Nuts and Bolts of Human Cytomegalovirus Lytic DNA Replication

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Contents

Abstract HCMV lytic DNA replication is complex and highly regulated. The *cis*-acting lytic origin of DNA replication (oriLyt) contains multiple repeat motifs that comprise two main functional domains. The first is a bidirectional promoter element that is responsive to UL84 and IE2. The second appears to be an RNA/DNA hybrid region that is a substrate for UL84. UL84 is required for oriLyt-dependent DNA replication along with the six core proteins, UL44 (DNA processivity factor), UL54 (DNA polymerase), UL70 (primase), UL105 (helicase), UL102 (primase-associated factor) and UL57 (single-stranded DNA-binding protein). UL84 is an early protein that shuttles from the nucleus to the cytoplasm, binds RNA, suppresses the transcriptional activation function of IE2, has UTPase activity and is proposed to be a member of the DExH/D box family of proteins. UL84 is a key factor that may act in concert with the other core replication proteins to initiate lytic replication by altering the conformation of an RNA stem loop structure within oriLyt. In addition, new data suggests that UL84 interacts with at least one member of the viral replication proteins and several cellular encoded proteins.

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Introduction

Human cytomegalovirus lytic DNA replication appears to be a complex and highly regulated event. Although immediate early gene expression occurs shortly after infection, the onset of viral DNA synthesis does not take place until approximately 24 h postinfection in cell culture. HCMV DNA replication is thought to involve circularization and concatemer formation (McVoy and Adler 1994). Viral DNA synthesis, which occurs in the nucleus, requires the HCMV core replication machinery and may be regulated by multifunctional immediate early proteins that were originally thought to have a role only in gene regulation. As for the structure of replicating viral DNA, head-to-tail concatemers are evident from examination of the replication products from cloned oriLyt in human fibroblasts. Although a rolling circle model is postulated for lytic DNA replication, there is limited evidence to support this method for HCMV DNA synthesis and actual events may be more complex (McVoy and Adler 1994).

The *cis*-acting lytic origin of replication, oriLyt, is located within the unique long (U_L) region of the genome between AD169 nucleotides 90,500 and 93,930. This region, which is situated between ORFs 57 and 69, was originally determined to contain an origin based on the results of both the transient replication assay, where cloned regions of the HCMV genome were amplified upon transfection and subsequent infection of permissive cells (Anders and Punturieri 1991; Anders et al. 1992; Masse et al. 1992), and a method that took advantage of the chain termination effects of the drug ganciclovir (Hamzeh et al. 1990). Later studies confirmed that the oriLyt identified form both studies was the only functional replicator in the virus genome (Borst and Messerle 2005). Both approaches confirmed that a complex DNA region within the HCMV genome was responsible for the propagation of viral DNA during the lytic phase of viral growth. With the cloned lytic origin in hand, a cotransfection replication assay was developed in an effort to elucidate the viral-encoded *trans*-acting factors that contribute to oriLyt-dependent DNA replication. The transient cotransfection replication assay revealed that eleven loci are required for efficient oriLyt-dependent DNA replication. These genes are: UL36-38, UL44 (pol accessory protein), UL54 (DNA polymerase), UL57 (single-stranded DNA binding protein), UL70 (primase), UL102 (primase-associated factor), UL105 (helicase), UL112/113 (early proteins), IRS1/TRS1 (immediate early RNA-binding), IE1/2 (transactivator) and UL84. Several of these proteins were determined to play ancillary roles in DNA replication (Sarisky and Hayward 1996; Xu et al. 2004b).

Initiation of lytic DNA replication occurs at a single origin of replication and appears to be initially mediated by the viral encoded proteins UL84 and IE2. As our understanding of HCMV lytic DNA matures, it is clear that viral encoded proteins may function in a regulatory as well as enzymatic role such as recognition and unwinding of a distinct structure within oriLyt. What this suggests is that replication proteins themselves may serve to control the onset of DNA synthesis by interacting with, or acting as *trans*-acting factors themselves.

