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FUNCTIONAL ANALYSIS OF ACCESSORY FACTORS INVOLVED IN HUMAN CYTOMEGALOVIRUS DNA REPLICATION

A DISSERTATION
SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY AND
IMMUNOLOGY AND THE COMMITTEE ON GRADUATE STUDIES
OF STANFORD UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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Edward S. Mocarski, Jr.

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Ann M. Arvin

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Mark Krasnow

Approved for the University Committee on Graduate Studies:

Thomas Wasow

ABSTRACT

The mechanism involved in the initiation of human cytomegalovirus (HCMV) DNA replication and the switch for late phase viral DNA amplification are unknown. To understand the early events in HCMV DNA replication, I undertook a detailed characterization of a mutant in the HCMV uracil DNA glycosylase (UDG), UL114 and constructed a mutant in the HCMV core replication function, UL44. RC2620, made by Prichard *et al.*, exhibited a prolonged growth cycle corresponding to a restriction prior to the start of viral DNA replication, leading to the suggestion that UL114 was required for efficient viral DNA synthesis. I found that the defect in RC2620 growth was most dramatic in cells which were confluent. The requirement for the UL114 gene product was completely supplanted when cells were actively growing at the time of infection and correlated with the expression of the human UDG gene. Human UDG expression is induced by HCMV infection at times correlating with the onset of DNA synthesis in the mutant, implicating the human enzyme in the complementation of RC2620. To determine whether UL114 is required for uracil excision, I examined the genomic integrity of HCMV during infection. I found that the frequency of uracil incorporation into mutant virus particles was similar to that observed for wild-type virus particles, suggesting that UDG activity is not required prior to initiation of DNA replication but at a later step. Interestingly, wild-type HCMV incorporates uracils into its genome at 72 hpi leading to dramatic viral DNA amplification, while the UL114 mutant is unable to begin large scale DNA replication and does not incorporate uracil. These data suggest a role for UL114 in initiation of late phase DNA amplification through excision of uracils incorporated in the early rounds of DNA replication. Finally, to understand the role of the putative HCMV polymerase processivity factor (UL44) during infection I attempted to construct a mutant in this gene. I was unable to purify the UL44 mutant away from wild-type virus despite use of a UL44-expressing cell line.

ACKNOWLEDGEMENTS

I am indebted to a great number of individuals for their advise, support, encouragment and active participation. I would like to thank my adviser Edward Mocarski for providing an environment friendly to new ways of addressing scientific problems and for providing the growing room needed for my development as an independent researcher. I greatly appreciate the enjoyable discussions I had with my thesis committe, Ann Arvin, Karla Kirkegaard and Mark Krasnow, and thank them for their advise and support which were instrumental in the evolution of my thesis. I would like to thank my fellow Mocarskiites for their active interest in my project, for all their helpful comments and suggestions and for making the Mocarski lab a fun, interesting place to spend my graduate career. I am deeply indebted to Mark Prichard for laying down a solid foundation for a great proportion of my thesis work and for his mentoring from the time that I started as a rotation student in Ed's lab. My deepest thanks go to Shinya Watanabe, Dana Wolf and Louise McCormick for their generous spirits, the wonderfully stimulating intellectual discussions, the constant reminders that "Science is Fun!" and most importantly, for their friendship.

I consider myself fortunate to have spent my graduate career surrounded by friends. In particular, I am deeply grateful to my good friends, Diane McFadden and Anthea Lee, for the commiseration, laughter and trips to various food establishments. I would also like to thank Denise Monack and Amy Clewell for the exercise breaks which were important for my stress management.

I would like to thank my parents for their support, their constant encouragement in my academic pursuits and for all those children's science books which sparked my imagination and curiosity about the world around me.

Finally, I would like to thank my best friend, my husband, Justin for his love, support, words of encouragement and for his unwavering faith in me. His companionship has made the good times better and the hard times easier.

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Chapter 1: Introduction

The herpesvirus family infects a wide variey of animal species including man. Members of this family share many biological properties, including characteristics of their virion and genome structure, and the ability to establish lifelong persistant and latent infections. Eight human herpesviruses have been identified to date, herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus, human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7) and human herpesvirus 8 (HHV-8 or KSHV).

There are three subgroups within the herpesvirus family, α, β, and γ, classified according to host range, length of infectious cycle and sites of latency (Roizman *et al.*, 1981). Cytomegaloviruses (CMVs) are the prototype members of the betaherpesvirus family. This subgroup is defined by a narrow host range, restricted tissue tropism and slow replication cycle. In the past decade, sequence alignment between herpesvirus genomes has also allowed assignment of HHV-6 and HHV-7 to the betaherpesvirus family (Efstathiou *et al.*, 1988; Frenkel *et al.*, 1990; Lawrence *et al.*, 1990; Littler *et al.*, 1990).

