MOLECULAR MECHANISMS OF REPLICATION OF HERPES SIMPLEX VIRUS 1

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Received July10, 2000; accepted September 13, 2000

Summary. – The present review focuses on recent development in our understanding of the molecular mechanisms of DNA replication of herpes simplex virus 1 (HSV-1). Progress made in the characterization of the early events of viral DNA synthesis and of virus-host cell interactions, especially in the context with the formation of viral DNA replication sites, is highlighted. An up-dated overview is presented on important stages of lytic infection cycle, such as virion entry, viral DNA synthesis, viral DNA cleavage and packaging into preformed capsids, and maturation (nucleocapsid envelopment) and egress of virions. Many novel interactions are discussed that extend not only our knowledge of the biology of this virus but may represent possible new targets for antiherpesviral therapy.

Key words: host shutoff; nuclear bodies; viral DNA recombination; viral DNA replication; viral DNA transcription; virion egress; virion entry; virion maturation

General features of HSV-1

HSV-1, whose formal designation is human herpesvirus 1 (van Regenmortel *et al.*, 2000), belongs to the better studied representatives of the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family that display a neurotropic phenotype, and cause latent infections in humans affecting neuronal cells. Contradictory to its designation, HSV-1 is a complex virus. Its large genome (152 kbp) comprises 84 open reading frames (ORFs). More than half of them can be deleted without an apparent effect on virus replication ability in cell culture (Roizman, 1996). Its genes are designated UL or US indicating that they derived from the long (L) or short (S) unique component of the genome (Fig. 7); the genes are numbered starting from left to right of the respective component from the prototype configuration (McGeoch *et al.*, 1985, 1988a). The corresponding gene functions are named accordingly or alternatively, following nomenclatures of leading laboratories in the U.S.A. and Europe (Honess and Watson, 1974; Marsden *et al.*, 1976) and are frequently defined.

The double-stranded (ds) linear DNA genome encodes about 40 virion proteins and a variety of viral enzymes involved in nucleic acid metabolism, DNA synthesis and protein processing (protein kinases) that are essential for virus replication in cells, especially cells of the central nervous system, which are deficient in metabolic pathways

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Abbreviations: aa = amino acid; CD = cluster of differentiation antigens; CENP-C = centromeric protein component; DNA-PK = DNA-dependent protein kinase; DR1 = direct repeat 1; ds = double-stranded; EHV-1 = equine herpesvirus 1; HCMV = human cytomegalovirus; hnRNP-K = heterogenous RNP-K; ICP = infected cell protein; Kr = Krüppel bodies; mRNA = messenger RNA; ND10 = nuclear domain 10; NMR = nuclear magnetic resonance; non-snRNP = non-small nuclear RNP; nt = nucleotide; OBP = ORI-binding protein; ORF = open reading frame; ORI = origin of DNA replication; PAA = phosphonoacetic acid; PML = promyelotic leukemia protein; POD = PML oncogenic domain; PRV = pseudorabies virus; RNP = ribonucleoprotein; rRNA = ribosomal RNA; ss = single-stranded; ssDBP = ssDNA-binding protein; SUMO-1 = small ubiquitin-related modifier protein; TAP = transporter associated with antigen presentation; α-TNF = α trans-inducing factor; vhs = viral host shutoff; VP = virion polypeptide

Table 1. Capsid proteins of HSV-1

Capsid protein	Gene	M_r	Function
NC1, VP5	UL19	155 K	Penton and hexon formation
NC2, VP19C	UL38	50 K	Forms basic capsid shell with VP5 and VP23
NC3, VP21	UL26	40 K	Scaffolding proteins (interacting with themselves and VP5) released from mature B capsids by protease (VP24) cleavage at C-terminus
NC4, VP22a	UL26.5	38 K	
NC5, VP23	UL18	33 K	Forms triplexes with VP19C
NC6, VP24	UL26	26 K	Maturation protease, its selfcleavage generates VP21
NC7, VP26	UL35	12 K	Vertex protein, basic phosphoprotein (pI = 11.67)

NC = nucleocapsid protein.

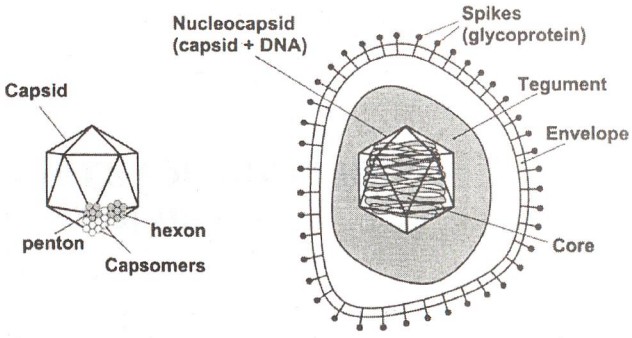

Fig. 1
Major components of HSV-1 virions

Table 2. Envelope proteins of HSV-1

Protein	Gene	Features
Glycoproteins		
gB	UL27	*syn* plaque phenotype, attachment and cell fusion
gC	UL44	Attachment, penetration
gD	US6	Attachment, penetration, strongest immunogenicity
gE	US8	IgG Fc-receptor binding, complexed with gI, cell-to-cell spread, plaque size, virulence, phosphorylated by UL13, acylated
gG	US4	Subtype-specific antigen
gH	UL22	Complexed with gL, penetration
gI	US7	Complexed with gE, modulates IgG binding, cell-to-cell spread
gJ	US5	?
gK	UL53	*syn* plaque phenotype, envelopment, egress
gL	UL1	Complexed with gH, *syn* plaque phenotype
gM	UL10	Cell-to-cell spread, plaque size, complexed with UL49.5
Unglycosylated membrane-associated proteins		
	UL11	Myristylated
	UL20	*syn* plaque phenotype, virus egress
	UL24	*syn* plaque phenotype
	UL43	?
	UL45	*syn* plaque phenotype, associated with endoplasmic reticulum
	UL49.5	Complexed by disulfide bond to gM

Data from: Adams *et al.*, 1998; Avitabile *et al.*, 1995; Barnett *et al.*, 1992; Cockrell and Muggeridge, 1998; MacLean *et al.*, 1989; Ng *et al.*, 1998; Peng *et al.*, 1998; Rajčáni and Vojvodová, 1998; Wu *et al.*, 1998. *syn* = syncytium; IgG = immunoglobulin G.

of DNA synthesis. Like other herpesviruses, HSV-1 has evolved genes that help the virus to evade immune responses, to prevent cell cycle progression from G1 to S phase, to block cellular mitosis, to interrupt activation of apoptotic pathways and to overcome intracellular defense mechanisms. Herpesviruses share a common virion structure consisting of an icosahedral capsid shell in which the dsDNA genome is embedded as a toroidal core structure of a diameter of about 75 nm (Fig. 1). A three-dimensional structure of the herpesvirus B capsid has been determined at the 0.85 nm resolution by electron cryomicroscopy (Zhou *et al.*, 2000). The capsid shell of a 125 nm diameter and a M_r of 192,000 K is formed by 4 proteins: VP5, VP26, VP23, and VP19C (Table 1). The major morphological units in the capsid are 12 pentons and 150 hexons, which contain five and six copies of VP5, respectively (Fig. 1). The hexon also contains six copies of VP26, the vertex protein, which form a ring on the top of the VP5 subunits (Zhou *et al.*, 1995). The capsid is surrounded by a glycoprotein-containing envelope. Between capsid and envelope is a proteinaceous region, designated tegument, which appears amorphous in electron micrographs. The envelope probably contains all glycoproteins (at least 11 in HSV-1, Table 2) and several unglycosylated membrane-associated proteins, which play a central role in viral infectivity and host range. Five envelope proteins were identified to be responsible for syncytial plaque formation (*syn*) caused by cell membrane fusion. These glycoproteins are found in three sets of heterodimer complexes (gE/gI, gH/gL, and gM/UL49.5). Whereas the herpesviruses are almost indistinguishable in electron micrographs, they can be discerned by a distinct organization of their genomes containing unique sequence stretches, intermingled with different kinds of direct and inverted repeat sequences. By restriction analysis and agarose gel electrophoresis it is possible to differentiate human herpesviruses on the basis of the nature of their DNA. To date, typing of herpesviruses is carried out using the polymerase chain reaction (Roizman and Sears, 1996; Rajčáni and Vojvodová, 1998; Subak-Sharpe and Dargan, 1998).

The lytic cycle of hsv-1 replication

In the following, a brief and updated version of the individual steps of HSV-1 replication is presented. The infection of a cell by HSV-1 can be divided into the following stages: (i) entry of virions, marked by specific cell membrane interactions such as adsorption (attachment and binding to the cell surface) and penetration (fusion of the virion envelope with the cell membrane), (ii) transport of the nucleocapsid to nuclear pores, (iii) uncoating of the nucleocapsid and entry of the core into the nuclear compartment, where viral transcriptional events take place, (iv) viral DNA replication and recombination, (v) assembly of capsids and cleavage and packaging of viral DNA into preformed capsids, and (vi) maturation of virions (envelopment of nucleocapsids) by budding through the nuclear membrane and egress of the mature virions.

Virion entry

Infection of cells by a herpesvirus is initiated by a contact between glycoproteins, gB and/or gC, decorating the viral envelope, and glycosoaminoglycan residues of cell surface proteins (Shukla *et al.*, 1999; Spear *et al.*, 1992). This first contact is designated "attachment". Fusion of the viral envelope with the cell membrane, resulting in penetration of the viral nucleocapsid, is then mediated by an interaction of viral glycoprotein gD with specific cellular receptor molecules. Table 3 provides an overview of known herpesvirus entry mediators (Geraghty *et al.*, 1998; Krummenacher *et al.*, 1998, 1999). Penetration through the cell membrane is assisted by two other viral glycoproteins, gH and gL, which form a 1:1 complex (Peng *et al.*, 1998). As an alternative route for penetration, endocytosis may be accomplished by the clathrin endocytic machinery (Brodin *et al.*, 2000; Harter and Reinhard, 2000).