Although the exact mechanism of initiation of HCMV lytic DNA synthesis remains unknown, much of the emphasis has been focused on the *cis*-acting sequences within oriLyt and the protein encoded by the UL84 open reading frame (ORF). UL84 is a unique protein that has no known homolog to any cellular or viral protein. UL84 is a multifunctional protein that is capable of regulating the transcriptional activation mediated by IE2, and evidence indicates that it is a member of the DExH/D-box family of proteins. This review will discuss (a) what is known about the *cis*-acting regions in oriLyt that contribute to DNA synthesis and (b) the properties of the *trans*-acting factors, specifically UL84, required for lytic DNA synthesis and how they relate to the regulation and initiation of HCMV lytic DNA replication.

Essential Region I: IE2-UL84 Responsive Promoter in oriLyt

The HCMV oriLyt region (Fig. 1) was defined according to the smallest fragments that supported amplification in the transient replication assay. The transient replication assay involves the transfection of oriLyt-containing plasmids into human fibroblasts followed by infection with HCMV. Total cellular DNA is harvested and cleaved with two restriction enzymes. The first cleaves input plasmid and cellular DNA. The second, DpnI, cleaves methylated (unreplicated) plasmid DNA. Plasmids propagated in bacterial Dam^+ methylase hosts will add a methyl group at an adenosine (which is one base within the DpnI recognition sequence). Once plasmid DNA goes through one round of semiconservative DNA synthesis in mammalian cells, it then becomes unmethylated, since mammalian cells lack an adenosine methylase. Replicated DNA is distinguished from input plasmid DNA based on the sensitivity or resistance to cleavage by DpnI where amplified plasmid DNA migrates more slowly through an agarose gel due to its larger size. Since DpnI has a 4-base pair recognition sequence, input unreplicated DNA migrates toward the bottom of the gel. This powerful assay allowed for the fine mapping of DNA sequences that are essential or contribute to amplification of oriLyt. These initial studies defined the oriLyt region between nts 90,504 and 93,930 on the AD169 genome.

Later studies identified a core domain for oriLyt that contained two essential regions (I and II). These essential regions were originally defined as those sequences that could not be mutated or deleted and still retain a functional oriLyt in transient assays (Zhu et al. 1998). Within essential region I, there are several DNA repeat elements and a highly prymidine-rich DNA sequence referred to as the Y-block (Zhu et al. 1998) as well as two 29-base pair repeat sequences that contain several transcription factor-binding sites. In addition, two IE2 binding sites are also present. The first is a consensus *cis* repression sequence (CRS) that was shown previously to interact with IE2 (Cherrington et al. 1991; Arlt et al. 1994; Tsai et al. 1997; Lashmit et al. 1998; Huang and Chen 2002). This consensus CRS element does not appear to have a functional role in oriLyt promoter activation/repression or DNA

