of replicating herpes simplex virus DNA.
Plos One. 2014 Nov 3;9(11):e111584.
- III. Tang KW, Zhao ZY, Samuelsson T, Elias P. Replication-dependent expression of herpes simplex virus 1 late genes is controlled by P-TEFb and DSIF.
Manuscript

Related publication:

Muylaert I, Tang KW, Elias P. Replication and recombination of herpes simplex virus DNA.

J Biol Chem. 2011 May 6;286(18):15619-24.

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Abbreviations

bp	base pair/s
CDK9	Cyclin-Dependent Kinase 9
CTD	Carboxy-Terminal Domain of RNA Polymerase II (Rbp1)
DRB	5,6-Dichlorobenzimidazole 1- β -D-ribofuranoside
DSB	Double-Stranded DNA Break
DSIF	DRB Sensitivity Inducing Factor
dsDNA	double-stranded DNA
E. coli	Escherichia coli
hpi	hour/s post infection
HR	Homologous Recombination
HSV-1	Herpes Simplex Virus 1
ICP	Infected Cell Protein
kb	Kilobase
NHEJ	Non-Homologous End-Joining
OBP	Origin Binding Protein
pol II	RNA Polymerase II
SSA	Single-Strand Annealing
ssDNA	single-stranded DNA
U _{L/S}	Unique Long/Short
VP	Viral Protein

1 INTRODUCTION

1.1 Herpes Simplex Virus 1

1.1.1 Clinical manifestations of disease

The recurring oral symptoms caused by herpes simplex virus have been described in ancient scriptures. But it was not until the 1930s that it was proven that the recurring lesions occurred in patients which already had neutralizing antibodies against HSV-1 [1]. Besides causing these common oral lesions HSV-1 may also cause severe encephalitis. Such untreated patients suffer a 70% mortality and the majority of patients do not regain full neurological function [2]. Other clinical manifestations include genital lesions, gingivostomatitis, neonatal infection, keratoconjunctivitis, eczema herpeticum, paronychia, erythema multiforme and facial paralysis [3].

1.1.2 Phylogeny

HSV-1 is classified into the order of Herpesvirales which contains viruses that infect a diverse variety of organisms from oysters to humans (Figure 1) [4]. The mammalian viruses all belong to the family of Herpesviridae and they are subdivided on the basis of their biological properties into the subfamilies alpha-, beta- and gamma-herpesvirinae. Common for all the nine human herpesviruses is that after an initial infection in an epithelial cell the viruses establish latency in a particular subset of cells. For alphaherpesvirinae latency is established in neuronal cells, betaherpesvirinae latency is established in secretory glands, lymphoreticular cells and in the kidney, and for gammaherpesvirinae, latency is established in lymphocytes [3]. Reactivation of latent virus is thought to be a balance between the viral properties and the immunological system which keeps the virus infected cells in a latent state. Therefore patients with a attenuated immune response such as immunosuppressed, AIDS and elderly patients have a higher degree of reactivation [5].

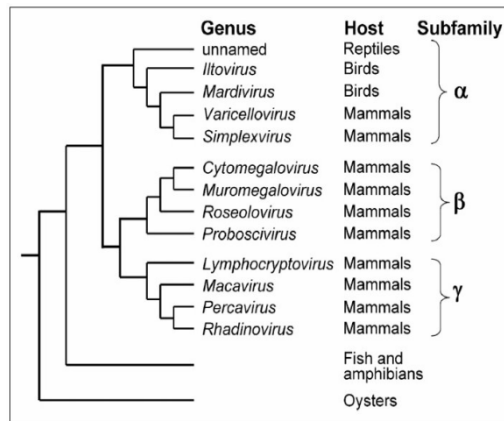


Figure 1. *Phylogenetic classification of Herpesvirales*. Printed with permission [3].

1.1.3 Nomenclature of genes

The nomenclature of HSV-1 genes is somewhat confusing. The initial steps towards studying the viral proteome using SDS-PAGE gels started in the 1960's [6]. Purification of radiolabeled virions and subsequent separation in polyacrylamide gels yielded 24 distinct bands which were denoted viral protein (VP) and a number according to their size [7]. In order to detect non-structural viral proteins a similar approach was used to distinguish 47 bands, which were designated infected cell polypeptides (ICP), in cellular extracts from HSV-1 infected cells [8]. The present nomenclature of HSV-1 proteins reflect the position of the open reading frame in the unique or repeated sequences in the long or short region respectively (U_L , R_L , U_S , R_S), however the old nomenclature e.g. VP16, ICP8 as well as functional description e.g. OBP (origin binding protein) still remains in the literature.

1.1.4 Virus Particle

Herpesviruses all share the common structural features. A dsDNA is packed into an icosahedral capsid which is surrounded by an amorphous layer called the tegument which is enveloped by a lipid bilayer containing glycoproteins (Figure 2) [9]. The average diameter of the spherical virion is 186nm with spikes extended to 225nm. The HSV-1 particle contains mostly virus-encoded proteins. However, by using mass spectrometry of highly purified virions, 49 distinct cellular proteins were found mainly in the tegument of the mature virus particle [10].

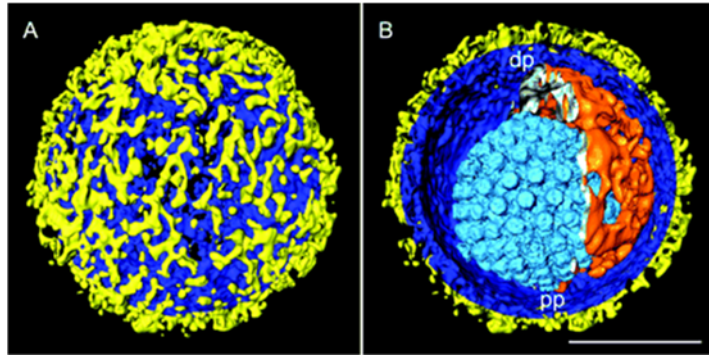


Figure 2. Single virion tomogram. A. Protruding glycoprotein spikes (yellow) anchored to the membrane (blue). B. Cutaway view of the virion interior. The capsid (light blue) containing the viral DNA is surrounded by the amorphous tegument (orange) inside the envelope (blue and yellow). pp, proximal pole; dp, distal pole. Scale bar, 100nm. From Grünewald 2003 [9]. Reprinted with permission from AAAS.

The lytic life-cycle of herpesviruses (Figure 3) begins with the virus attaching to the cell-surface with its glycoproteins. Following de-envelopment the capsid is transported by microtubuli to the outer side of the nucleus where it injects the viral DNA into the nuclear compartment [11]. Early studies using radiolabeled viruses revealed that following an infection at an MOI (multiplicity of infection) of 20 only 35% of the radioactive DNA reaches the nucleus [12]. Inside the nucleus, specific compartments, ND10 (also known as PML nuclear bodies), form around the viral genome containing viral as well as host proteins [13]. The viral DNA form an endless circular structure by ligation of the ends by DNA ligase IV/XRCC4 shortly after entering the nucleus [14]. Circularization has been shown to be essential for DNA replication [15]. The viral program for taking over the cell begins immediately upon de-envelopment and involves release of tegument proteins and transcription of a cascade of viral genes. The sequential transcription of $\alpha \rightarrow \beta \rightarrow \gamma$ genes (see 1.4.5) will produce proteins for host/virus regulation, DNA replication and finally components for the virus particle. The newly formed viral DNA will be packed inside a new capsid which will exit the nuclear compartment by envelopment and de-envelopment. In the cytoplasm the capsid will acquire an envelope and exit the cell through exocytosis. The complete cycle in cultured cells takes approximately 20 hours [3].

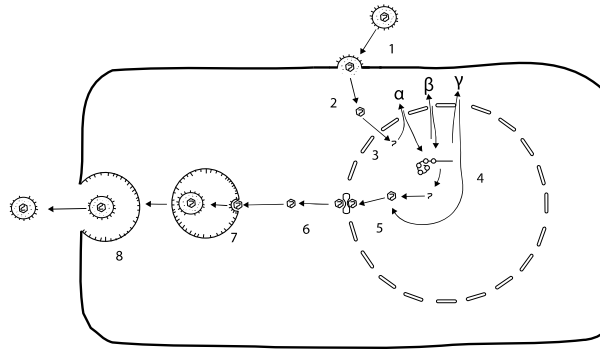


Figure 3. The HSV-1 lytic life-cycle. (1) Viral attachment and entry through fusion of the envelope with the cellular membrane. (2) Transport of capsid to nuclear pore. (3) Injection of viral genome into cell nucleus. (4) Sequential transcription of viral $\alpha \rightarrow \beta \rightarrow \gamma$ genes. (5) Assembly of viral structural proteins and encapsidation of viral DNA. (6) Capsid transport by envelopment and de-envelopment of the nuclear membranes. (7) Transport of capsid to exocytic vesicles and acquirement of viral envelope. (8) Virus release. (Artist: Zhiyuan Zhao, printed with permission)

Envelope

More than 10 different intrinsic and extrinsic proteins are anchored to the lipid bilayer, most of them being glycoproteins protruding outside the virion. The initial step for virus approach to the extracellular matrix is by glycoprotein B and glycoprotein C binding to heparan sulfate of glucose-amino-glycans [16]. Glycoprotein D will thereafter bind to the cell-surface proteins nectins [17]. Together with glycoprotein B, glycoprotein H and glycoprotein L fusion is initiated [18]. Great efforts have been made to find a surface antigen suitable as an effective vaccine. So far worldwide trials of a HSV-2 glycoprotein D vaccine have not yield desired results and a secondary trial with sero-negative women showed a complex picture with protection against HSV-1 genital infection, but no protection against HSV-2 infection [19].