Transport of viral nucleocapsid to cell nucleus (nuclear import)

After fusion of the virion envelope with the cell membrane, viral nucleocapsid associated with tegument proteins is deposited in the cytoplasm of the cell. This process seems to involve an interaction with the cellular cytoskeleton (Kristensson *et al.*, 1986). At initial times of infection a fragmentation of microtubules is observed at the periphery of the cell near the cell membrane (Ward *et al.*, 1998). Recently, it was suggested that migration of the viral nucleocapsid to the cell nucleus occurs along the microtubules by a dynein-mediated transport (Izaurralde *et al.*, 1999). In pseudorabies virus (PRV)-infected cells the incoming nucleocapsid co-localizes with cell microtubules during the nuclear transport (Kaelin *et al.*, 2000). As illustrated in Fig. 2, nucleocapsid, laden with tegument proteins, apparently binds to the dynein transporter (a minus-end directed, microtubule-dependent motor) and is rapidly transported along the microtubules towards their negatively charged end to the centriole. From there the nucleocapsid is transported to and binds to the nuclear pore. Probably, triggered by the tegument protein UL36 (Batterson *et al.*,

Table 3. Cell surface entry mediators for herpesviruses

Virus	Glycoprotein	Cellular receptor	Receptor family
HSV-1	gC (gB)	Heparan sulfate	GAG
	gD	3-O-sulfated heparan sulfate	GAG
	gD	HveA (HVEM)	TNF
	gD	HveC (PRR1, Nectin-1)	Ig
HSV-2	gB	Heparan sulfate	GAG
	gD	HveA (HVEM)	TNF
	gD	HveC (PRR1, Nectin-1)	Ig
	gD	HveB (PRR2, Nectin-2)	Ig
VZV	gB	Heparan sulfate	GAG
EBV	gp350/220	CD21 (CR2)	Ig
	gH, gL, gp42	HLA class II	Ig
HCMV	gB	Heparan sulfate	GAG
HHV-6, HHV-7	gB, gp65	Heparan sulfate	GAG
		CD46	Ig
PRV	gC	Heparan sulfate	GAG
	gD	HveC (PRR1)	Ig
	gD	HveB (PRR2)	Ig
	gD	HveD (PVR)	Ig
BoHV-1	gB, gC	Heparan sulfate	GAG
	gD	HveC (PRR1)	Ig
	gD	HveD (PVR)	Ig

Data from: Boyle and Compton, 1998; D'Addario *et al.*, 1999; Geraghty *et al.*, 1998; Krummenacher *et al.*, 1998, 1999; Lopez *et al.*, 2000; Perez and Fuller, 1998; Rajčáni and Vojvodová, 1998; Santoro *et al.*, 1999; Secchiero *et al.*, 1997; Shukla *et al.*, 1999; Spear *et al.*, 1992; Takahashi *et al.*, 1999; Wang *et al.*, 1998; Wang *et al.*, 2000.

BoHV-1 = bovine herpesvirus 1; CR2/CD21 = complement receptor type 2; CD46 = membrane cofactor protein receptor; GAG = glycosoaminoglycan; gB, gC, gD, etc. = glycoproteins B, C, D, etc.; gp = glycoprotein; HLA = human leukocyte antigen; HveA/B/C/D = herpesvirus entry mediator A/B/C/D; Ig = immunoglobulin; PRR1, PRR2 = poliovirus receptor-related protein 1 and 2; PVR = poliovirus receptor; TNF = tumor necrosis factor.

BoHV-1 = bovine herpesvirus 1; EBV = Epstein-Barr virus; HCMV = human cytomegalovirus; HHV-6 = human herpesvirus 6; HHV-7 = human herpesvirus 7; HSV-1 = herpes simplex virus 1; HSV-2 = herpes simplex virus 2; PRV = pseudorabies virus; VZV = varicella-zoster virus.

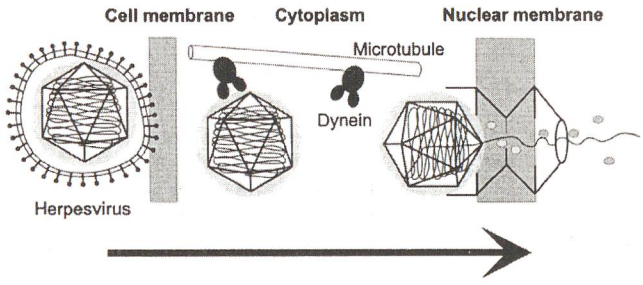

Fig. 2
HSV-1 entry and transport to cell nucleus
Scheme according to Izaurralde *et al.* (1999) and Ye *et al.* (2000).

1983), the viral genome-containing core together with some adherent tegument proteins is released into the nucleus and becomes circularized. The identification of the viral proteins, involved in the nuclear transport of the nucleocapsid/tegument structure, is just in beginning. Because of its co-localization with microtubules, when expressed in the absence of other viral proteins, the homologous UL25 protein of PRV was proposed to participate in the microtubular transport of the nucleocapsid to the nucleus (Kaelin *et al.*, 2000). By pull-down experiments and recombinant protein expression it was shown that the virion protein UL34, formerly characterized as a membrane-associated protein that is phosphorylated by the viral kinase US3, interacts with the neuronal isoform of the intermediate chain (IC-1a) of the dynein complex, as well as with the virion nuclear matrix protein UL31 and the major capsid protein UL19 (Ye *et al.*, 2000). The available data suggest that UL34 is more likely a tegument protein, which interacts with dynein and uses the microtubular network for retrograde transport of the nucleocapsid to the nuclear pore. At the late phase of virus replication, the newly synthesized UL34 is transported to the cell nuclear membrane, where it interacts with UL31 and UL19. This observation together with a recent mutational analysis (Roller *et al.*, 2000) suggest that UL34 also plays a significant role in viral envelopment.

Shutoff of cellular transcription and translation

Two important proteins having late gene functions are liberated during the uncoating process, namely vhs (viral host shutoff) protein (UL41 gene product) and a trans-inducing factor (α-TIF) UL48 gene product also called VP16 or Vmw65 (viral polypeptide with apparent M_r of 65 K). Whereas the vhs protein is responsible – nomen est omen – for the shutoff the cellular transcription machinery (Kwong *et al.*, 1988), α-TIF induces transcription of the very early viral protein functions by binding to the nucleotide sequence motif TAATGARAT (VP16-responsive element) of the promoter regulatory domain of the immediate-early genes (Whitton and Clements, 1984; Gaffney *et al.*, 1985). Interestingly, vhs protein was shown to exert an endoribonuclease activity, degrading messenger RNA (mRNA) but not ribosomal RNA (rRNA), preferably in the 5′-to-3′ direction (Elgadi *et al.*, 1999; Karr and Read, 1999). The insertion of an internal ribosome entry site (IRES) at any location within mRNA provokes the preferential endoribonucleolytic cleavage, suggesting that the cleavage is targeted by *cis*-acting secondary structural elements in the RNA substrate (Elgadi and Smiley, 1999). In contrast, a partially purified vhs protein is not restricted to mRNAs and cleaves target RNAs at various sites. This apparent discrepancy was solved by experiments demonstrating that the nuclease activity and target specificity of vhs protein is derived from the interaction with the translation factor eIF4His, that is itself a component of the cap-binding complex (G.S. Read, personal communication). This activity fits therefore nicely to the observed rapid degradation of cellular mRNA and the selective shutoff of cellular protein synthesis immediately after herpesvirus infection.

Viral transcription and protein synthesis

Transcription of the large, complex viral genome is sequentially regulated in a cascade fashion (Honess and Roizman, 1974). All of the viral transcripts are produced by host cell RNA polymerase II, whose large subunit is subject of specific phosphorylation events mediated by the UL13 protein kinase as well as the immediate-early gene function ICP22 (Long *et al.*, 1999). Three distinct classes of mRNA are made: (i) immediate-early or α transcripts which are translated to five *trans*-acting regulators of virus transcription. (ii) (delayed)-early or β transcripts from which enzymes, non-structural regulatory and a subfraction of structural proteins are derived, and (iii) late or γ transcripts that produce regulatory and major structural proteins. γ transcripts are further subgrouped in γ1 and γ2. The γ2 expression is more stringently dependent on ongoing viral DNA synthesis. From the α gene functions ICP0, ICP4, ICP22, ICP27, and ICP47, representing mainly multifunctional proteins, ICP27 (UL54 gene function) is the only protein essential for propagation of the virus in tissue culture. ICP27 is conserved throughout all the herpesviruses and acts as an additional host shutoff protein. It is differentially phosphorylated, modulates viral gene expression, affects viral DNA replication, inhibits host splicing by interacting with essential non-small nuclear ribonucleoprotein (non-snRNP) splicing factors, is required for the prevention of apoptosis (Aubert and Blaho, 1999) and shuttles between the nucleus and the cytoplasm (Soliman

et al., 1 . Phelan and Clements, 1997; Mears and Rice, 1998; S ti-Goldin, 1998a, 1998b). ICP37 ressembles in its fun n heterogeneous nuclear RNP-K (hnRNP-K), which l lso able to shuttle from the nucleus to the cytopla with a possible role in processing and transport of pre-mRNA (Matunis *et al.,* 1992; Ostareck-Lederer *et al.,* 199). Interestingly, ICP27 was recently shown to interact with hnRNP-K and the casein kinase 2ß subunit (Wadd *et al.,* 1999). This finding suggests that the ICP27-mediated phosphorylation of hnRNP-K by casein kinase 2 prevents the binding of hnRNP-K to RNA, thus affecting transport of cellular RNAs or altering the subcellular localization of hnRNP-K. Whereas ICP4 and ICP27 are found to be associated with the sites of viral DNA replication, ICP22 and ICP0 do not enter the replication compartments (Zhong and Hayward, 1997). A special role in the regulation of viral protein expression play the gene products of ORF O and P (Roizman, 1999). ORF O binds ICP4 and prevents it from binding to DNA. ORF P localizes in spliceosomes and blocks the synthesis of viral proteins derived from spliced mRNA. ORF P may also act synergistically with ICP22 and ICP0 during the late gene expression (Bruni and Roizman, 1996).

ICP47 effectively interferes with the major histocompatibility complex class I antigen presentation pathway. It binds with high affinity to the human transporter associated with antigen presentation (TAP) and blocks the binding of antigenic peptides (York *et al.,* 1994; Hill *et al.,* 1995). The structure of an active domain of ICP47 in a lipid-like environment has been determined by nuclear magnetic resonance spectroscopy (Pfander *et al.,* 1999).