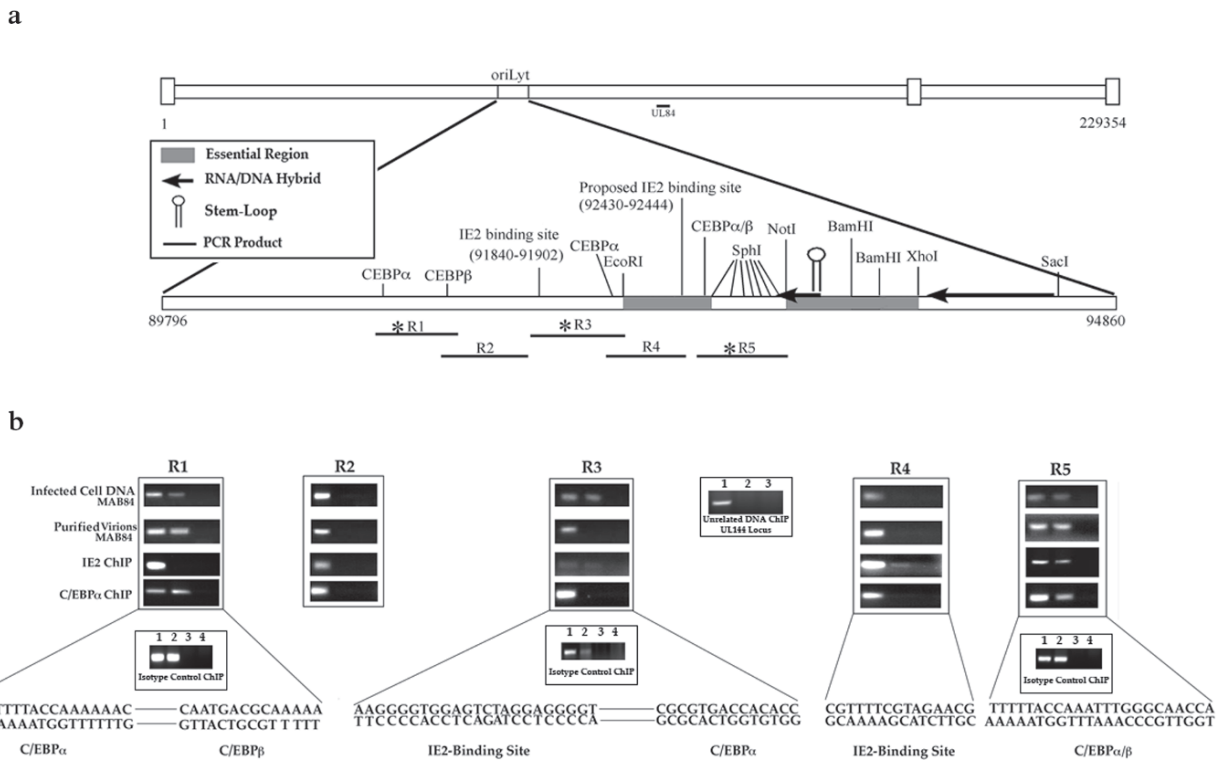

Fig. 1 *Cis*- and *trans*- acting factors interacting with HCMV oriLyt. **a** Schematic of HCMV oriLyt showing the relative position of two essential regions, C/EBPα transcription factor-binding sites, IE2-binding sites, vRNA and the RNA stem loop structure. **b** Chromatin immunoprecipitation (*ChIP*) assay showing the interaction of UL84, IE2 and C/EBPα with oriLyt. Lane one of each set is the PCR product from input DNA, lane two is the PCR product from immunoprecipitation with the anti-UL84 antibody and lane 3 is the PCR product from immunoprecipitations using an isotype matched unrelated antibody

synthesis since it can be deleted without affecting oriLyt amplification. A second IE2-binding site is nonconventional and was discovered using CASTing and SAAB methods (Huang and Chen 2002). This second site appears to be functional in that it was shown to interact with HCMV IE2 (IE86) in vivo using the chromatin immunoprecipitation assay (ChIP) (Xu et al. 2004b). In addition, the DNA sequence containing the nonconventional IE2-binding site was shown to have promoter activity, driving the expression of a luciferase reporter upon HCMV infection, or in the presence of UL84 and IE2 in transient assays in HFF cells (Xu et al. 2004b). The nonconventional IE2-binding site does contain a CRS-like element that is similar to those found in other IE2-responsive promoters, and this DNA motif was shown to be required for efficient promoter activity and oriLyt amplification. In the context of oriLyt, it appears that this promoter element may participate in the initial events surrounding the regulation of DNA synthesis and could serve as the trigger for initiation of lytic DNA synthesis. For example, one such scenario could be that when UL84 protein is produced it interacts with IE2 and facilitates the binding of the protein complex to oriLyt, thereby initiating transcription and signaling the onset of lytic DNA replication.