Tegument

Upon de-envelopment the tegument content is released into the cytoplasm. Important viral proteins such as VP16 (also known as Vmw65, α -TIF and UL48) and VHS (virion host shutoff, UL41) will initiate the take-over of the cell by initiating viral α gene transcription and degrading host cell mRNA respectively [20, 21]. Other tegument components will assist the release of viral DNA into the nucleus and selected viral/cellular transcripts play a role in the hostile take-over of the cell [22].

Capsid

The icosahedral structure of the capsid consists primarily of the four proteins VP5 (UL19), VP26 (UL35), VP23 (UL18) and VP19C (UL38) which forms the repeated subunits [23]. A channel through which DNA is packaged is formed by a dodecamer of UL6 protein [24].

Genome

The 152 kb linear dsDNA genome is divided by three repetitive genomic elements called *a*-sequences, two are located at the ends and one in the middle. These *a*-sequences are bracketed by repetitive elements and the sequences in between these repeats are called U_L and U_S . The arrangement of the U_L and U_S elements is variable and equimolar amounts of all possible four combinations can be found in a virus population (Figure 4) [25].

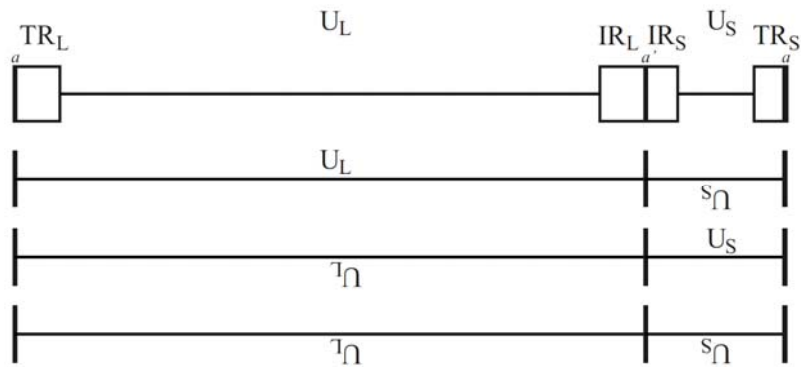


Figure 4. *The bipartite HSV-1 genome with repeated regions.* Upper, the genome is divided into two regions, U_L and U_S , bracketed by repeated sequences. TR_L and TR_S denote terminal repeats and IR_L and IR_S are internal repeats. The *a*-sequence repeats (black bars) are located at the genome ends and in between the internal repeats. Lower, three isomeric configurations of the HSV-1 genome besides the top standard arrangement.

1.2 DNA replication

1.2.1 E. Coli

The initial steps towards understanding the mechanism behind DNA replication was through the discovery of DNA pol I in *E. coli* [26]. Subsequent studies by using e.g. enzyme fractionation and thermosensitive mutants have revealed a complex multi-subunit replication machinery [27]. According to the replicon model, DNA replication is initiated by the binding of an initiator protein to an origin of replication (replicator), in the case of *E. coli* the *oriC* [28]. The replisome is recruited sequentially and is initiated by the binding of a DnaA molecule to each of the five 9-mer box within the *oriC* sequence (~240 bp). By ATP-hydrolysis DnaA will unwind *oriC* and melt an AT-rich sequence upstream of the DnaA binding sites. DnaA will also recruit a complex consisting of a helicase, DnaB, bound to a negative regulator DnaC to each of the separated strand. The primase DnaG will interact with DnaB which will in turn dissociate DnaC from DnaB and thereby activate the helicase. DnaB will continuously unwind duplex DNA in a 5'→3' direction and DnaG will simultaneously leave primers for synthesis of the lagging strand [29]. A γ -complex (consisting of five proteins; γ , δ , δ' , χ and ψ) will recognize primed ssDNA and load the β sliding clamp which will surround the ssDNA like a ring ensuring processivity. The core-complex (consisting of three proteins; α (DNA polymerase), ϵ (proofreading 3'→5' exonuclease) and θ (accessory subunit)) will then bind to the loaded β -clamp. The simultaneous synthesis of both strands is mediated by τ -dimer which binds a core-complex each. The τ -dimer is bound together with a γ -dimer which will recruit the rest of the γ -complex subunits to form a clamp-loader on the lagging strand. Together these two core-complexes are bound to a τ -dimer and a γ -complex form the DNA polymerase III holoenzyme (pol III). The replication complex will proceed with a primosome melting the dsDNA and leaving primers on the lagging strand and the pol III following it. The pol III will continuously polymerise DNA on the leading strand while the core polymerase on the lagging strand will have to process ~1-2kb Okazaki fragments at a time and then disassociate from the DNA and loaded onto a new clamp which was put in place by the γ -complex [30].

1.2.2 Eukaryote

The sequence of events leading to replication initiation in the single-cell eukaryote *Saccharomyces cerevisiae* (budding yeast) start at the autonomously replicating sequences (ARS, ~150 bp), containing the ACS (ARS consensus sequences, 11 bp) [31]. In contrast to *E. coli*, which only

contains one origin of replication (oriC), budding yeast has several hundred copies of ARS in order to copy the larger genome at a reasonable speed. According to sequence homology there are approximately 12000 ACS, of which approximately 400 are functional [32]. This implies that there are important surrounding factors that support the ACS, such as the A and B2-element as well as nucleosomal occupancy [33]. In contrast to budding yeast, few origins have been discovered and no consensus sequence has been found for fission yeast or multicellular eukaryotes. Genome-wide studies have shown that human origins are associated to transcription start sites and AT-rich regions [34]. The six-subunit initiator origin recognition complex (ORC) was discovered in budding yeast, but can be found in all eukaryotes [35]. In contrast to ORC from budding yeast, which binds sequence-specifically to elements in the ARS, human ORC has the ability to restore replication in *Xenopus* egg extracts where the endogenous ORC was depleted. Also, human ORC can initiate replication on bacterial plasmids without an human origin sequence with the same efficiency as plasmids containing an origin [36]. Following ORC-binding a pre-replicative complex will form by recruitment of the first replisome components, MCM 2-7, a hexameric helicase, by Cdt1 and Cdc6 during G1-phase. S-phase CDK and Cdc7 kinase will phosphorylate a wide range of initiation proteins, thereamong MCM10, RP-A and Cdc45 which will activate the MCM helicase [37]. The unwound DNA will subsequently be primed by DNA polymerase α . The RNA primer will then be recognized by RF-C (clamp loader) which will switch the polymerase by recruitment of PCNA (sliding clamp) upon which the processive DNA polymerase δ and ϵ will bind. Lagging strand ssDNA will be bound by RP-A and Okazaki-fragments will be processed by the PCNA-bound endonuclease Fen-1 and ligated by DNA ligase 1 [38]. Studies using mutant polymerases with specific error signatures have shown that DNA polymerase ϵ is responsible for polymerization on the leading strand while DNA polymerase δ fills the gaps between the primers on the lagging strand [39]. Recently it has been suggested that polymerase δ may be responsible for replication on both strands and that polymerase ϵ may only be involved in post-replicative repair [40]. However, this observation still remains controversial.

1.2.3 Model DNA virus replication

The simian virus 40 (SV40) DNA replication has been extensively studied. This small (5kb) dsDNA encodes a single viral protein, T-antigen, required for initiation of replication. T-antigen forces the cell to enter S-phase by inactivating various host cell factors such as the well-known tumor suppressor genes p53 and Rb [41]. The origin recognized by T-antigen contains a palindromic array of four GAGCC pentanucleotides which is

flanked by an AT-rich region and an easily denatured imperfect palindrome [42]. Cellular replication machinery is thereafter recruited to the activated origin by T-antigen. A complete in vitro SV40 replication system of both leading and lagging strand has been reconstituted using purified T-antigen and the cellular proteins RPA, DNA polymerase α and δ , RFC, PCNA, RNase H and DNA ligase I [43]. In a similar manner human adenovirus (22-48kb dsDNA) induces S-phase by inhibiting p53 and Rb [44]. However, the DNA replication machinery consists of three viral proteins, terminal protein, DNA-binding protein and DNA polymerase, which synthesize continuously on one strand by strand displacement [45].