Important regulatory functions can be assigned to ICP0 (Vmw110), another major transactivator, that stimulates the onset of virus infection in a multiplicity-dependent manner and is required for efficient reactivation from latency (Cai *et al.,* 1993). HSV-1 infection blocks the cell cycle in early to mid-G1 phase by inhibiting specific phosphorylation of proteins of the retinoblastoma family (pRB, p107, and p130) that is required for the progression of cells to the S phase (de Bruyn Kops and Knipe, 1988; Song *et al.,* 2000). Involvement of ICP0 in this G1 cell cycle block is likely, because ICP0, expressed by transfection, possesses the capabilities both to prevent transfected cells moving from G_1 to S phase and to block infected cells at an unusual stage of mitosis defined as pseudo-prometaphase (Lomonte and Everett, 1999). The latter property correlates with the ICP0-induced proteasome-dependent degradation of a centromeric protein component of the inner plate of human kinetochores (CENP-C, de Bruyn Kops and Knipe, 1988; Everett *et al.,* 1999a). The mitotic block is a specific property of ICP0, and more particularly of its so-called RING finger (C3HC4 zinc-binding motif) domain (Everett *et al.,* 1999a; Lomonte and Everett, 1999).

Viral DNA replication

Initiation of viral DNA replication

Both α and β genes are required for genome replication. A special role in the onset of viral DNA replication play viral interactions with specific nuclear matrix-associated domains, recently described as nuclear bodies, ND10s (nuclear dots 10, since they occur at an average of 10 per nucleus), Kr (Krüppel)-bodies, PML (promyelotic leukemia protein)-associated bodies, and PODs (PML oncogenic domains) (Maul *et al.,* 1993). These small subnuclear punctate structures have been shown to be sites for input viral DNA accumulation in adenovirus, simian virus 40, HSV-1, and human cytomegalovirus (HCMV) infection as well as for immediate-early transcription in HCMV (Ishov and Maul, 1996; Ishov *et al.,* 1997; Ahn *et al.,* 1999). ND10s representing multiprotein complexes were originally described as an autoantigenic target (SP100) in patients with primary biliary cirrhosis (Szostecki *et al.,* 1987) and are also known to play a role in development of acute promyelocytic leukemia and possibly other forms of neoplasia (Kakizuka *et al.,* 1991). The ND10-associated proteins, PML and SP100, are involved in regulation of cell growth and are subjects of cell cycle-controlled biochemical modifications. The both proteins are posttranslationally modified by covalent linkage to the small ubiquitin-related modifier protein SUMO-1 (Sternsdorf *et al.,* 1997) and this conjugation seems to be essential for association of both proteins with ND10. During mitosis, PML and SP100 become de-conjugated as well as separated, and a novel phosphorylated isoform of PML emerges (Everett *et al.,* 1999b). This demodification of PML correlates with a complete loss of normal ND10 structures. The SUMO-1 de-conjugation probably involves human homologs of the recently identified ubiquitin proteases in yeast, Ulp1 and Ulp2 (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000). In HSV-1-infected cells, proteasome-dependent degradation by an ubiquitin-specific protease (HAUSP) appears to play a role in the modification of the ND10 structures (Everett *et al.,* 1998; Robinson *et al.,* 1998). Re-conjugation of SUMO-1 at the end of mitosis may be carried out by the ubiquitin-conjugating enzyme Ubc9 (Seufert *et al.,* 1995). Thus the SUMO-1 modification is emerging as an important pathway in the control of cell status and proliferation.

In HSV-1 the ND10 domains were proposed to be essential for the formation of viral DNA replication compartments. Already in the 80s, small punctate structures have been observed in the nuclei of HSV-1-infected cells by immunofluorescence microscopy, in which the major DNA binding protein (ICP8, UL29 gene product) was localized even in the presence of the viral DNA polymerase

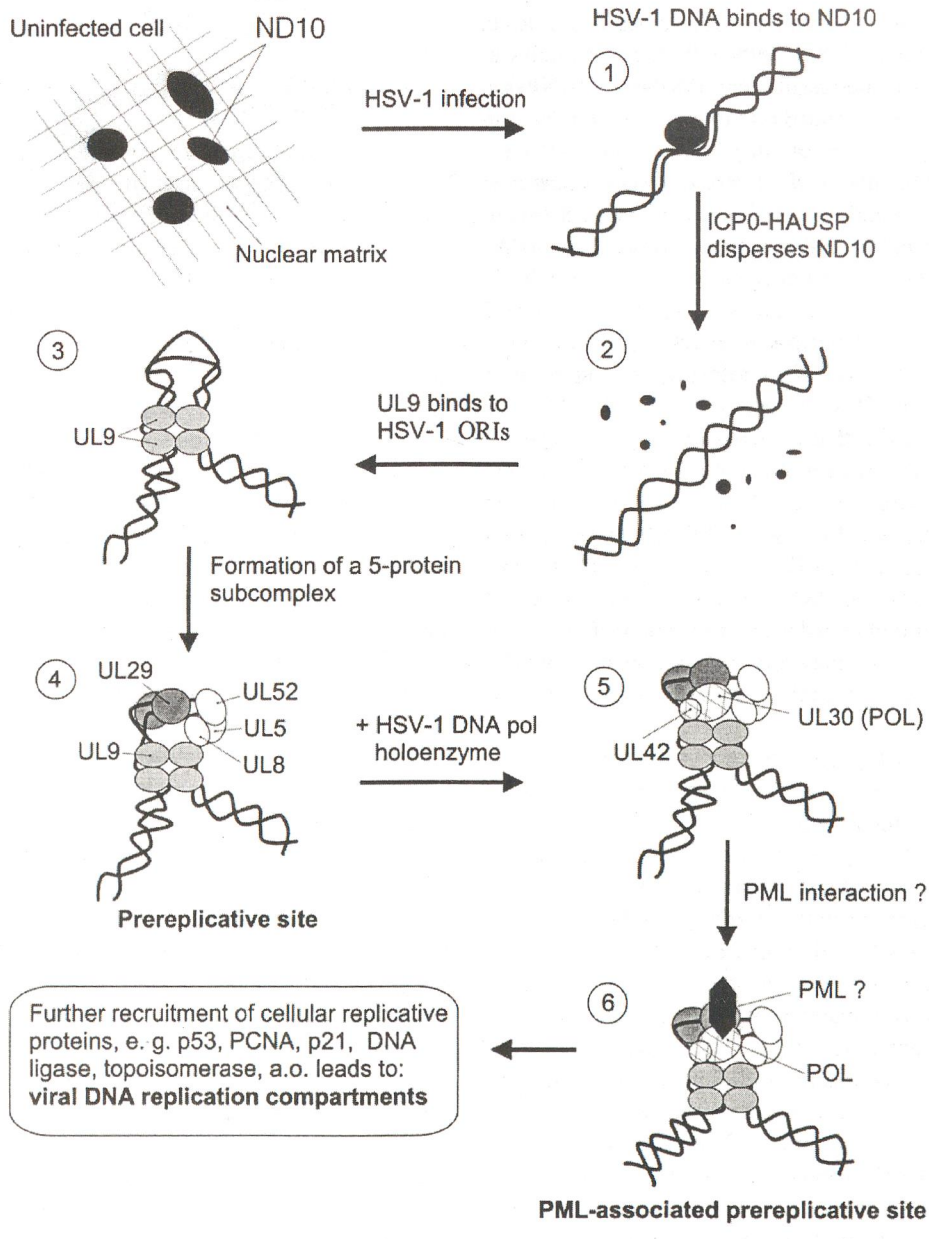

Fig. 3
Formation of HSV-1 DNA replication compartments
Schematic view of the individual stages required for the recruitment of replication compartments according to Burkham *et al.* (1998). POL = catalytic subunit of HSV-1 DNA polymerase; PCNA = proliferating cell nuclear antigen.

inhibitor, phosphonoacetic acid (PAA) (Quinlan *et al.*, 1984). These nuclear structures were designated prereplicative sites. At early times of infection, ICP8 also forms numerous small punctate structures in the absence of PAA (de Bruyn Kops and Knipe, 1988). Studies with mutant viruses and DNA-transfection experiments have shown that the micropunctate structures generate in the presence of ICP8, the DNA helicase/primase complex (UL5, UL8, and UL52), with and without the ORI-binding protein (OBP) (UL9) (Liptak *et al.*, 1996; Lukonis and Weller, 1997; Zhong and Hayward, 1997). In contrast, in cells co-transfected with an HSV-1 ORI-bearing DNA plasmid all seven essential DNA replication proteins are required to form a much smaller number of spherical prereplication foci, that can develop into large globular and kidney-shaped DNA replication compartments, in which ongoing viral DNA synthesis was

successfully demonstrated (Zhong and Hayward, 1997). This second type of prereplication sites is presumed to represent true replication intermediates (Lukonis *et al.*, 1997; Lukonis and Weller, 1997; Uprichard and Knipe, 1997; Zhong and Hayward, 1997).

The so far identified individual steps of ND10 interactions with HSV-1 gene functions during replication and formation of viral replication compartments (Burkham *et al.*, 1998) are schematically illustrated in Fig. 3. Soon after infection with HSV-1, the circularized viral DNA binds to ND10s (Fig. 3, step 1). The viral transactivator ICP0 migrates to ND10s and is believed to initiate the dispersal of PML, SP100 and other ND10-associated proteins by specific abrogating of the SUMO-1 modification (Muller and Dejean, 1999). For the disruption of ND10 by ICP0 the binding to the HAUSP protease is essential (Everett *et al.*, 1999c). Only the SUMO-1-modified ND10 proteins are attacked (Fig. 3, step 2). In addition to the degradation of CENP-C, another important proteasome-mediated activity of ICP0 is the induction of degradation of the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) that is accomplished by the RING-finger domain of ICP0 (Parkinson *et al.*, 1999). The ICP0-induced abolishment of DNA-PK activity appears to be beneficial to HSV-1 infection, as virus replication is more efficient in cells lacking the latter activity, especially at low multiplicity of infection.