Cytomegalovirus Infection and Clinical Significance

CMV derives its name from the characteristic cytopathic effect induced by this virus — namely cell enlargement with the presence of intranuclear inclusions (Alford and Britt, 1995). Human CMV (HCMV) is a ubiquitous pathogen that infects 40-80% of the population worldwide with a higher incidence in underdeveloped nations and a lower incidence in most developed nations (Gold and Nankervis, 1982; Reynolds *et al.*, 1981). Healthy individuals infected with HCMV infrequently present with clinically apparent disease and the host immune response involving both cellular immunity and antibody response is thought to provide a protective effect (Rasmussen, 1990; Riddell and Greenberg, 1997). Importantly, virus secretion in various bodily fluids including breast milk, urine and saliva persists for an extended time following primary infection (Reynolds *et al.*, 1973; Stagno *et al.*, 1980). This prolonged shedding of virus is an important source for virus transmission among healthy and immunocompromised individuals (Alford and Britt, 1995).

While infection in healthy individuals rarely causes disease, HCMV infection is severe and even life-threatening to neonates and immunocompromised individuals. It has been estimated that approximately

1% of infants born in the United States are infected with HCMV *in utero* (Stagno *et al.,* 1983; Stagno *et al.,* 1982). As such, HCMV is the most common congenital viral infection among those born in this country (Alford and Britt, 1995). Greater than 90% of these infected neonates are born with no apparent disease (Stagno *et al.,* 1986; Stagno *et al.,* 1982). The severity of the clinical outcome seems most closely associated with viral load and gestational age at the time of infection (Alford and Britt, 1995). Human CMV congenital infection has long-reaching effects extending past the time of birth. Many affected infants display lasting neurological disease with about 10% showing substantial loss of hearing and IQ within the first few years of life (Hanshaw *et al.,* 1976).

In addition to neonates, individuals undergoing immunosuppressive therapy for organ and bone marrow transplantation are at a great risk of developing HCMV-related disease. In solid organ transplantation cases, HCMV disease arises from primary infection or reactivation of latent virus from transplanted tissue and results in increased graft rejection, decreased graft survival independent of rejection, pneumonia and increased risk to bacterial, fungal and protozoal secondary infections (Alford and Britt, 1995; Rand *et al.,* 1978; Vierling and Fennell, 1985).

Efforts to treat HCMV have met with limited success. Current antiviral therapies including the HCMV DNA polymerase inhibitor phosphonoformic acid (foscarnet) and the nucleoside analog, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG/ganciclovir) have been efficacious; however, both drugs are associated with considerable toxicity. Additionally, the appearance of drug resistant strains of HCMV has become common in immunocompromised individuals undergoing long-term treatment with foscarnet and ganciclovir (Alford and Britt, 1995). Additional antiviral compounds are currently under investigation as possible HCMV therapies. Finally, the search for a potent HCMV vaccine is ongoing. Increased understanding of HCMV genes essential for the replication of this virus may provide valuable information for the development of a live attenuated vaccine.

Genome Structure

The human CMV genome is a 230 kilobase linear double-stranded DNA molecule with at least 200 genes based on open reading frame analysis

(Chee *et al.*, 1990; Mocarski, 1995). The HCMV genome consists of a complex arrangement of unique and repeated regions (Mocarski, 1995). The two unique regions of HCMV, U_L (unique long) and U_S (unique short), are flanked by repeated *a*, *b* and *c* sequences. Varying copies of the *a* sequence are found as direct repeats at the termini of the HCMV genome. These direct repeats contain cleavage and packaging signals and are important for assembly of the HCMV genome into mature virions (Kemble and Mocarski, 1989; Mocarski *et al.*, 1987; Spaete and Mocarski, 1985). The *a* sequences also facilitate recombination and as a consequence of this, HCMV exists as a population of four equimolar isomers produced by inversion of the U_L and U_S regions relative to one another (Mocarski, 1995).

Overview of Events in HCMV Infection

Many parallels can be drawn between the HCMV and HSV life cycles. In the lytic phase of infection, both viruses attach to cell surface receptors via virion glycoproteins, resulting in fusion between the lipid membranes (Compton *et al.*, 1992; Roizman and Sears, 1995). Naked virions are translocated to the nucleus where viral DNA is delivered. Following nuclear penetration, HCMV viral gene expression is activated as a coordinately regulated temporal cascade that can be divided into at least three kinetic classes, immediate early, early and late (Chua *et al.*, 1981; DeMarchi *et al.*, 1980; McDonough and Spector, 1983; McDonough *et al.*, 1985; Wathen and Stinski, 1982; Wathen *et al.*, 1981).