1.2.4 HSV-1 replication

Unlike many other viruses HSV-1 DNA replication is independent of host cell cycle stage and the protein machinery carrying out the process is encoded by its own genome [3]. Temperature-sensitive mutants harbouring defective genes required for viral propagation were created using mutagenic chemicals. These could then be assigned to a complementation group and mapped to genomic loci such as syn [46]. Another approach to find proteins required for viral replication was by using a plasmid containing oriS as a reporter and expression plasmids encoding the genes of the replication proteins [47]. Seven essential replication genes (Table 1) were discovered and their function established by biochemical studies. It turns out one protein, the origin binding protein, OBP, is required for initiation of DNA replication. Six other proteins are necessary and sufficient for DNA replication of both leading and lagging [48].

Table 1. HSV-1 essential replication proteins.

Name	Gene	Function
OBP	UL9	Origin binding protein, 3'→5' DNA helicase
ICP8	UL29	ssDNA binding protein, interacts with OBP and helicase-primase
DNA helicase-primase	UL5/UL8/UL52	5'→3' DNA helicase, DNA primase
DNA polymerase	UL30/UL42	5'→3' DNA polymerase, 3'→5' exonuclease, apyriminic/apyrimidinic and 5'- deoxyribose phosphate lyase

Origin of replication

The search for the origins of replication in HSV-1 by employing electron microscopy revealed three origins, one in U_L , between UL29 and UL30, and the other two within each of the bracketing repeated regions of U_S [49]. It was also shown by passage of HSV-1 at high MOI that fragments containing the same sequences could accumulate in virus particles [50]. By cloning in these fragments into reporter plasmids and through transfection and coinfection with HSV-1 the oriS-sequence was defined. The product of this virally amplified reporter plasmid yielded a high molecular DNA which was about the same size as the viral genome itself [51]. Due to the palindromic sequence in the oriL-sequence bacterial amplification was not possible, but was later achieved in a yeast system [52]. Both origins contain palindromic sequences and a neighbouring AT-rich sequence (Figure 5). Mutant virus containing deletions of oriS or oriL sustain regular replication indicating overlapping function [53, 54].

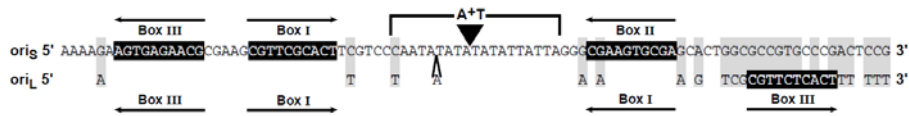


Figure 5. **The HSV-1 origins.** Sequence of oriS. Underlying bases in gray box and triangle indicate sequence differences between oriS and oriL. Arrows above sequence shows orientation of OBP recognition sites box I, II and III. A+T denotes AT-rich sequence. Printed with permission [55].

UL9/OBP – Origin Binding protein

The UL9 gene encodes an 851-amino acid polypeptide, OBP, which exists as a homodimer and initiates DNA replication by sequence specific binding to the origins. OBP contains an N-terminal Superfamily II DNA helicase and ATPase activities. It has a C-terminal DNA-binding domain, Δ OBP (~317 amino acids), that binds with high affinity to the consensus sequence TTCGCAC (Box I). Binding to Box II is ten times weaker and binding to Box III is only observed with full-length OBP and requires cooperative protein-protein interactions [54, 56, 57]. The OBP 3'→5' DNA helicase is greatly enhanced by a direct interaction with ICP8 [58]. Using OBP ts-mutants it was shown that OBP was only essential for the initiation of replication, but not elongation, and mutations in either conserved amino acids in the helicase or DNA-binding domain disrupts DNA replication [59-61]. OBP was shown by electron microscopy to extrude hairpins of DNA containing oriS [62]. Further on it was shown that OBP in the presence of ICP8 can specifically unwind an 80bp dsDNA containing oriS into a hairpin consisting of Box I and Box III. This newly formed heteroduplex termed

oriS* binds OBP with high affinity and increases its ATPase activity which is thought to denature the AT-rich sequence and pave the way for recruitment of other replication proteins (Figure 6) [63].

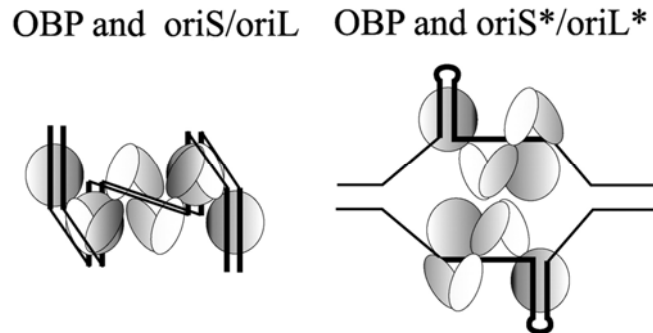


Figure 6. Proposed model for origin activation by OBP. Dimer of OBP binds to oriS/oriL through C-terminal domain (circles) to BoxI and BoxII. Subsequent activation of origin leads to formation of a hairpin/s, oriS/oriL*. Helicase (ovals) expose ssDNA which will lead to recruitment of replisome. Printed with permission [64].*

UL29/ICP8 – single-strand DNA binding protein

The gene product of UL29, ICP8, is an ATP-independent ssDNA-binding protein [65, 66]. Its ability to form nucleoprotein filament has been shown by crystallography and electron microscopy [67]. Such filaments not only stabilize ssDNA for DNA replication, but they may also be substrates for strand-annealing in homology mediated repair-pathways, possibly in collaboration with UL12, a 5'→3' exonuclease [68]. ICP8 has also been implied to act in a vast variety of viral pathways such as regulation of viral gene expression and intranuclear distribution [69, 70].

UL5/UL8/UL52 – DNA helicase-primase

The helicase-primase heterotrimer consists of the protein products encoded by the viral genes UL5, UL8 and UL52 and exist in a 1:1:1 ratio [71]. UL5 contains conserved helicase motifs and UL52 contains conserved primase motifs [72]. UL8 lacks enzymatic activity, but it is strictly required for in vivo replication [48]. It has been proposed to interact with several other viral proteins and bioinformatically characterized as an inactivated polymerase, however its function still remains poorly characterized [73-75]. The N-terminal domain of UL52 binds to UL5 and the middle domain binds to UL8 and ICP8 [76]. Functional complexes of UL5 and UL52 have been shown to retain their DNA-dependent ATPase as well as DNA helicase and primase activity in vitro [77]. Binding of ATP or GTP propels the helicase in a 5'→3' direction along the template for lagging strand synthesis while the primase

synthesize 8-12 base long primers for extension in the lagging strand [71]. It is debated if the initial priming step after origin activation is carried out by the human DNA polymerase α and/or the HSV-1 helicase-primase, but recent studies have found no indication that DNA polymerase α resides on replicating HSV-1 DNA [78].

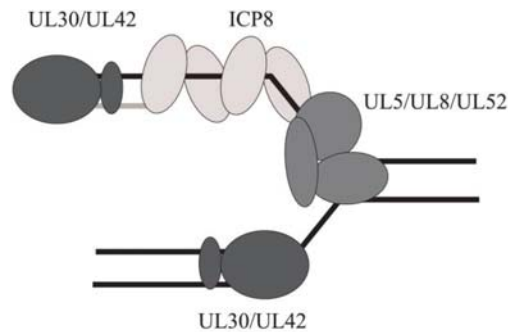


Figure 7. **HSV-1 replisome.** Simultaneous leading and lagging strand replication by DNA polymerase (UL30/UL42), ssDNA binding protein (ICP8) and helicase-primase (UL5/UL8/UL52).

UL30/42 – DNA polymerase

Following priming, the HSV-1 DNA polymerase, a heterodimer consisting of UL30 and UL42 performs replication in a theta-type bi-directional manner [79]. The helicase-primase interacts functionally with the polymerase and it may also interact directly with UL52 and/or possibly also through ICP8 and the C-terminus of UL8 [80, 81]. The UL30 protein, which contains the enzymatic polymerase activity, increases its processivity and reduces its dissociation rate from DNA by interaction between its C-terminal domain to the essential accessory protein UL42 [82, 83]. Purified UL30 has shown to contain intrinsic proof-reading 3'→5' exonuclease activity and apurinic/aprimidinic and 5'-deoxyribose phosphate lyase activities [84, 85].