The initiation of DNA synthesis takes place at specific palindromic sequences that function as ORIs. The HSV-1 genome contains three of these cis-acting signals: two identical copies of 46 nt in the S component, designated ORI_S, and a larger related sequence (144 nt) in the middle of the L component, designated ORI_L (Vlazny and Frenkel, 1981; Stow, 1982; Weller *et al.*, 1985). The presence of at least one ORI signal is required and sufficient for DNA replication (Longnecker and Roizman, 1986; Polvino Bodnar *et al.*, 1987; Igarashi *et al.*, 1993). E.g., ORI_S, contains at least five functional domains: two high affinity binding sites (designated sites I and II or boxes I and II) for UL9, a virus-encoded OBP that is essential for viral DNA replication, exhibiting a 3′-to-5′ DNA helicase activity with structural similarity to superfamily II helicases (Bruckner *et al.*, 1991; Boehmer *et al.*, 1993; Gustafsson *et al.*, 1994), an A/T-rich region, a sequence homologous to site I with much lower affinity for UL9 (designated site III or box III), and binding sites for cellular transcription activator proteins such as Sp1 and Sp3 (Nguyen Huynh and Schaffer, 1998). Sites I and II are located on the arms of a 46 bp palindrome and separated by the A/T-rich region. The affinity recognition sequence for site I is the 10 bp sequence 5′-CGTTCGCACT-3′ (Elias and Lehman, 1988; Deb and Deb, 1989). Site II differs from this sequence at two nucleotide positions, resulting in a reduced binding affinity for OBP to about one-fifth that of site I. OBP is a homodimer in solution and binds to ORI_S in a cooperative manner at sites I and II (step 3) (Fierer and Challberg, 1995). The C-terminal amino acids (aa) 534–851 of UL9 were identified as the DNA binding domain including a nuclear localization signal (Malik *et al.*, 1996). Lysines in this region (aa 780–786) are responsible for the sequence-specific binding. The peculiar symmetry of the ORI sequences and the strong conservation of the inverted repeat sequences, flanking the A/T-rich spacer region, was interpreted to mean that DNA initiation *in vivo* is involved with the formation of specific hairpin structures (Knopf *et al.*, 1986). Recent *in vitro* studies support this notion showing that (i) the linear ds form of ORI_S can be stably converted by heat denaturation to hairpin molecules, in which the A/T-rich region forms the loop, and boxes I, II, and III are alternatively base-paired at the bottom of the stem, (ii) OBP binds stronger to this hairpin structure, and

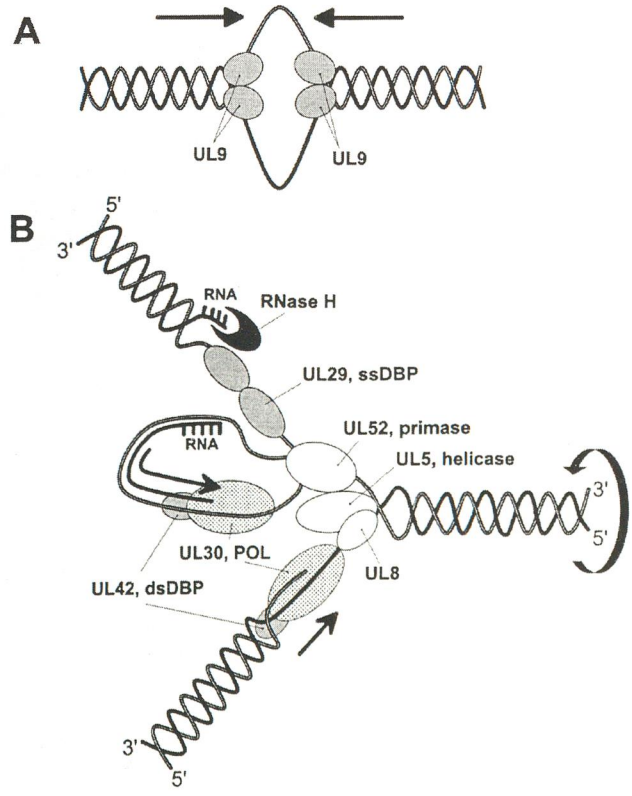

Fig. 4
Proteins and enzymes involved in initiation and elongation stages of HSV-1 DNA synthesis
(A) Binding of UL9 to ORI. Arrows indicate the DNA bending force driven by the protein interaction of the UL9 dimers. (B) Schematic replication fork with the herpesvirus functions required for leading and lagging strand synthesis. Arrows indicate the polymerization direction as well as the unwinding of the dsDNA by the helicase/primase complex. POL = catalytic subunit of HSV-1 DNA polymerase, DBP = DNA binding protein.

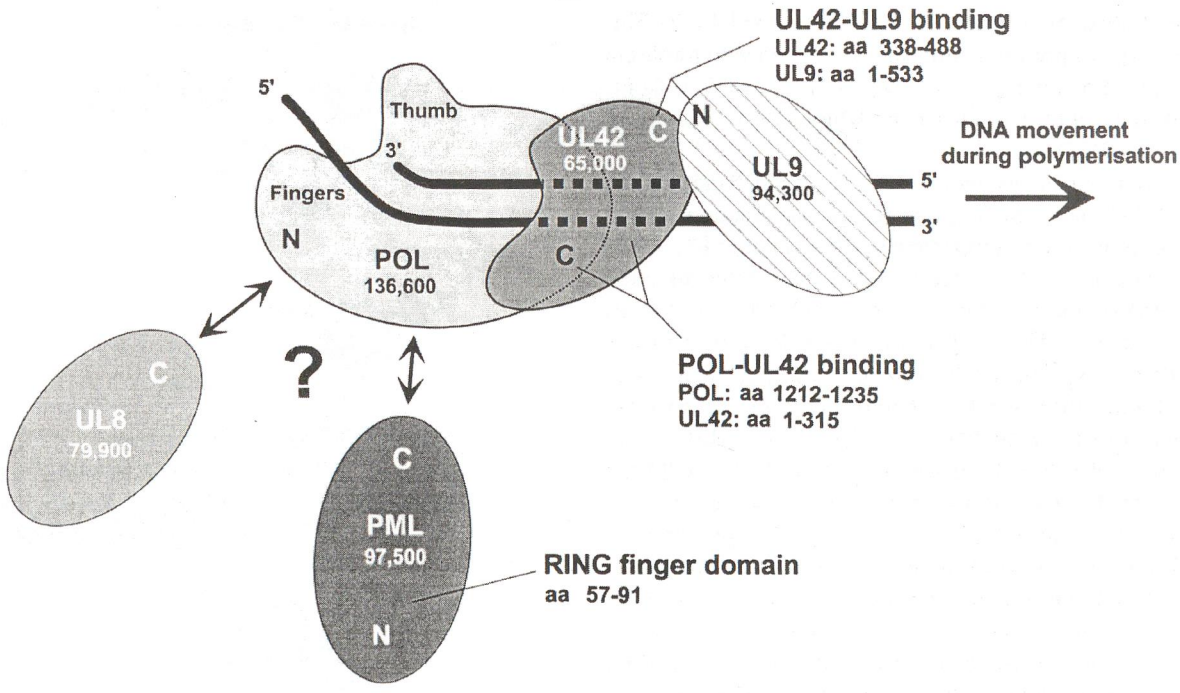

Fig. 5
Protein-DNA interactions of HSV-1 DNA polymerase
Schematic representation of the DNA polymerase bound to a linear DNA primer substrate. N- and C-termini of the interacting proteins with their M_r are indicated and the identified amino acids involved in polymerase protein binding are provided. Data from Digard *et al.* (1993), Stow (1993), Tenney *et al.* (1993), Everett *et al.* (1995), and Monahan *et al.* (1998).

preferentially to that derived from the upper strand, and (iii) the structure of the 5′-flanking sequences, comprising box III, are crucial for OBP binding (Aslani *et al.*, 2000). Taking this and former studies into consideration, virus-specific DNA initiation may begin with the sequence-specific binding of the OBP dimer to boxes I and II, found in both ORI_S and ORI_L signals. Through the interaction of both OBP pairs, the A/T-rich DNA loop region may become extruded, so that an intermediate hairpin structure is formed by the flanking inverted repeats of the ORI palindromes (Fig. 4A). Further unwinding of the ORI region by OBP may be assisted by the single-stranded (ss)DNA binding protein (ssDBP) ICP8 (Boehmer *et al.*, 1994; He and Lehman, 2000). ICP8 can cooperatively bind to short stretches of ssDNA, becoming exposed at the top or base of the stem-loop structure. By this means, ICP8 enlarges the single-stranded region so that loading of the DNA helicase/primase complex consisting of UL5, UL52, and UL8 in equal molarity (Crute *et al.*, 1989) is enabled (Fig. 3, step 4). The docking of the helicase/primase complex, termed primosome (Falkenberg *et al.*, 1997), is facilitated by the direct interaction of UL8 with N-terminal amino acids of UL9 (McLean *et al.*, 1994). The UL8 binding region of UL9 has recently been mapped to aa 161–211, containing a putative leucine zipper (F. Hendry, H. Marsden, G. McLean, M. Murphy, M. McElwee, N. Stow, personal communication). According to sequence alignment, UL5 is considered to represent the DNA helicase subunit, and UL52 the DNA primase subunit (Holmes *et al.*, 1988; Zhu and Weller, 1992). A subcomplex consisting of UL5 and UL52 but not singly expressed proteins display helicase and primase activity (Dodson and Lehman, 1991). Within the UL5/UL52 subcomplex there are two different domains for nucleoside triphosphatase activity. Site I hydrolyzes both ATP and GTP, while site II only ATP (Crute *et al.*, 1991). The primase component synthesizes oligoribonucleotide (8–12 nt) primers (Crute and Lehman, 1991). The DNA unwinding reaction proceeds in the presence of ICP8 with a displacement rate of 60–65 bp/sec and with a 5′-3′ polarity (Crute *et al.*, 1988; Falkenberg *et al.*, 1998). The unwinding of large dsDNA requires the UL5/UL8/UL52 heterotrimer as well as the presence of ICP8 and UL8 in a 1:1 ratio (Makhov *et al.*, 1996; Falkenberg *et al.*, 1997; Boehmer, 1998). The UL5/UL52 subcomplex does not interact with ICP8 (Falkenberg *et al.*, 1997). UL8 has two separate binding sites for UL5 and UL52. By two-hybrid screening it was shown that UL8 interacts more strongly with UL52 and specifically with a peptide region within aa 366–914