The presence of an IE2-UL84 responsive promoter suggested that transcription plays an important role in the activation of lytic DNA synthesis. This is not unlike other herpesviruses such as EBV and HHV-8, where promoter elements are part of lytic origins and their presence is essential for efficient oriLyt activity (Hammerschmidt and Sugden 1988; Aiyar et al. 1998; Lin et al. 2003; AuCoin et al. 2004; Wang et al. 2004). The HCMV oriLyt promoter element extends from AD169 nts 91495-92888, and appears to be bidirectional in that transcription driven in either direction can accommodate oriLyt amplification. Transient reporter assays demonstrated that either orientation of the oriLyt promoter region could drive the expression of luciferase. The activity of this promoter is cell type-dependent since transient transfection of luciferase reporter plasmids containing the oriLyt promoter were constitutively active in Vero cells, whereas transcriptional activation in human fibroblasts (HFs) was only achieved by subsequent viral infection or the cotransfection of IE2 and UL84. The constitutively active oriLyt promoter in Vero cells led to the observation that IE2 was not required for oriLyt amplification in the cotransfection replication assay in those cells (Sarisky and Hayward 1996). In HFs however, amplification of oriLyt requires both IE2 and UL84, presumably to activate the oriLyt promoter. Interestingly, when the cotransfection replication assay was performed in HFs constitutively expressing the catalytic subunit of telomerase, these life-expanded cells supported oriLyt amplification without the need for the proteins expressed from the UL36-38 loci. This suggests that in the context of the cotransfection replication assay, ORFs UL36-38 may serve to enhance cell survival.

In an effort to further define the role of transcription in HCMV oriLyt activation, a strong constitutive promoter was inserted in oriLyt in place of the defined oriLyt promoter (Xu et al. 2004b) in a manner similar to that done with the EBV oriLyt (Hammerschmidt and Sugden 1988). The SV40 early promoter was able to functionally substitute for the HCMV oriLyt promoter in transient assays and alleviated

the need for IE2, but not UL84, in the replication assay. This strongly suggested that transcription somehow triggers initiation of DNA synthesis. In addition, these experiments identified UL84 as the factor most likely to act as an initiator protein and, since the leftward region of oriLyt is a promoter, the rightward region must contain a substrate for direct or indirect UL84 interaction.

Essential Region II: RNA/DNA Hybrid Structure

In addition to the IE2-UL84 responsive promoter region found in essential region I, the downstream region of oriLyt contains RNA/DNA hybrid structures. These structures were discovered while investigating the presence of alkali-labile regions within the HCMV genome. This RNA/DNA hybrid region stretches from approximately the Not I site (nts 92888) to 300 bp upstream of the BamHI site (93513) (Prichard et al. 1998). Interestingly, this region of the origin is variably reiterated via tandem duplication in the laboratory strain AD169, resulting in about 300 bp of extra sequence in the viral genome. The RNA/DNA hybrids are present within the packaged viral genome and appear to be a stable part of the genome. The RNA/DNA hybrid region contains several G+C-rich repeat sequences, one of which has the capacity to form a stem loop configuration. Since this region can be repeated many times within the viral genome, this stem loop may also be amplified. Although the significance of the RNA/DNA hybrid and the stem loop remains to be seen, it has been postulated that essential region II may be the area of oriLyt that interacts directly with UL84. In vitro RNA binding assays determined that UL84 does interact with a synthetic RNA stem loop oligonucleotide that is the same sequence as that present within the RNA/DNA hybrid region of oriLyt. In addition, UL84 appears to change the conformation of this oligonucleotide in that the UL84-RNA stem loop complex migrates faster in a native gel when more UL84 protein is added. This yields a staircase pattern of binding observed only when RNA stem loop oligonucleotide substrates are used in the binding assay (Colletti et al. 2007).

With respect to UL84 interactions with oriLyt in the infected cell environment, our laboratory used the ChIP assay to demonstrate that UL84 does interact with the RNA/DNA hybrid region of oriLyt in infected cells, as well as in packaged virions. In addition, we demonstrated that UL84 interacts directly or indirectly with regions of oriLyt that contain a CAAT enhancer-binding site (C/EBPα). This interaction suggests that UL84 may cooperate with C/EBPα or use these consensus sequences to facilitate DNA synthesis. This scenario would be consistent with HHV-8 where K-bZIP interacts with C/EBPα and binds to these transcription factor-binding sites within HHV-8 oriLyt (Wang et al. 2003). Another plausible scenario is that UL84 uses the consensus C/EBPα binding sites independent of the C/EBPα transcription factor interacting with oriLyt. Although ChIP results do show C/EBPα binding to this region, we were unable to detect a UL84-C/EBPα protein interaction within infected cells.