Structures of replication intermediates

Following the first-round θ -replication the two daughter molecules will be separated by topoisomerase II [86]. Subsequent replication generates head-to-tail concatemers of genomic DNA, most likely through rolling circle replication. This was showed by the increased concentration of DNA fragments containing the junction of the L and S components in relative to other fragments in the nucleus [87]. Efficient rolling-circle in vitro replication of both leading and lagging strand using primed templates have been achieved by using the replisome components described above (Figure 7) [48, 88]. Electron microscopy extracted replicating HSV-1 DNA display a variety

of different conformations; circular, multiunit length, single-stranded, lariats, loops and large replicating forms [12]. Similarly, 2D DNA gels show highly branched DNA products [89]. Replicating HSV-1 DNA cannot be monomerized using restriction enzymes and most replicated DNA is unable to enter pulsed-field gel electrophoresis [90]. The extensive branching as well as the equimolar configurations of the genome suggests a high degree of HR in the replicating HSV-1 DNA. However, mutant virus, containing deletions of the internal inverted repeats at the junction between the U_L and U_S , produce viable progeny of only one isoform [91].

1.3 DNA Recombination

Recombination between homologous DNA strands is an error-free repair pathway for dsDNA breaks employed when a homologous sequence is available e.g. during DNA replication. As ssDNA nicks and gaps in the DNA template are readily converted to dsDNA-breaks during DNA replication it is reasonable to assume a tight coupling between DNA replication and homologous recombination [92].

1.3.1 Homologous recombination in *E. coli*

The majority of DSB in *E. coli* is repaired through RecBCD-pathway. RecBCD contains a 3'→5' helicase (RecB), a 5'→3' helicase (RecD), a sequence-specific recognition of Chi-site (RecC) and a 3'→5'/5'→3' exonuclease (RecB). This complex will degrade the dsDNA end until a Chi-site is found whereupon RecC binds the Chi-sequence, RecD helicase halts, 5'→3' exonuclease of RecB increase its activity and a 3'-overhang will be released and loaded with RecA [93]. RecA forms a nucleoprotein filament of the 3'-overhang in a 5'→3' direction and disassembles in the same order. RecA bound to ATP invades homologous sequence by base flipping [94]. Structures of recombination intermediates have been elucidated by X-ray crystallography [95]. ATP-hydrolysis is required for disassembly, bypass of heterologous sequence and strand exchange >3kb in length [96]. Following strand invasion, a Holliday junction consisting of four strands will form and migration is mediated by a tetramer of RuvA, which interacts directly with DNA and manages base-pair rearrangements, and RuvB that functions as the motor protein. Together, RuvAB drives branch migration preferably in a 5'→3' direction. To repair double-stranded breaks Holliday intermediates are established in both sides of the break and residual gaps after recombination are filled by DNA synthesis. Finally RuvC endonuclease resolves the junction [97].

1.3.2 Eukaryote

DSB can arise from e.g. DNA-damaging chemicals, ionizing radiation or nuclease activity. Repair of DSB is essential and failure to complete this type of repair often results in apoptosis. Most of the studies in DSB-repair has been done in yeast models [98]. Three major pathways are thought to mainly responsible for the repair of DSB in eukaryotic cells; a non-homology-driven non-conservative pathway, NHEJ, a non-conservative homology-driven mutagenic pathway, SSA and, a homology-driven non-mutagenic pathway, HR (Figure 8) [99]. Besides these established pathways, two of them with clear physiological function, NHEJ in VDJ-recombination and HR in

meiosis, other less well characterized repair-mechanisms using similar machineries have been suggested, such as micro-homology mediated end-joining and alternative-NHEJ. Competition between repair pathways and preference of method depends upon cell type and stage as well as availability of proteins and sister chromatids [100, 101].

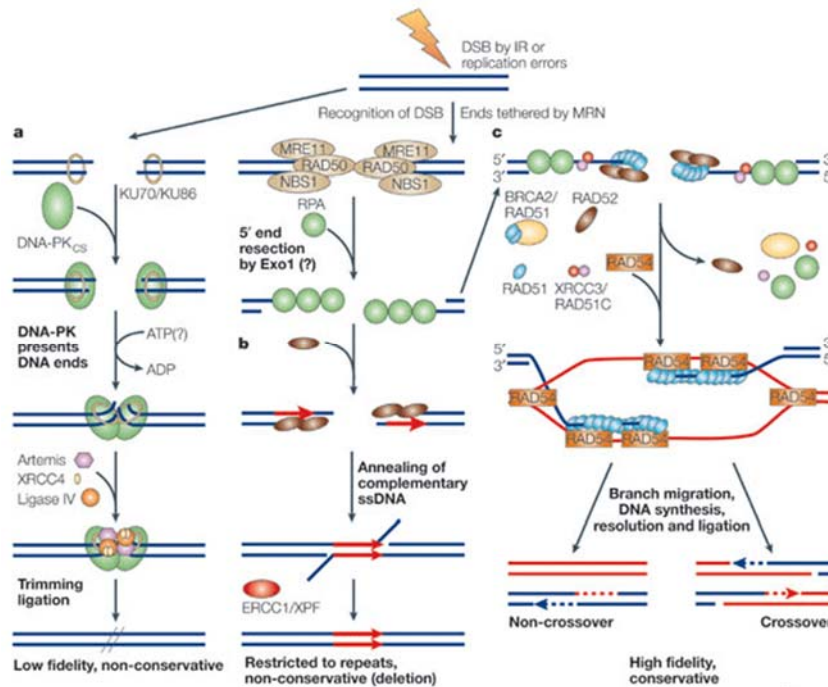


Figure 8. Three major DSB repair pathways. (a) NHEJ. (b) SSA. (c) HR. Printed with permission (Vasileva 2005).

Non-Homology End-Joining

NHEJ is considered to be the most prominent DSB repair pathway in non-dividing cells. DSB-ends are recognized by Ku heterodimer and brought together by DNA-PKcs and polished by nuclease/polymerase e.g. Artemis to finally be ligated by DNA ligase IV/XRCC4/XLF [102]. NHEJ results in an altered DNA sequence and it is not suitable for repair in coding sequences.

Single-Strand Annealing

DSB-ends are recognized by the MRN-complex (MRE11-RAD50-NBS1), which has been described as a molecular Velcro that brings together DNA-ends [103]. MRE11 contains a 3'→5' exonuclease and an endonuclease activity. It has been suggested that dimerized MRE11 holding two ends of a DSB could facilitate micro-homology dependent joining [104]. RAD50 is the

proposed “fishing rod” which protrudes from the DNA-ends in search of a matching partner. It contains ATP-binding domains at the ends, a hinge containing a zinc hook in the middle responsible for homomeric interaction and long coiled-coil regions flanking the flexible hinge [103]. NBS1 (Nijmegen breakage syndrome 1) contains phosphopeptide-binding domains for interaction with other components in signalling and repair and have been shown in conjunction with NHEJ-complex during VDJ-recombination [100]. Since MRE11 has an exonuclease in the opposite direction, the responsible exonuclease for creating a free 3'-end is most likely EXO1 or BLM-DNA2 [105]. The exposed ssDNA is then stabilized by RPA, annealing is carried out by RAD52 and ends are cleaved by ERCC1/XPF [105, 106]. RAD52 exists in a heptameric ring shape and has a circular positively charged DNA binding groove that interacts with the phosphate-backbone of DNA. It has been suggested to mediate annealing of ssDNA possibly by rolling motion of RAD52 rings [107]. Intriguingly the MRN-complex is inactivated by the adenovirus oncoprotein E4orf3 and the E4orf6-E1b55K complex in order to prevent formation of adenovirus DNA concatemers during virus replication [108].

Homologous Recombination

Similar to the SSA repair pathway a 3'-filament is essential for initiating the process. RPA-binding stabilizes ssDNA, but blocks at the same time RAD51 recombinase from binding. This block is ameliorated by a huge protein (3418 amino acids), BRCA2 (BRCA2 (Breast Cancer susceptibility gene 2)), which has a DNA-binding C-terminal and RAD51-binding domains in the middle region. Mutations in BRCA2 are known to cause breast and ovary cancer. BRCA2 binds up to eight RAD51 molecules and enhance their ability to bind to ssDNA, reduce their ability to bind dsDNA, reduce their ATPase activity and assists in displacement of RPA. BRCA2 interacts with BRCA1 for which the function still is unclear [109, 110]. RAD51 binds dsDNA and ssDNA, the latter with higher affinity. It has a low ssDNA-dependent ATPase activity and forms nucleoprotein filaments in a 3'→5' direction, covering 3bp per monomer. Short strand-exchanges are dependent on ATP and Mg²⁺, and are stimulated by RPA addition, but RAD51 is unable to perform longer (3kb) strand exchanges in vitro by itself. RAD51 can also facilitate end-to-end and end-to-tail ligation of DNA-substrates, which might explain its presence in high concentration in lymphoid tissue where NHEJ is a prominent pathway [111]. Interestingly, in contrast to yeast, RAD51 mutations in mice are embryonically lethal and greatly impair proliferation [112]. RAD51 paralogs such as RAD51B, C and D share sequence similarities and have been found on filaments as well as in Holliday junctions, but exact roles for these proteins are still elusive [109]. Subsequent annealing is mediated by RAD52.