Similar to other DNA viruses, HCMV DNA is deposited to distinct sites within the infected cell nucleus following nuclear entry (Ahn and Hayward, 1997; Ishov and Maul, 1996; Ishov *et al.*, 1997; Maul *et al.*, 1996). These nuclear regions, known as nuclear domain 10 (ND10), contain several cellular proteins including PML and SP100. ND10 contain components of the mRNA splicing machinery, including the spliceosome assembly factor (Fu and Maniatis, 1990; Spector, 1990). HCMV immediate early gene transcription appears to initiate at the periphery of these nuclear bodies (Ishov *et al.*, 1997).

Immediate early gene expression has been mapped to a total of five regions in the HCMV genome including *ie1*/*ie2*, TRS1/IRS1, UL36-38, UL69 and US3 (Stamminger and Fleckenstein, 1990; Stasiak and Mocarski, 1992; Stenberg *et al.*, 1989; Stenberg *et al.*, 1984; Stenberg *et al.*, 1985; Weston, 1988). This class of viral genes is expressed within a few hours of viral infection independent of *de novo* protein synthesis. The predominant transcripts expressed within this class arise from the major immediate early gene locus (Stenberg *et al.*, 1984; Stenberg *et al.*, 1985). The gene products encoded in this region, *ie*1 and *ie*2, are expressed abundantly from a set of differentially spliced transcripts (Akrigg *et al.*, 1985; Boshart *et al.*, 1985; Stenberg *et al.*, 1989; Stenberg and Stinski, 1985; Stenberg *et al.*, 1984; Stenberg *et al.*, 1985; Thomsen *et al.*, 1984). IE1 and IE2, along with the other immediate early gene products TRS1/IRS1 and some of the proteins encoded by UL36-38 region, serve as viral transactivators for expression of early and late viral genes (Mocarski, 1995).

Expression of the early genes of HCMV requires synthesis of functional immediate early proteins. Gene products encoded by this class of genes include proteins involved in replication of the HCMV genome and DNA metabolism (Mocarski, 1993; Mocarski, 1995). Following the accumulation of replication proteins, viral DNA synthesis is initiated, with the majority of HCMV DNA replicated during the late stage of infection (Morin *et al.*, 1996).

The HCMV early and true late genes are distinguished based on sensitivity of viral gene expression to DNA replication inhibitors. True late gene expression begins only after the onset of DNA replication (Stinski, 1978). The late genes of HCMV encode the structural proteins of the virion as well as packaging functions. Once viral DNA is replicated, packaging of the full-length HCMV genome into preformed nucleocapsids proceeds. *Cis* signals present in the termini of the HCMV genome mediate the proper cleavage and packaging of the newly synthesized viral DNA in a manner likely similar to HSV-1 (Booy *et al.*, 1991; Kemble and Mocarski, 1989; Mocarski, 1995; Mocarski *et al.*, 1987; Spaete and Mocarski, 1985). Following packaging, the full nucleocapsid matures into progeny virion by an as yet poorly understood mechanism.

HCMV DNA Replication

Much of our understanding of HCMV DNA replication comes from transient replication studies and by analogy to HSV-1 replication. As in HSV (Roizman and Sears, 1995), the HCMV genome circularizes very early in infection and is found in an "endless" configuration (LaFemina and Hayward, 1983). This circularization process is most likely mediated by joining or recombination between the repeat sequences found at the termini of the HCMV genome.