Viral-Encoded *trans*-Acting Factors Required for Lytic Replication

Most of the information concerning the *trans*-acting factors involved in HCMV lytic DNA synthesis was elucidated from the cotransfection replication assay. This assay, which is similar to the assay that elucidated the HCMV cloned oriLyt only using a set of plasmids that encode putative replication factors, revealed that eleven loci were required to achieve efficient amplification of cloned oriLyt. The original assay used plasmid clones that expressed the putative replication proteins under the control of each of their native promoters. This meant that those factors that contributed to the efficient expression or activation of replication proteins were also identified. Therefore the complete set of enzymes involved in the mechanics of DNA synthesis, as well as any ancillary factors, were identified using this assay (Pari and Anders 1993; Pari et al. 1993).

Table 1 lists the ORFs identified from the initial cotransfection replication assay performed in human fibroblasts. Six of these genes identified are common to all herpesviruses and are designated the core replication proteins, which comprise a DNA polymerase, polymerase accessory protein, single-stranded DNA binding protein, helicase, primase and primase associated factor. Interestingly, efficient oriLyt-dependent DNA replication can occur when core proteins from one herpesvirus, for example EBV, is used with a different herpesvirus species oriLyt, for example HCMV, in the cotransfection replication assay (Sarisky and Hayward 1996; Xu et al. 2004b). In all cases, the only additional factor that is needed is the initiator protein unique to each herpesvirus. This fact suggests that a set of core enzymes can carry out DNA synthesis on a primed oriLyt substrate independent of the mechanism of initiation. It also suggests that the initial events in DNA synthesis can be performed independent of the enzymes involved in DNA replication, at least in the context of transient assays.

Table 1 Essential ORFs elucidated from the cotransfection-replication assay

Open reading frame	Proposed function
UL54	DNA polymerase
UL44	Pol accessory, binds to UL84
UL105	Helicase
UL70	Primase
UL102	Primase-associated factor
UL57	Single-stranded DNA binding
UL84	UTPase, RNA-binding within oriLyt, regulatory, shuttling protein
IRS1	RNA binding, transactivation
UL112/113	Early proteins, transactivation
UL36–38	Anti-apoptotic
IE2	Transactivation, binds to UL84

Many of the core replication proteins remain poorly characterized at the biochemical level with the exception of UL44 and UL54, which are the best studied of the DNA replication enzymes. UL44 in infected cells is very abundant and found in replication compartments in early and late time of infection. UL44 encodes the polymerase accessory factor and appears to form a C-shaped clamp that interacts with DNA with increased specificity when bound to UL54 (Loregian et al. 2004; Appleton et al. 2006). The C-terminal end of UL54 appears to be the point of interaction with UL44 and a small peptide corresponding to a portion of the UL54 protein efficiently interferes with binding to UL44 in vitro (Loregian et al. 2003). UL44 was shown to interact with UL54 in the cellular environment and this interaction can influence subcellular localization (Alvisi et al. 2006). The crystal structure of UL44 is known and its interaction with UL54 was shown to enhance the affinity of UL44 for DNA (Appleton et al. 2006). UL44 is a substrate for the HCMV-encoded protein kinase UL97 (Krosky et al. 2003) as well as other cellular encoded kinases. This suggests that UL44 activity or interaction with other viral/cellular proteins may be regulated by both viral as well as cellular kinases (Krosky et al. 2003).

UL54 was initially purified from insect cells along with UL44 and found to synthesize DNA in vitro (Ertl and Powell 1992; Ertl et al. 1994). Expression of UL54 is regulated by several different promoter elements depending on the time of infection. In addition, it appears that several regulatory proteins contribute to efficient expression of UL54 in the context of the virus genome (Kerry et al. 1994, 1996).

The helicase-primase complex, composed of UL105, UL70 and UL102, has been studied using recombinant expression systems as well as limited characterization and identification from infected cells (Smith and Pari 1995; Smith et al. 1996). Although this group of proteins comprises the proposed counterparts of the herpes simplex helicase primase proteins, no data has been published demonstrating helicase or primase activity in vitro. Elucidation of the transcription units for UL102 and UL105 revealed that these transcripts are unspliced and approximately 2.7 and 3.4 kb, respectively (Smith and Pari 1995; Smith et al. 1996). The UL105 protein product is a 110-kDa species that is present at early times postinfection. It was demonstrated that all three proteins form a complex and each member of the complex is in contact with every other protein (McMahon and Anders 2002).