Branch migration of the Holliday junction have been shown to be carried out by several potential proteins e.g. RAD54, BLM and RECQ1, which preferably drive the migration in a 3'→5' direction. RAD54 lacks helicase activity, but is able to perform branch migration through longer stretches of heterology in comparison to BLM and RECQ1 [113, 114]. In contrast to RAD51, RAD54 knockout mice are viable, but display sensitivity for ionizing radiation [115]. Resolving the Holliday junction can be carried out by a number of nucleases e.g. MUS81-EME1, GEN1 and SLX1-SLX4, and is often dependent on interaction with branch migration proteins [116].

1.3.3 HSV-1

Replication of HSV-1 DNA has been shown to be tightly coupled to DNA recombination as well as DNA repair. Upon entry of the HSV-1 genome into the nucleus, the DNA is immediately circularized by DNA ligase IV/XRCC4, which are involved in NHEJ [14]. DNA isolated from virus particles contains a large number of nicks, gaps as well as ribonucleotides randomly located on both strands suggesting that DNA repair may have to precede DNA replication in newly infected cells. [117, 118]. During the replicative phase (8 hpi), incorporation assay using radiolabeled nucleotides showed that 40% was incorporated into replicating DNA and 30% was incorporated into viral DNA undergoing repair [12]. The ND10 formed after viral infection have been shown to contain DNA repair proteins such as RPA, Rad51 and NBS1 [119]. Using UV-irradiated HSV-1, it was shown that transcription-coupled repair, nucleoside-excision repair and HR are essential for propagation of these viruses [120]. It has also been observed that HSV-1 infection increases SSA-mediated repair 2-fold of a genome-integrated reporter-gene. In the same study, infection led to a reduction of HR and NHEJ with analogous reporters [68]. However, reporter plasmids containing linearized *a*-sequences at the ends displayed SSA-like repair in ERCC4-deficient cells and *a*-sequences have shown to increase recombination frequency two-fold in a similar assay [121, 122].

HSV-1 has its own uracil glycosylase, UL2, which is thought to be associated to UL30 and possibly carry out replication-coupled base-excision repair [123]. ICP8 has been shown to share some properties with classical recombinases such as RecA and Rad51 mentioned above. ICP8 has been shown in vitro able to melt and anneal dsDNA as well as efficiently transfer ssDNA from a dsDNA template to an ssDNA circle with the same sequence [66, 124, 125]. ICP8 has also been shown to work in conjunction with the 5'→3' exonuclease, UL12, also known as the alkaline nuclease [126]. It has been shown that UL12 interacts with the MRN-complex [127]. HSV-1 UL12

null-mutants propagate poorly, but with only minor impairment of DNA replication, and genomic inversion appears to be undisturbed [90, 128]. UL12 protein has been shown to enhance SSA of integrated gene-reporter in conjunction with Rad52 or ICP8. However, SSA has not been shown to occur in the HSV-1 genome in itself [68].

1.4 RNA transcription

1.4.1 Transcription initiation

Eukaryotic RNA polymerase II (pol II) transcription is separated into three stages; initiation, elongation and termination. The initiation process starts at the promoter DNA-sequence elements e.g. TATA-box, BRE (TFIIB recognition element), Inr (initiator) and DPE (downstream promoter element) [129]. However no promoter contains all elements and many promoters contain one or none of these sequences. The global transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIIF and TFIIH are required for all pol II transcription, but variations of subcomponents can be found depending on the sequence of the promoter [130]. The order of global transcription factors recruitment to a model promoter is initiated by the binding of TFIID, which is built up by TBP (TATA-binding protein) and ~14 TAFs (TBP-associated factors). TBP will bind the TATA-box in the promoter and will induce a sharp bend in the DNA, which will facilitate the forthcoming interactions. Next TAF1 and TAF2 will bind the Inr and TAF6 and TAF9 will bind the DPE [131]. TFIIA will then bind to TFIID and stabilize the DNA-TFIID complex and block transcriptional inhibitors. TFIIB will be recruited to bind BRE. A motif in TFIIB, the B-finger, will later be inserted into the exit channel for newly synthesized mRNA in pol II and thereby regulating the promoter clearance of pol II. TFIIIF will bind pol II and escort it to the other bound factors and finally TFIIE and TFIIH will join the complex [132]. These components together are called the pre-initiation complex and recruitment of factors for initiation is thought to be the rate-limiting step for transcription [133]. Components of TFIIH includes helicases XPB and XPD, which will facilitate the initial melting of the DNA-duplex, and a kinase component, CDK7, which will phosphorylate one of the twelve subunits of pol II, Rbp1, on the Serine-5 residue of the CTD (C-terminal domain) and this event will kick-start the pol II to leave the pre-initiation complex and begin the transcription [132]. The CTD contains dozens of repeats of a heptapeptide with the sequence $Y_1S_2P_3T_4S_5P_6S_7$. It is thought to function like a scaffold for signalling and interactions, such as splicing factors and DNA-repair, by modulation of the amino acids either by phosphorylation (tyrosine, threonine or serine) or isomerization (proline). Stimulation of initiation by phosphorylation of Serine-5 and elongation by phosphorylation of Serine-2 appears to be conserved throughout eukaryotes. Other CTD-patterns appears to be less general and elusive [134, 135].

1.4.2 Transcription elongation

During elongation a Positive Transcription Elongation Factor b (P-TEFb), consisting of CDK9 and CyclinT is recruited to the initiated pol II. Phosphorylation by CDK9 of CTD at Serine-2, and the evolutionarily conserved transcription factor SPT5, which together with SPT4 forms the elongation complex DSIF (DRB Sensitivity Inducing Factor), establishes the elongating complex [136]. Interestingly DSIF is the only transcription elongation factor conserved in all three domains of life. Crystallization experiments in yeast and archaea have shown that Spt4 and Spt5 closes the RNA polymerase clamp surrounding the transcribed DNA and interacts with upstream DNA as well as the non-template strand during transcription [136, 137]. The C-terminal domain of Spt5 has been suggested to interact with nascent RNA at the exit tunnel from RNA polymerase [137]. As the complex transcribes towards the 3'-end, maintenance and expansion of Serine-2 is carried out by CDK12 in complex with CyclinK. The location of CDK9 and CDK12 at opposite end of transcribing genes have been shown convincingly using ChIP-Seq [138, 139].

1.4.3 Promoter proximal stalling

Many human genes regulate transcription at the elongation phase by activation of a stalled pol II. This has been extensively studied for heat shock protein 70 and HIV-1 [140, 141]. The negative elongation factors, NELF A-E binds through DSIF to pol II and have been shown to pause the complex and await a signal for continued transcription. In the case of HIV-1, pol II is activated by the viral protein Tat (Trans-Activator of Transcription) in collaboration with P-TEFb [142]. CDK9 phosphorylates the NELF-complex, which then dissociates from the transcription complex, Serine-2 on CTD, and SPT5, at which transcription is resumed [143].

1.4.4 Transcription termination and export

Transcription termination is initiated by the recognition of the highly conserved polyadenylation signal AAUAAA by CPSF (Cleavage/Polyadenylation Specific Factor). CPSF has been shown to interact with TFIID as well as CTD and can be found at the beginning and the end of transcription [144]. Downstream of the polyA-site, a U/GU-rich sequence is recognized by CstF (Cleavage stimulating Factor). Together in a complex, CPSF, CstF, CTD of pol II, PAP (poly-adenylation polymerase) and CF (Cleavage Factor) I_m and II_m, a cleavage is induced at the poly-A site. Further polymerization will only require CPSF, PAP and a poly-A binding protein PABII. Export of nascent mRNA has shown to be linked to splicing

by binding of hnRNP-complexes [145]. However many viruses such as HSV-1, hepatitis B virus and HIV, as well as intronless genes in human cells, contain cis-acting sequence elements in nascent mRNA which promotes export and poly-adenylation [146].