(Const and Dodson, 1999). UL8 seems to stabilize the *de nov* nthesized RNA primer molecules (Sherman *et al.*, 19 and to be responsible for attracting the DNA polyme e to the ORI complex (Fig. 3, step 5). A specific bindin the UL8 C-terminus with the catalytic subunit of DNA po merase (UL30) has been convincingly shown by *in vitro* udies (Marsden *et al.*, 1997). Docking of the DNA polyme ase to the ORI complex is further promoted by some amino acids within the N-terminal 533 amino acids of UL9, that bind to UL42, the small subunit and accessory factor of the DNA polymerase (Monahan *et al.*, 1998) (Fig. 5). As mentioned before, there are now recognized two types of prereplicative sites which were identified by immunofluorescence microscopy: (i) "numerous sites", thought to represent foci of ICP8 and other viral proteins at cellular replication forks in S-phase cells, and (ii) "PML-associated sites", thought to represent actual intermediates in the formation of replication compartments (Burkham *et al.*, 1998). The presence of the catalytic DNA polymerase subunit (UL30) but not UL42 is required to recruit the isoform of PML, supporting the notion that only PML-containing nuclear dots represent true viral prereplicative sites. Since PML has never been implicated directly in cellular DNA replication, its role in viral DNA synthesis remains obscure. It is worth mentioning that an antibody (PG-M3, Santa Cruz Biotechnology) used to characterize the fate of PML and ND10 during HSV-1 infection by Burkham *et al.* (1998) was shown to cross-react with ICP4, which itself is a component of replication compartments (Boulware and Weber, 2000). In awareness of this fact it seems to be important to reexamine the role of PML in herpesvirus DNA replication.

Two current models may be envisaged for the role of ND10 disruption. (i) A selective degradation of cellular proteins could lead to a stimulation of viral gene expression, whereby the likelihood increases that viral infection will progress efficiently (Everett *et al.*, 1998). (ii) Viral DNA replication may require association with nuclear sites that are marked by ND10 (Maul, 1998). Disruption of ND10 and degradation of some isoforms of PML upon infection may expose underlying sites, allowing the viral genome or viral replication proteins to become associated. According to the latter model, that is supported by a recent independent study (Everett *et al.*, 1999b), the presence of intact ND10 may inhibit viral DNA replication by masking important nuclear attachment sites.

Participation of other cellular DNA polymerases in the initiation of viral DNA replication has not yet been defined. There is only one report showing that UL9 can bind to the 180 K catalytic subunit of DNA polymerase α (Lee *et al.*, 1995). This cellular DNA polymerase, which carries a DNA primase subunit, is responsible for the initiation of cellular DNA synthesis. It is intriguing to speculate, whether DNA polymerase α is involved in initiation of viral DNA synthesis under restricted conditions of viral growth, e.g. latent infection.

Elongation of viral DNA

After the successful assembly of the viral replisome, the helicase/primase complex further unwinds viral dsDNA, and at the same time the primase subunit synthesizes short RNA stretches (8–12 nt) on ssDNA portions in the 5′-to-3′ direction. The RNA initiator molecules are then further extended to the length of Okazaki fragments by the DNA polymerase holoenzyme. Fig. 4B depicts the viral replisome acting at a replication fork, namely the viral replicative functions recently identified to be essential and sufficient to replicate HSV-1 ORI-bearing plasmids in a transient replication system *in vitro* (McGeoch *et al.*, 1988b; Wu *et al.*, 1988); UL9 is not shown as it is thought to be replaced by ongoing bidirectional fork movement shortly after its origin binding interaction. Removal of the RNA portion of the Okazaki fragments has to occur in the 5′-to-3′ direction. This is most likely being achieved by a cellular RNase H activity. The produced gaps are then filled with deoxynucleotides by the viral DNA polymerase, and the extended DNA fragments are joined to the preceding DNA strands by a cellular DNA ligase. It has formerly been questioned whether a DNA polymerase-associated RNase H activity could perform the RNA hydrolysis function (Crute and Lehman, 1989). However, the herpesvirus DNA polymerase has been demonstrated to possess only a 3′-to-5′ exonuclease for proofreading, which is capable to remove both RNA and DNA moieties from dsDNA templates, but in the opposite (3′-to-5′) direction (Knopf and Weisshart, 1990; Hall *et al.*, 1996). There are two other possible viral candidates for the removal of RNA, the alkaline DNase, which was shown to be an endoexonuclease, and the vhs protein. Both proteins are capable to degrade RNA in the 5′-to-3′ direction. The endoexonuclease is more likely to be involved in DNA recombination events (Kehm *et al.*, 1998) and, as shown above, the major task of vhs protein is the degradation of cellular mRNA. The synthesis of leading and lagging strands is believed to be carried out solely by the DNA polymerase holoenzyme. The elongation rate and the processivity of this enzyme is strongly influenced by its cofactor, the UL42 protein (Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990). Some amino acids within the N-terminal 315 amino acids of UL42 bind to the 25 C-terminal amino acids of the polymerase subunit UL30, and this essential interaction has emerged as a promising novel target for antiviral therapy (Digard *et al.*, 1993; Stow, 1993; Tenney *et al.*, 1993; Knopf and Strick, 1994). Nuclear magnetic resonance (NMR) analysis of C-terminal peptides (18-mer, and 36-mer) from HSV-1 DNA polymerase, that successfully

inhibit the UL42 binding, as well as studies on the crystal structure of a complex between the 36 amino acids long polymerase peptide and aa 1–319 of UL42 provide a basis for the rational design of new antiviral drugs (Bridges *et al.*, 2000; Zuccola *et al.*, 2000). Conceivably, every essential and virus-specific interaction of a herpesvirus DNA polymerase during the replication cycle represents a potential antiviral target. Many of the abovementioned protein interactions of HSV-1 DNA polymerase, summarized in Fig. 5, can be regarded as such targets.

A salient feature of HSV-1 DNA polymerase is its apparent preference for high salt, provided the activity is assayed *in vitro* (Knopf, 1979). This property could be related to the interaction of its accessory factor, UL42 protein, with DNA (Hart and Boehme, 1992). UL42 displays a strong binding to dsDNA and this binding is loosened by high salt. Consequently, the forward polymerase reaction as well as the proofreading activity is enhanced (Franz *et al.*, 1999). The situation *in vivo* is probably different, as the components driving the fork movement (helicase/primase complex) and keeping the single-stranded portions of the DNA extended (UL29, ICP8) are the limiting factors of the polymerization rate.

HSV-1 DNA polymerase, like a true major DNA replicase, has an associated proofreading 3′-to-5′ exonucleolytic activity (Knopf, 1979). This activity eliminates mismatched nucleotides at the 3′-terminus very efficiently, even during the forward polymerase reaction (Kuehn and Knopf, 1996; Strick and Knopf, 1998). Like the bacteriophage T4 DNA polymerase, the HSV-1 DNA polymerase does not leave the DNA template while switching from proofreading (backward reaction) to polymerase mode (Strick and Knopf, 1998). Even though HSV-1 DNA polymerase exhibits a clear proofreading function towards mispaired natural nucleotides, its error rate during nucleotide incorporation is considerably higher than those reported for the major cellular DNA polymerases (Hall *et al.*, 1985). This circumstance has contributed significantly to the fact that the herpesvirus DNA polymerase has become the main target of antiviral drug therapy (Furman *et al.*, 1979). Two classes of inhibitors have been shown to be very effective in blocking herpesvirus infection: (i) base analogs (e.g. acyclovir, ganciclovir, etc.) and (ii) pyrophosphate competitors (e.g. PAA, foscarnet, etc.) (Honess and Watson, 1977; Coen and Schaffer, 1980; Furman *et al.*, 1984). In its replicative function the HSV-1 DNA polymerase resembles by large the cellular DNA polymerase d, the major cellular DNA replicase engaged in the leading strand synthesis (Knopf and Strick, 1994). From an evolutionary point of view the viral enzyme is also closely related to the latter enzyme, belonging to the polymerase superfamily (family B) with homology to the *E. coli* DNA polymerase II (Knopf, 1998). In the absence of physicochemical data on the HSV-1 DNA polymerase structure, the striking similarity to the cellular DNA polymerase d can be documented by comparing their modular organization with that of already crystallized polymerases of family A (*E. coli* Pol I) and B (*Thermococcus gorgonarius*, RB69) as shown in Fig. 6 (Ollis *et al.*, 1985; Wang *et al.*, 1997; Hopfner *et al.*, 1999). Each of these polymerases is endowed with a N-terminal domain, encoding a proofreading exonuclease activity (3′ EXO). Whereas the *E. coli* Pol I carries an additional 5′-exonuclease domain (5′-EXO) in the very N-terminal sequences, a similar function could not be assigned to the variably sized N-terminal sequences in the family B polymerases. From recent crystallographic data major differences between family A and B DNA polymerases became apparent: (i) only the conserved polymerase motifs I and II, involved in metal binding and representing a characteristic feature of the palm domain according to the united right hand model of DNA polymerases (Brautigam and Steitz, 1998), are conserved in both families, (ii) in the family B polymerases, exonuclease and polymerase domains are separated by a helical interdomain insertion of various size, (iii) the polypeptide region recruiting the so-called thumb function is located in *E. coli* Pol I right behind the exonuclease domain, and in the family B polymerases at the C-terminus of the enzyme, respectively, (iv) HSV-1, RB69 and d DNA polymerases contain a binding site for an accessory factor at the very C-terminus. From this comparison (Fig. 6) it is evident that the modular structure of the HSV-1 DNA polymerase, except for a larger helical interdomain insertion, is strikingly similar to that of the DNA polymerase δ, and correlates quite well with the functional and evolutionary relatedness of both enzymes.