The requirements for initiation of viral DNA synthesis and the mechanism for transition to late phase DNA amplification are two areas of herpesvirus replication that are still poorly understood. Herpesvirus DNA replication is thought to occur as a biphasic process (Igarashi *et al.*, 1993; Lehman and Boehmer, 1999; Roizman *et al.*, 1965; St Jeor and Hutt, 1977; Stinski, 1978) with the bulk of DNA replicated during the late stages of infection (Igarashi *et al.*, 1993; Morin *et al.*, 1996). The structure of replicating DNA has been inferred by restriction mapping and by detection of long concatameric DNA. Based on these studies, some researchers have proposed a model in which early phase herpesvirus DNA replication occurs by *theta* mode. Although *theta* forms have never been isolated, evidence that HSV-1 replication requires circularization and initiates from an origin of replication following recognition by an initiator protein are consistent with this idea (Lehman and Boehmer, 1999). At later times of infection, HSV-1 DNA replication switches to a rolling circle process by a heretofore unknown mechanism (Ben-Porat and Tokazewski, 1977; Jacob *et al.*, 1979; Lehman and Boehmer, 1999; Roizman and Sears, 1995). This process is thought to be analogous to the replication mode observed in the late phases of bacteriophage λ infection (Enquist and Skalka, 1973). However, the form of herpesvirus replication may be more complex than rolling circle as replicating HSV-1 DNA has been found as a branched structure by electron microscopy (Fig. 1.1) (Severini *et al.*, 1994; Shlomai *et al.*, 1976). A simple rolling circle process cannot explain the rapid, large scale DNA amplification observed in late phase herpesvirus replication. In addition, it has been demonstrated that recombination is intimately associated with herpesvirus replication (Dutch *et al.*, 1992; Sarisky and Weber, 1994; Zhang *et al.*, 1994). Thus, some researchers have proposed that herpesvirus replication is a recombinagenic process (Goldstein and Weller, 1998; Martinez *et al.*, 1996; Severini *et al.*, 1994; Severini *et al.*, 1996). According to such a model, breaks in herpesvirus DNA allow strand invasion and the generation of branched replication intermediates. Importantly, this same strand invasion may also initiate recombination-dependent herpesvirus DNA replication at multiple sites of the viral genome in a manner analogous to late phase replication in bacteriophage T4 (Mosig, 1998). Human CMV DNA replication is predicted to proceed through these same replicative intermediates based on its shared properties with HSV-1 (Hamzeh *et al.*, 1990; LaFemina and Hayward, 1983; McVoy and Adler, 1994; Pari and Anders, 1993; Sarisky and Hayward, 1996).

Human CMV encodes viral functions that impact on the growth cycle of infected cells and thus creates an environment favorable to virus replication. Human CMV DNA replication occurs independently of cellular DNA synthesis (DeMarchi and Kaplan, 1976). To ensure its survival, HCMV induces cellular factors including cyclin E and cyclin E kinase that are associated with cell cycle progression to late G_1 phase (Bresnahan *et al.*, 1996; Jault *et al.*, 1995). HCMV activates enzymes associated with cellular proliferation such as proliferating cell nuclear antigen (PCNA) (Dittmer and Mocarski, 1997), proteins involved in nucleotide metabolism like thymidine kinase and dihydrofolate reductase (Estes and Huang, 1977; Wade *et al.*, 1992) and completes its subversion of the cell cycle by stimulating expression of p53 (Muganda *et al.*, 1994; Speir *et al.*, 1994). Thus, HCMV selectively primes the host cell to produce conditions favorable for DNA synthesis — increased nucleotide pools and cellular replication functions —and then subsequently blocks host cell entry into S phase (Bresnahan *et al.*, 1996; Dittmer and Mocarski, 1997; Lu and Shenk, 1996). This induced cell cycle dysfunction may serve to set up a host cell environment in which HCMV DNA is preferentially replicated (Albrecht *et al.*, 1989; Ihara *et al.*, 1980; Morin *et al.*, 1996).

The HCMV genome contains a single origin of replication located within the U_L region and adjacent to the open reading frame encoding the single-stranded DNA binding protein (Anders *et al.*, 1992; Anders and Punturieri, 1991; Hamzeh *et al.*, 1990; Masse *et al.*, 1992). The close pairing of this *cis*-acting replication element with the single-stranded DNA binding protein is analogous to the arrangement observed for HSV-1 ori_L (Spaete and Frenkel, 1985). Using a DNA chain termination method, Hamzeh et al. (Hamzeh *et al.*, 1990) found that HCMV DNA replication proceeded bidirectionally beginning from this region, demonstrating that this sequence functions as a classical origin of replication (Huberman and Riggs, 1968; Huberman and Tsai, 1973). The region containing HCMV oriLyt contains many repeated and inverted sequences, as well as binding sites for cellular transcription factors (Anders *et al.*, 1992; Hamzeh *et al.*, 1990; Masse *et al.*, 1992). In contrast to the HSV-1 origin, HCMV oriLyt does not have a recognizable central A+T-rich region (Anders *et al.*, 1992; Masse *et al.*, 1992). It has been hypothesized that the presence of a A+T-rich sequence in the HSV-1 origin allows initial unwinding by the origin-binding protein and subsequent loading of DNA replication machinery (Lehman and Boehmer, 1999). The difference in the structure of the HCMV and HSV-1 origins leaves open the possibility that HCMV initiates DNA replication differently than HSV-1. A further difference between these viruses is the finding that HSV-1 contains three origins of replication (Roizman and Sears, 1995) compared with a single identified origin sequence in HCMV.