1.4.5 HSV-1 transcription

HSV-1 utilizes the pol II to express its genes. It was observed that transcription of HSV-1 genes could start in the absence of any protein synthesis by adding cyclohexamide to infected cells [147]. Following up on this observation a combination of cyclohexamide and actinomycin D was used at different time-points [148]. By infecting cells at an MOI of 20 the results showed three distinct temporal groups of ICP (Figure 9).

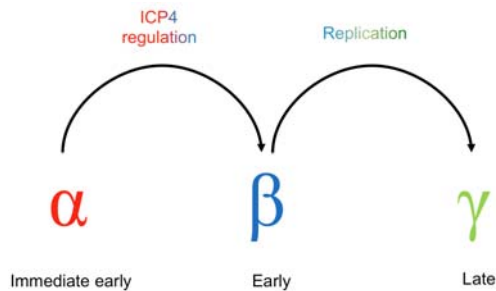


Figure 9. **HSV-1 transcription.** Sequential activation of HSV-1 alpha/Immediate Early, beta/Early and gamma/Late genes.

The alpha group (also known as Immediate-Early genes) are transcribed without prior protein synthesis and the protein products are synthesized at highest rates from 3-4 hours post-infection and at diminishing rates thereafter. VP16 (UL48) which is delivered to the cell through the incorporation of the tegument to the cytoplasm activates transcription of the alpha genes by associating to the cellular protein HCF-1. Together this complex binds to Oct-1 and recognizes the motif TAATGARAT present upstream in the promoters of alpha genes [20]. The complex will then recruit transcription factors and the pol II. Five of the six alpha genes regulate expression of themselves as well as the beta and gamma genes. The alpha-proteins ICP0 and ICP4 are required for activation/derepression of transcription of the next set of genes, the beta-group (also known as Early genes) [149]. The beta-proteins are synthesized at highest rates from 5-7 hours post-infection and at decreasing rates thereafter. The majority of the beta genes encode replication proteins (Table 1), and the DNA replication process does in turn activate the gamma-group (also known as Late genes). In later studies the gamma-group was subdivided into gamma1 and gamma2,

and they have the kinetics of moderate synthesis or low to no synthesis prior to replication respectively. The gamma-proteins represent the majority of the structural proteins. Upon DNA replication the transcription of the gamma-genes is greatly amplified. Besides DNA replication, the viral proteins ICP0, ICP4, ICP22 (US1.5) and ICP27 are required for proper expression of gamma genes, however the mechanisms have not been completely elucidated [3]. This remarkable order of transcription allows the virus to first produce the DNA before producing the structural components in which the DNA will be packaged into.

ICP0 contains a RING domain, which functions as an E3 ligase, indicating a role in ubiquitination of target proteins. ICP0 is thought to indiscriminately activate viral genes by derepression of histones bound to the viral genome [3]. It is proposed that ICP0 carries out this function by inhibiting HDAC (histone deacetylase) and mutant virus with ICP0-deletions have been shown to be partially rescued by HDAC-inhibitors [150]. ICP4 is considered to be the main activator of post-alpha genes and is required for all beta and gamma genes [149]. ICP4 has a sequence-specific binding domain, which appears to correlate with repression of its own gene and ORF P. Non-sequence specific binding however appears to correlate with trans-activation of beta and gamma genes. By binding to promoters and interacting with transcription factors and the mediator complex ICP4 stimulates transcription together with other viral proteins. ICP22 encoded by the US1 gene, which also encodes a truncated C-terminal version US1.5 has been shown to be required for appropriate expression of certain gamma genes and has been shown to be co-localized with pol II [151]. Another important viral factor for post-alpha gene expression is ICP27. Mutant virus with ICP27 deletions lead to less beta-(UL29 and UL42) and gamma-gene expression and a virus mutant carrying a truncated version of ICP27 lacking the last eight amino acids in its C-terminal tail is deficient in expressing gamma-genes despite seemingly normal replication [152, 153]. ICP27 has been proposed to be the link between replication and late gene expression by its ability to interact with ICP8 and CTD [154, 155]. The effect ICP27 has on gene expression is through its inhibition of host expression by inhibiting splicing and its post-transcriptional role mediating mRNA export and enhancing poly-adenylation of transcripts [156]. ICP27 mutants that are unable to interact with mRNA nuclear export factors have been shown to have a general effect on poly-A transcript accumulation in the nucleus [157].

Replication-coupled transcription

The notion that the separation of DNA-strands during replication can increase transcription is puzzling and several factors have been described to be

essential for this process in different organisms. This phenomenon is found in RNA and DNA-viruses as well as eukaryotic cells. In *Saccharomyces cerevisiae* 3.5% of 4270 genes are directly regulated by DNA replication and in human cells a set of replication-dependent histones have been extensively studied [158, 159]. Many different mechanisms have been suggested to be responsible for replication-coupled transcription such as; addition of a factor, removal of a factor, cell cycle stage, increased copies of promoter and replacement of modified nucleotides.

It has been shown *in vitro* in the T4-phage system that the sliding clamp protein gp45 left behind on the lagging strand interacts with the RNA polymerase-bound gp33 and gp55 to elicit transcription. The transcription direction is decided by which strand that contains the ssDNA nick [160]. Human viruses e.g. adenovirus, Epstein-Barr virus (EBV), human papillomavirus (HPV) and SV40 also display replication-coupled transcription. Inhibition of replication with chemical inhibitors, superinfection with similar viruses and the use of temperature-sensitive mutants with defects in replication proteins have often been used to study this phenomenon [158, 161, 162]. In the adenovirus system, removal of an inhibiting factor by replication is one of the mechanisms for replication-coupled transcription. Transcription of the adenoviral gene IVa₂ is normally inhibited by binding of IVa₂-RF, which exists at saturating levels before replication. With the increase of genomes during replication the unbound genomes will be able to transcribe the IVa₂ gene [163]. Switch from early to late transcription by transcriptional regulation has been described in adenovirus and HPV. Adenovirus E4orf4 has been shown to mediate the early to late switch by dephosphorylation of cellular SR proteins and thereby modulating splicing and HPV-16 E2 inhibit termination at an early poly-adenylation signal and result in a readthrough to the late poly-adenylation signal [164, 165].

HSV-1 gamma gene expression can be inhibited by addition of replication inhibitors. In order to show that activation of gamma2-genes in fact are activated by DNA replication *in cis* and not by trans-activating factors Roizman and colleagues sequentially infected cells using viruses containing promoters representing the three different temporal classes coupled to the thymidine kinase (tk) gene. Cells infected with tk-null mutant virus were superinfected with a gamma2-tk mutant virus at a time point when the first virus had started its replication. By measuring the tk-activity they found no trans-activating factors sufficient for inducing a gamma2-tk production [166]. Characterizations of the gamma2-genes have mainly been focused on UL38 and gC (UL44). It has been observed that many gamma2-genes have

exchangeable downstream activation signals, which have been shown to be associated with a component of TFIID [167-169]. It has also been shown that a cell line containing a temperature sensitive mutant version of TAF250, the major component of TFIID is required for gamma2 gene transcription [170]. Also, an ICP4-mutant virus with 2/5 of its C-terminus removed is unable to interact with TAF1 and TFIID in vitro and is unable to induce gamma-gene transcription [171]. The gC-gene contains a cis-acting sequence that functions as a negative regulative silencing element which requires ICP27 to overcome [172].

Organization of genes such as UL29 and UL30 which flank ori_L in a divergent fashion (also seen in SV40, polyomavirus, bovine papillomavirus 1, adenovirus and EBV) as well as the direction of beta genes in relationship to ori_L indicate functional coupling between replication and transcription [173-175]. This arrangement removes the possibilities for a head-to-head collision between replication and transcription of beta genes on U_L and is believed to prevent replication fork collapse [176]. In stark contrast the gamma genes are distributed randomly in both directions relative to the replication origin.

2 RESULTS AND DISCUSSION

2.1 Paper I

In the herpesvirus-family only the alphaherpesvirus and the roseolovirus of the betaherpesvirus have the origin binding protein (Figure 1). To characterize the binding of C-terminal OBP (Δ OBP) to the origin sequence oriS in vitro we created truncated versions as well as mutant variants of Δ OBP. Different truncated versions of Δ OBP displayed diverse solubility and binding capacity. A minimal construct containing amino acids 536-813 was needed to obtain a soluble protein which binds oriS in electromobility shift assays (EMSA). Conserved amino acids in the N-terminus of this truncated version contain a conserved F553XXKYL-motif. The lysine in this motif is mutated into an alanine in a temperature-sensitive mutant virus [177]. A phylogenetic analysis of Δ OBP-sequences revealed conserved regions in the C-terminal domain as well as the absolute C-terminal tail. The C-terminal domain of Δ OBP in alphaherpesviruses contains the motif R756VKNL and site-directed mutagenesis replacing the arginine, lysine or leucine with alanine greatly reduced the binding capacity to oriS, while replacing the valine or asparagine had little or no effect on binding. Additional replacement with alanines of a conserved phenylalanine-751 upstream and two leucines-768/769 downstream of RVKNL also displayed impaired binding while mutations of upstream of a proline-glycine-752/753 dipeptide only reduced binding moderately. With the exception of CHV1 (cercopithecine herpes virus 1) the recognition sequence in oriS is TTCGCAC, which in CHV1 is TCCGCAC. Expressing both HSV-1 and CHV1 Δ OBP we examined the binding properties of these proteins to respective oriS-sequence, however no difference was found in the binding affinity to either oriS-sequence. Together our results define a series of conserved amino acids, in particular the RVKNL-motif required for sequence specific binding of DNA. On the other hand the FXKKYL-motif seems to be required for stable folding.