The mechanism of viral DNA replication remains largely unexplored. After the nuclear entry and circularization of the viral genome, DNA replication proceeds very likely in two distinct phases: the initial phase is characteristic by a bidirectional (theta) replication, initiated at one or more ORIs with bidirectional fork movement; in the later phase a rolling-circle mode of DNA replication proceeds (Boehmer and Lehman, 1997). Whereas exploration of the bidirectional replication phase remains a challenge for future studies, work with defective genomes and plasmid replication *in vitro* (Deiss *et al.*, 1986) is in favor for a rolling-circle mode of DNA synthesis. More recent studies of DNA replication intermediates of HSV-1 or related equine herpesvirus 1 (EHV-1) infection by pulse-field gel electrophoresis (Severini *et al.*, 1994, 1996; Bataille and Epstein, 1997; Slobedman and Simmons, 1997) have provided evidence for a more complex pattern of herpesvirus DNA replication. On the other hand, both replication modes can actually lead to the generation of branched concatemeric DNA, that constitutes a major fraction at both the early and late times of HSV-1 infection.

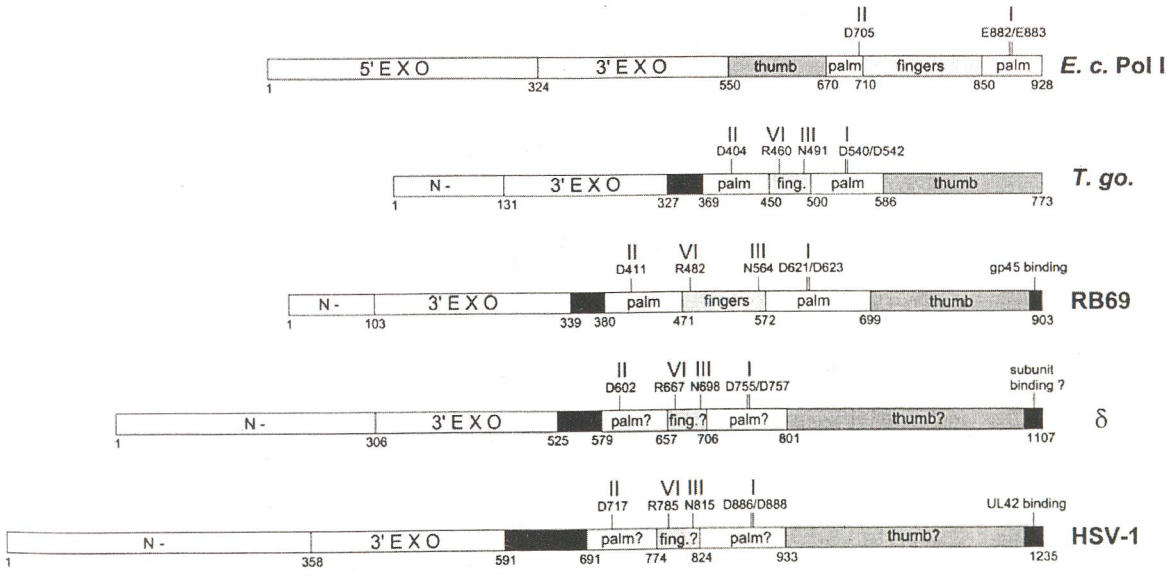

Fig. 6

Modular organization of family A and B DNA polymerases

The domains for 5′-exonuclease (5′-EXO) and 3′-exonuclease (3′-EXO) and the domains for palm, fingers, and thumb of the polymerase function derived from the crystallographic analysis of *E. coli* Pol I (*E. c.* Pol I), archaebacterial (*Thermococcus gorgonarius*, *T. go.*) and bacteriophage RB69 DNA polymerases (RB69) are shown. Conserved amino acids (given in single-letter code and number) in motifs I, II, III, and IV of the palm and fingers regions are indicated that were used to assess the polymerase domains in DNA polymerase d (d) and HSV-1 DNA polymerase (HSV-1) by multiple sequence alignment and secondary structure prediction using the Wisconsin Package Version 10.0 (Genetics Computer Group, Madison, WI) as described previously (Knopf, 1998). Numbers refer to amino acids.

Cleavage and packaging of viral DNA into preformed capsids

After completion of viral DNA synthesis, resulting in concatemeric DNA, unit-length genomes are cleaved and packaged into preformed capsids. DNA maturation involves two separate cleavage events at sites distal to the recognition sequences (Varmuza and Smiley, 1985). Specific signals, termed *pac1* and *pac2,* were identified as recognition sites for cleavage and packaging within the *a* sequences, that are found in random copy numbers as direct repeats at both ends of the genome, and internally as inverted repeats, separating L and S components (Deiss and Frenkel, 1986). Cleavage occurs asymmetrically within a 20 bp direct repeat sequence (DR1) flanking the *a* sequence, and generates an unit-length genome with defined staggered termini (Mocarski and Roizman, 1982). The left end contains 18 bp and the right end the residual bp of DR1. Both ends contain a 3′-nucleotide overhang. Circularization of the genome restores the original DR1. The left genome end terminates with *pac2* sequences, and the right end with *pac1* sequences, respectively. HSV-1 particles contain four isomeric forms of the genome, with L and S regions linked together in different orientation, and in equimolar amounts. Analysis by pulse-field gel electrophoresis of replication intermediates, digested with single-cutter restriction enzymes, clearly demonstrated that replicating DNA has a concatemeric structure, and inversions of the unique sequence components L and S are observed even at early times of infection. The major fraction of the replicating viral DNA is found in a network of branched DNA that is only partially resolved with single-cutter enzymes, suggesting that the network DNA contains non-linear structures. That DNA concatemers arise, in which the four possible configurations of L and S components are present at equimolar amounts and at any time during viral replication, cannot be explained by simple bidirectional or rolling-circle mode of replication, without including recombination. A pure rolling-circle mode may comply better with the replication of defective genomes or virus mutants, lacking the internal *a* sequences, where inversions are not observed (Kaerner *et al.,* 1981; Poffenberger and Roizman, 1985; Poffenberger *et al.,* 1983; Stow and McMonagle, 1983). However, non-linear DNA structures were observed also during replication of amplicon vectors indicating a more complex way of replication (Bataille and Epstein, 1997). Using HSV-1 amplicon vectors and recombinant virus that did not contain inverted repeats it was demonstrated that the presence of inverted repeat sequences in the herpesvirus genome is a prerequisite for the isomerization event, and a

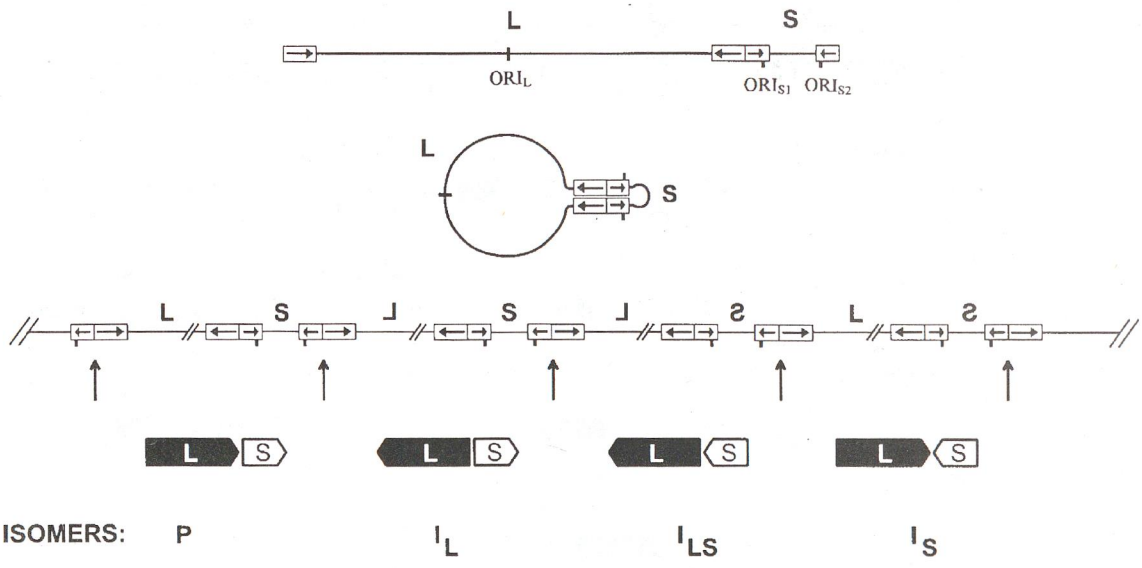

Fig. 7
Isomerization of the HSV-1 genome

The HSV-1 DNA molecule is shown both in linear and figure eight form. The orientation of inverted repeat sequences is given by boxed arrows and the ORIs are marked. Generation of four different genome isomers, designated P (prototype), I_L (inverted L component), I_{LS} (inverted L and S component), and I_S (inverted S component) is shown to occur by cleavage of concatemeric DNA that is replicated using a figure eight structure, in which crossing-over can take place between homologous inverted repeat sequences.

respective model was proposed (Bataille and Epstein, 1997) in which genome isomers are generated during packaging by alternative cleavage within the *a* sequences, as suggested earlier by Smiley *et al.* (1990). In this model, the generation of isomers is alleged to the inversion of the L component. Since there is no obvious reason to exclude an inversion of the S component in DNA concatemers according to the present model (Bataille and Epstein, 1997), we alternatively propose that both L and S components are subject of inversion. Considering the peculiar symmetry of the HSV-1 genome (McGeoch, 1987) displaying a figure 8 shape (Sheldrick and Berthelot, 1975), inversion of L or S components may simply occur during bidirectional or rolling-circle replication by homologous recombination between inverted repeats encompassing both ORI_S signals. Crossing-over events can take place at a constant rate at the onset of viral DNA replication and at the beginning of each new round of genome synthesis. Only one crossing-over is necessary for generating a L or S component inversion. Fig. 7 depicts as an example a concatemer molecule which comprises all four genome isomers. Specific, but not alternative cleavage within the *a* sequence will yield correctly sized genome isomers in equimolar amounts. E.g., generation of linear concatemers by rolling-circle mode of replication would require: (i) the introduction of a nick in one of the two parental DNA strands, (ii) the displacement of one end of the nicked strand, and (iii) priming at the exposed single-strand region for consecutive polymerization of the complementary strand by either leading or lagging strand synthesis. The rolling-circle replication would then lead to the formation of large linear DNA concatemers but also, due to recombination of the inverted repeat sequences, to a branched DNA. Linear and branched DNA structures would possess only one end of the herpesvirus genome. A recent study confirms that the ends formed on replicative concatemers of four different herpesviruses (HSV-1, EHV-1, guinea pig cytomegalovirus, and murine cytomegalovirus) are unique, consisting of terminal *pac2* sequences (McVoy *et al.*, 2000). It remains to be shown, how the very first end of a DNA concatemer is generated and modified into a well defined left HSV-1 genome terminus. The synthesis of DNA concatemers with only one type of terminus speaks for the directionality of the herpesvirus DNA packaging process that is controlled by *cis*-acting sequences.