HCMV UL57 encodes the single-stranded DNA-binding protein. Initial studies show that UL57 encodes a 140-kDa protein that is confined to the nucleus and associates with replication compartments (Penfold and Mocarski 1997). It is assumed that UL57 acts to facilitate strand separation during DNA synthesis. Interestingly, the HSV-1 counterpart, UL29, was shown to exhibit RNA-binding and aid in the formation of R-loops and participate in helix destabilization (Boehmer 2004).

The proteins encoded by the UL112-113 locus were required in the initial cotransfection replication assay and appear to act as transcriptional enhancers. It was recently demonstrated that the UL112-113 proteins may influence the intranuclear targeting of UL44 to foci involved in DNA replication (Park et al. 2006).

The IRS1/TRS1 locus encode proteins that are part of the HCMV virion. At least one locus is required for viral growth and a deletion of TRS1 results in a decrease in production of infectious virus (Romanowski et al. 1997; Blankenship and Shenk

2002). TRS1 was shown to interact with dsRNA and both IRS1 and TRS1 can block the host protein shutoff response mediated by HSV-1 (Child et al. 2004; Cassady 2005; Hakki and Geballe 2005). The role of IRS1/TRS1 in the initial cotransfection replication assay was assumed to be that of a transcriptional activator based on the findings that TRS1 and IE2 enhanced the expression of UL44 in transient assays (Stasiak and Mocarski 1992; Colberg-Poley 1996).

UL84 and IE2

Since the initial elucidation of the *trans*-acting factors required for oriLyt-dependent DNA replication, the most complex interaction is that of UL84 and IE2. This interaction has become the focus of much of the research with respect to the characterization of a dual assignment for UL84 as a replication and regulatory factor for gene expression. UL84 was first described as the product of a 1,761-bp ORF encoding a 65-kDa protein (He et al. 1992). The mRNA transcript encoding UL84 can be detected as early as 2.5 h postinfection and the protein product is clearly detectable at about 20 h postinfection. Later studies showed that UL84 was found to be associated with IE2 in infected cells (Spector and Tevethia 1994). This association can be found throughout the virus infectious cycle and the only time point where UL84 is not found bound to IE2 is at very early times after infection before the production of UL84. At this time, it is not clear how much of the UL84 or IE2 within infected cells is part of the complex. This is an important point since UL84 has a regulatory effect on IE2-mediated transactivation. Transient assays show that at least some IE2-responsive promoters can be efficiently silenced by the addition of a UL84 expression plasmid (Gebert et al. 1997). The overexpression of UL84 prior to HCMV infection leads to the complete shut down of virus replication (Gebert et al. 1997). This phenomenon is thought to arise from the ability of UL84 to suppress the transactivation function of IE2; however, other mechanisms for inhibition of virus replication are possible.

In the context of the virus genome, UL84 is required for efficient viral replication and regulated gene expression (Xu et al. 2004a). A recombinant HCMV BAC with UL84 deleted results in an aberrant gene expression pattern, especially with respect to late gene transcription. This suggests that UL84 may regulate the effects of IE2, as implicated by the results of the transient reporter assays. In addition, lack of UL84 expression resulted in failure of the formation of replication compartments in infected cells, also consistent with what was observed in transient assays (Sarisky and Hayward 1996; Xu et al. 2004a).