The absolute C-terminus of Δ OBP is known to interact with ICP8 and conserved amino acids form the motif WPXXXGAXXFXL. By replacing conserved amino acids with alanine as above and adding ICP8 to the EMSA we showed that the tryptophane and phenylalanine are essential for binding while replacement of the proline or phenylalanine did not affect the interaction. Interestingly, replacement of the HSV-1 OBP C-terminus with the varicella-zoster virus version still preserved the interaction albeit with altered mobility in the gel. This C-terminus interaction with ICP8 is absent in

the OBP of distantly related alphaherpesvirus that infects birds, gallid herpesvirus (GHV1) and psittacine herpesvirus (PsHV1), and sea turtles, Chelonid fibropapillomatosis herpesvirus (CFPHV), as well as the human betaherpesvirus (roseolovirus) HHV6A, HHV6B and HHV7. Interestingly, when studying the origin of replication for all alphaherpesvirus, these six distantly related viruses as well as VZV and CHV9 do not have the ability to form a BoxI-BoxIII hairpin (Figure 5). Furthermore, computational phylogenetic analysis using multiple sequence alignment of all OBP-sequences unbiasedly assigned these eight viruses into three separate evolutionary branches. NMR-experiments revealed that ssDNA oligo containing the oriS-sequence indeed form stable B-DNA conformation hairpin-structures.

To conclude, it appears that the evolutionary development of the OBP-oriS and OBP-ICP8 interaction has evolved in a stepwise manner. From an original herpesvirus, the first step appears to be the separation between betaherpesvirus (roseolovirus) and alphaherpesvirus, during which the RVKNL-motif in OBP was acquired and made possible the sequence specific binding to TTCGCAC in oriS. The following step involves the development of the C-terminus interaction with ICP8 found in all alphaherpesvirus except for GHV1, PsHV1 and CFPHV. A final step, separate from the ancestor of VZV and CHV9, a subset of herpesvirus acquired a hairpin in the oriS-sequence (Figure 10). These steps display a mechanistic co-evolution that might be coupled to biological changes such as host cell selection and establishing an evolutionary niche.

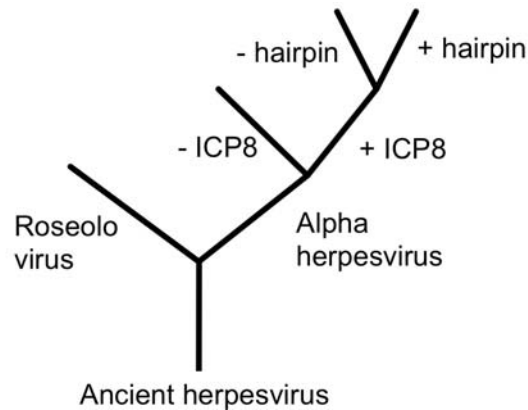


Figure 10. Stepwise evolution of OBP and oriS. From the ancient herpesvirus containing OBP (bottom), the first branching occurred with the alphaherpesvirus acquisition of the binding capacity to oriS. Subsequent branching followed with the acquisition of binding properties to ICP8 and a hairpin-structure in the oriS.

2.2 Paper II

In this study we focused on homology-driven recombination in HSV-1. Firstly we indirectly studied the isomer population (Figure 4) of HSV-1 by co-infecting two different temperature sensitive mutant viruses. Mutant virus tsK harbours mutations in the ICP4 gene located at the terminal repeats of U_S and the tsS virus contains mutations in the UL9/OBP gene (Figure 11). Theoretically if a cell would be infected by one virus of each strain and the viruses would undergo infinite number of recombination the chances to produce a non-temperature sensitive wild type virus would be 25% (i.e. carrying the UL9 from tsK and the ICP4 from tsS). We employed this assay using a control cell line as well as a cell line, GM16097, derived from a patient with a deficiency in ligase I function, resulting in a retarded ligation of Okazaki-fragments and aberrant DNA repair [178]. We measured the number of recombined progeny displaying wild type features i.e. replicating at the non-permissive temperature. For both cell lines the recombination rate was 17%. The inability to reach the maximum theoretical rate of recombination is most likely due to different replication foci in the nucleus and a Gaussian distribution that leads to unequal number of viruses infecting the same cell. In order to examine whether the recombination proteins Rad51 and Rad52 are involved in this recombination assay we pre-treated the cells with siRNA directed against these proteins. Rad51-knockdown reduced recombination rates to 11% in wild type cells and to 3% in GM16097 cells. Rad52-knockdown reduced recombination rates to 5% in both wild type cells and GM16097 cells. These results indicate that both Rad51 and Rad52 are essential in the recombination between the U_L and U_S , however, regarding the differences between the cell lines the data should be interpreted with caution since knock-down levels vary from cell lines and experiments.

Secondly we studied the recombination within a 3kb region of U_S by co-infecting the cell types mentioned above with three viruses harbouring different restriction enzyme markers in the region of interest. By isolating 329 plaques of the virus yield and subsequent PCR reactions of the specific regions, isolates were classified as recombinants or non-recombinants [179]. For a few isolates we also performed sequencing for validation. In the wild type cells 2% of the virus progeny were classified as recombinants, and in GM16097 cells 12% were recombinants, indicating that the increased amounts of single-strand nicks available on the HSV-1 replicating DNA correlates with increased recombination, most likely serving as recombination substrates.

Finally we studied a 21-nucleotide tandem repeat in the US7 gene which has been shown to vary between strains and also within the same infected individual implying that this region might be highly prone for recombination. Virus yield from the 3kb recombination assay mentioned above was passaged ten times at which recombination rates were 9% in wild type cells and 30% in GM16097 cells. Tandem repeats were calculated by measuring PCR product lengths. Unexpectedly we found no recombinants within the tandem repeat region in 20 plaque-isolated viruses. Intra-viral uneven recombination events of the tandem repeat region was simultaneously studied by infecting cells at low multiplicity of infection and passage of progeny 25 times. No recombinants were found under these premises either, indicating that recombination events leading to variable number of repeats is a rare occurrence in cell-based assays.

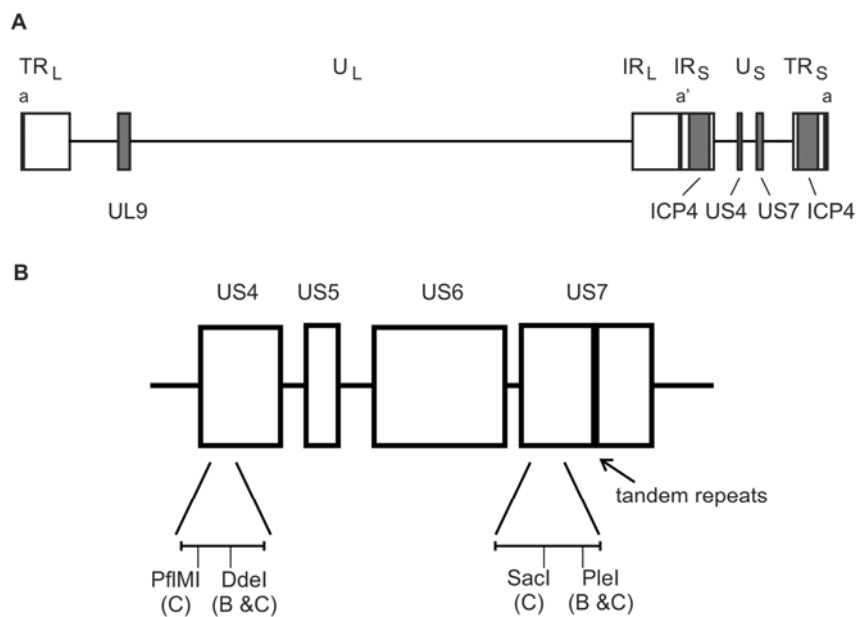


Figure 11. HSV-1 genome and markers for recombination experiments. (A) The *tsS* mutation is mapped to the UL9 gene and the *tsK* mutation is mapped to the ICP4 gene. A cluster of glycoprotein genes containing gG (US4) and gI (US7) in the U_S segment is separated by 3kb. (B) Enlargement of the glycoprotein gene cluster. The location of the 21 nucleotide tandem repeats is highlighted. Restriction enzyme polymorphism between the clade A (no cuts), B and C is shown under respective PCR-fragments.