Concomitantly with the onset of viral DNA replication (about 2–4 hrs after infection), the synthesis of the true late gene products (γ2) takes place (Fig. 8). They comprise most of the glycoproteins and membrane-associated proteins; the latter appear in the inner nuclear membrane but also in the cytoplasmic membrane (Table 2). At about the same time capsid proteins and proteins required for DNA packaging and cleavage are produced and transported to the DNA replication compartments. VP5 (UL19), VP19c (UL38), VP23 (UL18), pre-VP22a (UL26.5), and pre-VP21 (UL26)

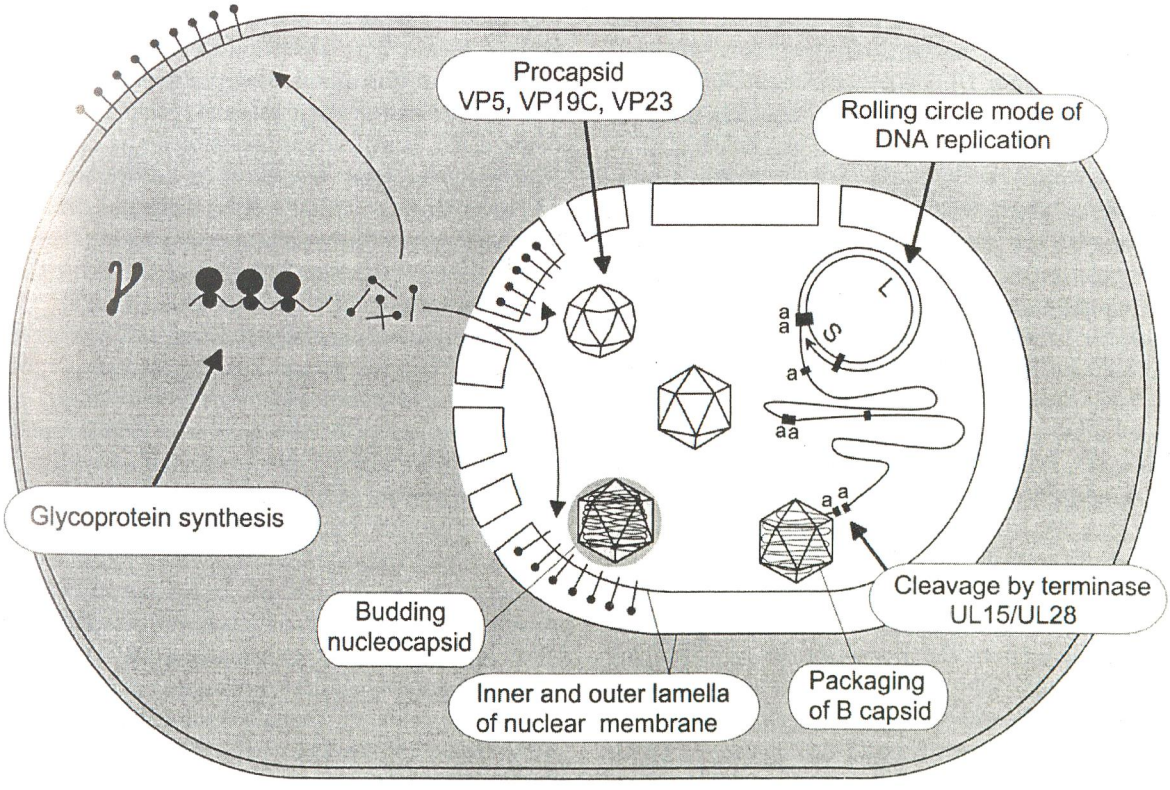

Fig. 8
Capsid formation, DNA concatemer cleavage and packaging into preformed capsids

assemble into fragile roundish procapsids, which mature into robust polyhedral capsids in a transition similar to that undergone by bacteriophage proheads (Trus *et al.,* 1996). The major capsid protein, VP5, and the scaffolding protein, pre-VP22a, condense to form structures that are extended into procapsids by addition of the triplex proteins, VP19C and VP23 (Spencer *et al.,* 1998). Three-dimensional reconstruction of cryo-electron microscopy revealed that inside the procapsid is the hollow spherical scaffold, consisting of the major scaffold protein pre-VP22a and forming a protein micelle with a preferred radius of curvature (Table 1) (Trus *et al.,* 1996). In the nuclei of HSV-1-infected cells three types of capsids are found: B or intermediate capsids containing a core composed of the scaffolding proteins (UL26, UL26.5 gene products) that is analogous to the scaffolded proheads of phage λ (Hohn, 1979), C capsids or nucleocapsids, in which the viral genome replaced the scaffold core of the intermediate capsid that is analogous to DNA filled proheads of phage λ, and A or abortive capsids containing neither DNA nor the scaffolding protein. Transport of procapsids and capsid proteins to the replication compartments seems to involve UL17, a tegument protein, and the DNA packaging protein UL32 (Lamberti and Weller, 1998; Taus *et al.,* 1998). Consistent with this, UL17 was recently found to be associated with B and C capsids, and localized together with the scaffold protein ICP35 and VP5 at early times of infection, when procapsid structures are not formed yet (Goshima *et al.,* 2000). Self-assembly experiments in a cell-free system indicated that the procapsids were similar in morphology and protein composition to procapsids formed *in vitro* from cell extracts containing HSV-1 proteins, representing initially partial capsids and, after longer incubation, predominantly closed spherical capsids with T=16 icosahedral symmetry (Newcomb *et al.,* 1996, 1999). After association of the capsid shell with UL35 (VP26), which binds to UL19 in a 1:1 ratio, yielding a hexameric ring on top of the hexons (vertex), and proteolytic processing of the scaffolding protein (Rixon *et al.,* 1988; Davison *et al.,* 1992), the virus genome is packaged into procapsids in a similar head-full manner known from bacteriophages (Black, 1989). The DNA packaging process apparently does not require ongoing DNA synthesis (Church *et al.,* 1998). Similar to the excision of bacteriophage l genomes from concatemeric DNA precursor molecules (Woods *et al.,* 1997), HSV-1 DNA is cleaved and packaged utilizing a sequence-specific and size-oriented

mechanism (Roizman and Sears, 1996). In addition to UL17 and UL32, UL25 is dispensible for cleavage (McNab *et al.*, 1998); four other genes, UL6, UL15, UL28, and UL33, have been shown to be required for DNA cleavage and packaging by the analysis of temperature-sensitive and deletion HSV-1 mutants (Lamberti and Weller, 1996; Taus and Baines, 1998; Reynolds *et al.*, 2000). As mentioned before, the *a* sequence plays a key role in replication, embodying the *cis*-acting signals for cleavage and packaging. Cleavage and packaging of DNA appear to be linked processes (Ladin *et al.*, 1980). An evidence comes from sequence homology, functional analysis and inhibitor (benzimidazole ribosides) studies, that herpesviruses and bacteriophages utilize a similar mechanism to package their monomeric genomes (Addison *et al.*, 1990; Davison, 1992; Tengelsen *et al.*, 1993; Bogner *et al.*, 1998; Mettenleiter *et al.*, 1993; Yu and Weller, 1998a; Krosky *et al.*, 1998; Underwood *et al.*, 1998; Bogner, 1999). A major role plays the terminase, an enzyme that is highly conserved in bacteriophages and herpesviruses. In HCMV-infected cells, the gene products UL56 and UL89 were identified as potential terminase subunits (Bogner, 1999; Bogner *et al.*, 1998). Lysates from UL56-expressing baculovirus- as well as HCMV-infected cells were shown to form specific complexes with the AT-rich sequences in *pac1* or *pac2* motif, and to cleave the *a* sequence endonucleolytically and in an ATP-independent manner (Bogner *et al.*, 1998). With lysates from UL89-expressing baculovirus-infected insect cells a similar result was obtained (Bogner, 1999), but these lysates exhibited a less specific endonuclease activity resembling that of phage T4 terminase (Black, 1986). In HSV-1, the UL28 and UL15 gene functions represent homologous counterparts of HCMV UL56 and UL89, respectively. The HSV-1 homologs were obtained as 1:1 heterodimers after three different purification steps (Koslowski *et al.*, 1999), but their functional activity remains to be shown. Preliminary data from the analysis of crude lysates of recombinant baculovirus-infected cells indicate, that the UL28 subunit is associated with an ATP-independent endonuclease activity (Bogner, 1999). The putative UL15 terminase subunit of HSV-1, like the UL89 protein of HCMV, consists of two exons, UL15A and UL15B (Baines *et al.*, 1997), and possesses two different nucleotide binding motifs, known as Walker boxes A (GKT) and B (DE) (Walker *et al.*, 1982). The apparent ATP-independence of the endonuclease activities, associated with both subunits of HCMV terminase, appears to point to a different utilization of ATP, known from phage DNA packaging (Fujisawa and Morita, 1997). Likewise the herpesviral terminase may utilize ATP predominantly for DNA packaging, more precisely, for the translocation of the viral genome into the procapsid shell. It needs to be shown, that the UL15 subunit features an additional helicase activity, that was reported to be associated with bacteriophage λ terminase (Yang and Catalano, 1997). Nonetheless, the UL15 terminase subunit tends to be involved primarily in DNA translocation, and the UL28 subunit in the specific cleavage of the *a* sequence. The intranuclear site of DNA cleavage and packaging is currently controversial. Localization studies of the UL28 homolog in HCMV-infected cells (Giesen *et al.*, 2000), and of major and minor capsid proteins in HSV-1-infected cells (Taus *et al.*, 1998) support the hypothesis that capsid assembly and DNA cleavage/packaging takes place at viral replication compartments.