UL84 was shown be a phosphorylated protein and to exhibit UTPase activity in vitro. This activity occurred independent of the presence of DNA or RNA (Colletti et al. 2005). This utilization of UTP suggests the use of an energy-generating system for an as yet unidentified enzymatic activity for UL84. UL84 contains amino acid motifs that are consistent with it belonging to the DExH/D Box family of proteins. This class of proteins has a broad range of activities. DExH/D Box

proteins interact with the RNA component of ribonucleoproteins, unwind RNA, shuttle RNA from the nucleus to the cytoplasm, can either up- or downregulate certain promoters, and can directly and actively displace stably bound proteins from RNA (Fairman et al. 2004; Jankowsky and Bowers 2006). There is overwhelming functional evidence that UL84 performs many of the activities similar to those described for DExH/D Box proteins. One of the most interesting features of UL84, mentioned above, also consistent with DExH/D Box protein activity, is that it was shown to shuttle between the nucleus and the cytoplasm (Lischka et al. 2006). This activity serves presumably to increase the accumulation of mRNA in the cytoplasm, although no substrate RNAs were identified. This novel shuttling property of UL84 suggests that UL84 can function in some as yet undefined role in the cytoplasm. This role may involve the export of viral or cellular encoded RNA in order to increase the steady state level of an essential protein.

Viral and Cellular Encoded UL84 Binding Partners

Although it is known that UL84 interacts with IE2, other binding partners have been identified. Our laboratory has performed a proteomics analysis to identify other potential binding partners for UL84 in infected cells. This analysis identified HCMV UL44 and UL83 (pp65) as viral encoded factors that interact with UL84 (Y. Gao et al., 2008). Our analysis also identified CK2 alpha and beta subunits, as well as the cellular protein importin. These results are interesting in that this is the first time that UL84 has been shown to interact with a member of the viral DNA replication machinery as well as a protein (pp65) that is a component of the virion. Additionally, although it was previously reported that UL84 is phosphorylated in infected cells, this result strongly suggests that UL84 is a substrate for casein kinase II (CK2).

Another interesting finding from the proteomics analysis is that UL84 is ubiquitinated in infected cells. This was also discovered in the proteomics analysis of UL84, which revealed two protein spots for UL84 in the 2D gel. Although molecular weights of these two UL84 protein species varied only slightly, their respective pI indicated that one was more acidic than the other. Protein sequence data from one UL84 spot showed that ubiquitin E2-conjugating enzyme was also present. Further analysis using the proteosome inhibitor MG132 demonstrated that UL84 was monoubiquitinated. This apparent mono ubiquitination implicates UL84 as a factor that may influence the degradation of other proteins or may be involved in a multistep regulated degradation pathway.

The interaction of UL84 with UL44 (Pol accessory protein) is very interesting in that the initiator protein for herpes simplex virus type 1 (HSV-1), UL9, also interacts with its Pol accessory protein (Trego and Parris 2003). It is postulated that UL42 increases the ability of UL9 to load on DNA (Trego et al. 2005), a scenario that is also plausible for UL84 with respect to its interaction with UL44. The finding that UL44 interacts with UL84 opens up the possibility that UL44 may have a

dual role in HCMV DNA replication. UL44 may act early in the virus life cycle at the initiation of DNA synthesis and also later in its traditional role as a processivity factor along with UL54 at the replication fork.

Summary and Perspectives

HCMV lytic DNA replication is complex and evidence supports a reliance on several viral encoded regulatory proteins for the initiation of DNA synthesis. The preponderance of the evidence points to one key factor, UL84, as a multifunctional protein that has a central role in lytic DNA replication and is involved with both the *cis*- and *trans*-acting replication machinery. UL84 facilitates lytic replication by binding to and regulating the transcriptional activation of IE2 as well as interacting with DNA and RNA motifs within HCMV oriLyt. Indeed our initial understanding of IE2, solely as a transcriptional activator, must be redefined to reflect the fact that, in the cellular environment, UL84 is bound to IE2 and this interaction may influence the activity of both proteins. This IE2-UL84 proteome performs a complex regulatory role controlling viral gene expression as well as defining the timing of initiation of viral lytic DNA replication. Additionally, the interaction of UL84 with other viral and cellular encoded proteins opens up the possibility that replication, regulation of gene expression and RNA transport all act together to orchestrate the onset and timing of viral DNA synthesis.

We are beginning to have a better understanding of HCMV lytic DNA replication; however, the mechanism of initiation of DNA synthesis remains elusive. The questions of how the precise orchestration, assembly and regulation of all the participants necessary to initiate DNA synthesis are still unanswered. It is clear that many factors contribute to the overall mechanism and the function of these factors will be the focus of future studies.

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