In conclusion we have described DNA recombination of HSV-1 at three different levels of the genome. We have shown that Rad51 and Rad52 are important for interviral recombination, that single-strand nicks are most

likely recombinogenic and that tandem repeat expansion/contraction are rare events. These assays could serve as future models for studying DNA recombination by using mutated cell lines and experimental use of knock-down/knock-out technologies or inhibitors. Such studies may yield important insights into mechanisms and regulation of homologous recombination in mammalian cells.

2.3 Paper III

We started our studies on replication-coupled transcription in HSV-1 by first looking at the recruitment of pol II to the different classes of promoters (alpha, beta and gamma). Using tsK and tsS (described in the previous section), occupancy on model promoters using chromatin immunoprecipitation were studied at non-permissive temperatures (i.e. without beta and gamma transcription (tsK) and without replication (tsS)). We confirmed previous studies that ICP4 was dependent for pol II recruitment (tsK) and discovered that pol II was recruited to gamma2-promoters despite deficient replication (tsS) [180]. Following up on this we used the same method to study the pol II occupancy profile on a beta and a gamma gene. The gamma gene displayed a retarded occupancy compared to the beta gene, but there were no signs of promoter proximal stalling. Using DRB (5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole), an inhibitor of CDK9 we showed a prominent specific inhibition of gamma2-gene expression with minor effects on beta-genes and DNA replication. Continuous DRB-inhibition of CDK9 is required for the full effect and addition of DRB at 6hpi, a time-point at which replication has started, still blocks gamma2-gene expression. To verify the specific effect of CDK9-inhibition, DRB- and flavopiridol-titration experiments displayed IC₅₀-values of gamma2-genes, but not beta-genes, similar to in vitro assays of CDK9-inhibition. Also, siRNA-experiments against CDK9 (unpublished data) and Spt5 reduced gamma2-gene expression in a similar manner. Despite major inhibition on gamma2-gene expression with DRB, measurements of mRNA-levels using RT-qPCR and RNA-seq displayed minor differences. However a two-fold difference was shown in the nuclear/cytoplasm ratio when using subcellular fractionation. In summary, pol II-recruitment to gamma2-genes is not impaired despite absence of DNA replication. CDK9 and DSIF are required for expression of gamma2-genes and are likely to be coupled to post-transcriptional mechanisms such as maturation and export.

How do these results fit into the model of replication-coupled transcription? At least two plausible scenarios can be discussed. Firstly, DNA replication leads to an addition or removal of a factor at the promoter that allows for DSIF to bind to the pol II-complex. Analogous to the T4 phage-system a factor such as ICP8 could be left on ssDNA gaps on the lagging strand and recruit ICP27 as well as DSIF. In contrast inhibiting factors could be bound to a downstream activation sequence in gamma2-genes and these would be removed by the DNA replication. Secondly, DNA replication might be essential because the gamma2-promoters are extremely weak and expression can be detected through the increased number of copies. In this scenario the

beta genes would be considered the unconventional genes since they do not require DSIF and CDK9. This scenario is supported by the observation that plasmids containing gamma2-promoters behave like beta-genes when transfected. Alpha genes might still require CDK9 and DSIF to some extent and this might explain why we observe a slight retardation in the recruitment of pol II and DNA replication.

Regardless of the coupling to DNA replication our data shows that gamma2-genes produce a transcription-complex that recruits CDK9. CTD and/or DSIF and/or ICP27 phosphorylation most likely provide the appropriate scaffold for maturation factors for mRNA-capping, transcription termination and mRNA-export, which seems to be circumvented in beta genes by another mechanism. DSIF has been shown to be a processivity factor in mammalian genes for transcription of longer transcripts. This is however not the case for HSV-1 genes where beta genes generate relatively long transcripts. Further characterization of the transcription complex of beta- and gamma2-genes seems to be the appropriate step to elucidate the mechanism. The difficulty of separating the different pol II-complexes during an infection and the absence of a plasmid-based in vivo system could perhaps be overcome by creating an (alpha and) beta-free virus using bacterial artificial chromosomes and complementing them with stably transfected plasmids containing essential proteins for transcription and replication, but under different promoters.

It has been suggested that DRB exerts its effect through ICP22 and UL13, which together increase the transcription of a subset of gamma genes such as UL38. The evidence that these two proteins are involved has been shown by immunofluorescence where mutants lacking ICP22 or UL13 display reduced co-localization between CDK9 and pol II. In contrast to our results this study does not find any inhibition of gamma-genes gB, gC and gD in the presence of DRB [151]. It is an interesting alternative explanation model, but it would not explain how we in our experimental setting find clear reduction in all five tested gamma-genes and that no specific gamma mRNA-reduction is found in the transcriptome of infected cells.

It is tempting to speculate whether CDK9-inhibitors could be a complement in the antiviral arsenal. Inhibition of virus propagation in cultured cells could be visualised with the viral cytopathic effect using light microscopy. We have shown that 25 μ M DRB is sufficient to inhibit the cytopathic effect and GFP expression of a recombinant HSV-2 gG2-GFP virus (Figure 12). DRB has also been shown to inhibit transcription elongation in adenovirus [181]. In a recently published study, a CDK9-inhibitor was efficiently employed to inhibit the propagation of HSV-1, cytomegalovirus and adenovirus in cell

cultures. The same inhibitor was also shown effective for treatment of HSV-1 infected mice, more inspiringly it was efficient even in mice infected with an acyclovir-resistant HSV-1 strain [182]. Considering that flavopiridol has passed phase II trials, clinical data regarding chronic virus infections would be interesting to study in the treated patient groups [183].

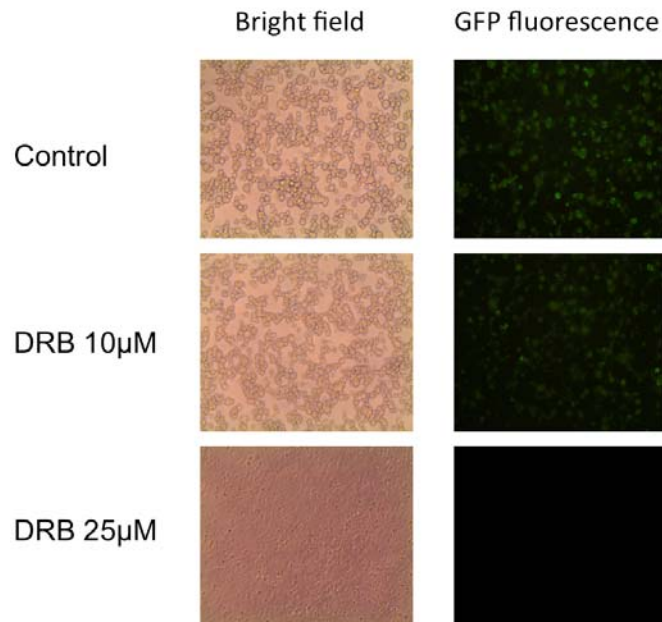


Figure 12. DRB inhibits cytopathic effect and HSV-2 gG2-GFP fusion protein expression. Human fibroblast cells were infected with recombinant HSV-2 gG2-GFP in absence or presence of DRB for 18 hours. Bright field microscopy in left column shows cytopathic effect and right column GFP-fluorescence.

3 CONCLUSIONS

3.1 Paper I

- A minimal soluble version of HSV-1 OBP which binds to oriS contains the amino acids 536-813.
- The evolutionary divergence of OBP-oriS has developed stepwise with the acquisition of an RVKNL-motif that binds to oriS, a C-terminus ICP8-interacting motif and finally an ability to form a hairpin in the oriS-sequence.

3.2 Paper II

- Rad51 and Rad52 are involved in isomeric HSV-1 recombination.
- Mutant cell line with increased amounts of ssDNA nicks display high rate of HSV-1 recombination events.
- Expansion or contraction of tandem repeats in US7 is rare despite high recombination frequency in surrounding sequences.

3.3 Paper III

- RNA polymerase II is recruited to gamma2-gene promoters even in the absence of DNA replication.
- CDK9 and DSIF are required for gamma2-gene expression.
- Only minor differences in the HSV-1 mRNA expression profile can be observed when inhibiting CDK9 with DRB.
- DRB-inhibition leads to accumulation of HSV-1 mRNA in the nucleus indicating a post-transcriptional role for CDK9.

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