In analogy to the assembly pathway of bacteriophage λ, initiation of HSV-1 genome packaging may require the formation of a catalytically-competent nucleoprotein complex (Hohn, 1979; Woods *et al.*, 1997). Since UL6, UL15 and UL28 were found to be associated with B capsids even in the absence of other DNA cleavage and packaging proteins (Yu and Weller, 1998b), it is likely that the packaging process of HSV-1 genomes initiates with the binding of the terminase to one of the *pac* signals of the *a* sequences found in DNA concatemers. The UL15 subunit of the terminase probably accomplishes the ATP-dependent DNA translocation into the procapsid shell, which is supported by the UL28-mediated interaction with UL6 (Taus and Baines, 1998). For successful packaging, DNA concatemers have to be cleaved into unit-length genomes. In the current model (Deiss *et al.*, 1986) it is assumed that excision of the viral genome from the large concatemeric DNA occurs after the packaging of viral DNA, and operates with an analogous synaptic mechanism as observed for the *cos* sequences in bacteriophage λ, requiring the interaction of two *a* sequences in *trans* at the portal of the procapsid (Hohn, 1979; Thomason *et al.*, 1997). At present, neither the precise composition of the terminase cleavage complex is known, nor at which stage of the packaging process DNA cleavage actually takes place. Maturation of the viral DNA into unit-length genomes seems to involve proteolytic processing of the amino terminus of the UL15 terminase subunit (Salmon *et al.*, 1999). We know that the DNA concatemers are cleaved ideally within two joined *a* sequences, but also within one *a* sequence (Deiss and Frenkel, 1986). Terminal *a* sequences can be amplified by illegitimate recombination via the flanking DR1 during viral DNA replication as discussed before (Fig. 7). From the analysis of the organization of defective genomes derives that the left end of the HSV-1 genome, containing the L component, is channelled first into the capsid shell (Deiss *et al.*, 1986). This is further supported by the analysis of HSV-1 DNA replication intermediates, demonstrating that high-molecular mass DNA concatemers were flanked exclusively by the L component of the viral genome and terminated with *pac2* elements only (McVoy *et al.*, 2000; Zhang *et al.*, 1994). At present it is unclear whether the latter terminus is generated at early times of HSV-1 DNA

replication while switching from bidirectional to rolling-circle mode of replication, or at late times by a specific cleavage event that involves the action of the terminase. In any case, the terminase/*a* sequence complex has to be transported to the procapsid, where it may dock onto a specific portal protein, following the phage assembly model (Hohn, 1979; Lin *et al.,* 1999). UL25 could possibly play the role of a portal vertex protein as proposed by Homa and Brown (1997). There is sufficient evidence that UL25 exerts an important function for nucleocapsid integrity as follows. UL25 is required for packaging and represents a minor yet essential constituent of all three forms of viral capsids (McNab *et al.,* 1998). Temperature-sensitive mutants in this gene accumulate abortive capsid shells, when the shift to the non-permissive temperature is performed after virus penetration (Addison *et al.,* 1984). The homologous UL25 of PRV was shown to remain associated with nucleocapsids during their microtubular transport to the nucleus (Kaelin *et al.,* 2000).

UL15 and UL6 may be required for DNA translocation. UL6 could have a function analogous to phage protein FI that is required for the reeling of the DNA into the prohead (Hohn, 1979). Whereas UL6 and UL25 remain to be associated with mature virions (Lamberti and Weller, 1996; McNab *et al.,* 1998), both terminase subunits are found only in nuclear B capsids (Yu and Weller, 1998b). This observation is consistent with the notion that, after the final cleavage event, both terminase subunits dissociate from the *a* sequence recognition site of the newly packaged viral genome. The cleaved free terminus of the linear DNA concatemer is then available for further encapsidation and processed following an identical packaging scheme.

The role of UL33 is less clear. UL33 represents a true late phosphoprotein. It acts like UL6 but is independent from the latter (Patel *et al.,* 1996). UL33 is apparently absent in capsids and virions; it was localized in DNA replication compartments. Therefore it may exert an important intermediate function in the DNA cleavage and packaging process (Reynolds *et al.,* 2000). The putative function in DNA cleavage and packaging of the very large tegument protein UL36, that was found in a protein complex binding to the terminal *a* sequence (Chou and Roizman, 1989), awaits to be identified.

Packaging of the large viral genome into B capsids is accomplished by a capsid scaffold that is formed by ICP35 and pre-VP22a, the products of the UL26 and UL26.5 genes. For successful DNA packaging, the scaffolding proteins, that are bound to VP5, are modified by two proteolytic cleavages, selfcleavage as well as cleavage in *trans*. Selfcleavage of scaffolding protein ICP35 generates a free protease activity (VP24) as well as a VP5-bound pre-VP21. pre-VP21 and pre-VP22a are then cleaved in *trans* generating VP21 and VP22a, leaving identical 25 amino acids C-terminal attached to VP5 that appear to be critical for the formation of the polyhedral capsid shell (Kennard *et al.,* 1995; Thomsen *et al.,* 1995). B capsids, the precursors of mature virions, contain only fully processed protease and scaffolding proteins (Robertson *et al.,* 1996). For completion of the packaging process a second cleavage event must take place within another *a* sequence, precisely at a distance of one genome length. Achieving this precise cleavage implies that a scanning mechanism for DNA packaging must exist that recognizes and cleaves *a* sequences only at each second L/S inverted repeat junction of a linear DNA concatemer as proposed by Deiss *et al.* (1986). As shown in Fig. 7, every second L/S junction displays an identical orientation in a DNA concatemer molecule, that makes the orientation of *a* sequence an appropriate measure for a scanning mechanism. Possible candidates for a scanning protein complex are the UL28 terminase subunit and UL6, because of their tight association with the B capsid shell (Taus and Baines, 1998). Assuming that the terminase cleavage complex is stably fixed at the portal of the procapsid, then only every second *a* sequence of the DNA, reeled into the capsid, has the appropriate sequence orientation for cleavage, that will allow to generate predominantly unit-length genomes. For cleavage and packaging of defective genomes and amplicons having only one *a* sequence but several direct repeats of it, in addition to the scanning mode a head-full recognition element has been proposed to generate unit-length genomes with terminal *a* sequences (Deiss *et al.,* 1986; Roizman and Sears, 1996), in which after near head-full packaging cleavage takes place at juxtaposed *a* sequences. The current assembly model cannot explain, whether packaging initiates randomly at internal *a* sequences of the DNA concatemers or in a preferred mode from the terminus of the linear concatenated DNA. The latter mode, that is observed in phage assembly, would facilitate the sizing of the DNA genomes for packaging by a head-full mechanism. A premise of this mode is that the terminus to be packaged meets the requirements for cleavage specificity by the terminase cleavage/packaging complex. Interestingly, the finding that HSV-1 DNA concatemers have a preferred genome terminus, is in support of such a phage-like assembly mechanism (McVoy *et al.,* 2000; Zhang *et al.,* 1994).

Summing up, the following gene functions appear to be major players in the DNA cleavage and packaging process: (1) UL18, UL19, UL35, and UL39 are required for capsid shell formation, (2) UL17 and UL32 for transport of capsids or capsid proteins to replication compartments, (3) UL26 and UL26.5 for the formation of the scaffold, (4) UL15 and UL28 for DNA cleavage and DNA translocation to the procapsid, and (5) UL6, UL25, and UL33 for the reeling of the DNA and the final stages of the packaging process.

Maturation and egress of virions (envelopment of nucleocapsids)

Mature B nucleocapsids are exported from the nucleus by budding through the inner lamella of the nuclear membrane, and become enveloped (Fig. 8). Briefly, the nucleocapsids have to be coated before envelopment with tegument proteins. We have probably to look for candidates amongst the latter proteins that trigger the attachment to the nuclear inner membrane and induce the envelope formation. As mentioned above, UL34 in concert with UL31 seems to play an important role in the association of the capsid structures to the inner nuclear membrane (Roller *et al.,* 2000; Ye *et al.,* 2000). Transport of viral transmembrane proteins from the rough endoplasmic reticulum to the nuclear inner membrane appears to be mediated by a specific signal sequence. The sequence DRLRHR in the C-terminal region of HCMV gB has recently been discovered to be a signal for nuclear translocation, and the same was shown for a comparable sequence stretch encompassing aa 832–867 in the cytoplasmic tail of HSV-1 gB (Meyer and Radsak, 2000). Virus particles accumulate in the perinuclear space between the inner and outer lamella. How virions are transported to the cell surface is presently not clear; this process may involve the endoplasmic reticulum and the trans-Golgi network apparatus (Alconada *et al.,* 1998; Elliott and O'Hare, 1999). Currently, two models are discussed concerning the anterograde transport of virions through the cytoplasm (Enquist *et al.,* 1998). In the lumenal model, viral capsids, after budding through the inner nuclear membrane, reach the cell surface via the secretory pathway (Johnson and Spear, 1982). In the re-envelopment model, the perinuclear virions lose their envelope by fusing with the outer nuclear membrane, and obtain a new envelope by budding into a compartment late in the exocytotic pathway, very likely the Golgi apparatus or membrane vesicles from it. In agreement with the latter model, firstly, unenveloped nucleocapsids, closely associated with microtubules, were found in the axons by analyzing anterograde axonal transport (Penfold *et al.,* 1994), and secondly, the composition of extra-cellular virions was shown to differ from that of perinuclear virions (Stackpole, 1969; van Genderen *et al.,* 1994). Virions accumulate in the perinuclear space, provided the cells are infected with deletion mutants in gene UL20 or UL48 (coding for VP16), suggesting that both gene functions are required for virus egress (Baines *et al.,* 1991; Mossman *et al.,* 2000). It was demonstrated in PRV infection, that the successful processing of gK, thought to play an essential role in virus egress (Klupp *et al.,* 1998), is dependent from the UL20 gene function (Dietz *et al.,* 2000).

It can be envisioned that studies with mutants, impaired in envelopment, as well as recombinants, designed for *in situ* detection, will advance our understanding of the yet obscure late events in HSV-1 biogenesis.

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