The identification of the HCMV minimal origin sequence (Anders *et al.*, 1992; Anders and Punturieri, 1991; Hamzeh *et al.*, 1990; Masse *et al.*, 1992; Watanabe and Yamaguchi, 1993) led to the development of a transient DNA replication assay and the description of *trans*-acting factors required for HCMV DNA synthesis (Pari and Anders, 1993; Pari *et al.*, 1993). A total of eleven HCMV loci were found to be required for replication of a plasmid bearing the minimal HCMV origin (Pari and Anders, 1993). Six of these loci were predicted based on functional conservation with known HSV-1 replication fork proteins (Coen, 1996; Roizman and Sears, 1995). These include the viral DNA polymerase (UL54), the DNA polymerase accessory factor (UL44), the single-stranded DNA binding protein (UL57) and the three subunit helicase-primase complex (UL70, UL102 and UL105).

In addition to these core replication proteins, three known regulatory functions were identified by this transient assay, IE1/IE2, UL36-38 and TRS1/IRS1. These are believed to be required for proper expression of the replication fork proteins, although a similar requirement for viral transactivators was not found for HSV-1 (Roizman and Sears, 1995). It is very likely that these proteins were uncovered as a result of the experimental system used; analysis of a HCMV IE1 mutant has demonstrated that this gene, at least, is dispensible for viral replication in tissue culture (Greaves and Mocarski, 1998; Mocarski *et al.*, 1996). Another of these identified genes, UL37, seems to be required as an anti-apoptotic function (Goldmacher *et al.*, 1999). Thus, the UL37 product may have been identified on the basis of its ability to counteract IE2 induction of p53 — and hence apoptosis — in transfected cells, rather than as a true replication protein. Lastly, two loci of unknown function (UL84, UL112-113) were also implicated as *trans*-acting factors for HCMV DNA synthesis.

Interestingly, a HCMV gene corresponding to the origin-binding protein of HSV-1 (Elias and Lehman, 1988; Elias *et al.*, 1986; Lehman and Boehmer, 1999; Olivo *et al.*, 1988; Roizman and Sears, 1995) or the betaherpesviruses, HHV-6 (Gompels *et al.*, 1995) and HHV-7 (Nicholas, 1996) has not been found. Some researchers have suggested that the product of the UL84 gene serves as an initiator protein. UL84 alone was found to facilitate the start of replication from the HCMV oriLyt with addition of replication fork proteins from Epstein-Barr virus (Sarisky and Hayward, 1996). However, the UL84 product was unable to initiate oriLyt-dependent replication with the HCMV core replication machinery in the same assay, implying that other factors are involved in initiation. Given that the mechanism of initiation is not known for herpesviruses, it is also possible that initiation of HCMV DNA replication may occur by a process independent of origin binding by an initiator protein. The origin sequence of HCMV differs greatly from HSV-1, consisting of multiple cellular transcription factor binding sites but no central A+T-rich region (Anders *et al.*, 1992; Hamzeh *et al.*, 1990). This observation suggests that early rounds of HCMV DNA synthesis may initiate by a transcription-dependent process similar to T4 bacteriophage (Mosig and Colowick, 1995) instead of through unwinding by an origin-binding protein. HCMV virions have been found to carry a stable RNA-DNA hybrid species mapping within the HCMV origin of replication (Prichard *et al.*, 1998) and a number of transcripts have also been identified within this region (Huang *et al.*, 1996; Prichard *et al.*, 1998), consistent with the idea that transcription plays a role in the initiation of HCMV DNA synthesis.

The single-stranded DNA binding protein of HCMV is encoded by the gene UL57 (Anders and Gibson, 1988; Kemble *et al.*, 1987). The product of UL57 is a 135 kDa protein that is expressed with early gene kinetics and binds to single-stranded DNA with high affinity (Anders and Gibson, 1988; Anders *et al.*, 1986; Kemble *et al.*, 1987). In addition, the UL57 product colocalizes with DNA in subnuclear regions of HCMV-infected cells similar to ICP-8, its functional homolog in HSV-1 (de Bruyn Kops and Knipe, 1988; Kemble *et al.*, 1987; Penfold and Mocarski, 1997; Quinlan *et al.*, 1984; Rixon *et al.*, 1983). By analogy to HSV-1 DNA replication, the product of UL57 is predicted to prevent reannealing of DNA strands following unwinding by the putative HCMV helicase-primase complex, UL70-UL102-UL105 (Lehman and Boehmer, 1999).

Human CMV encodes a DNA-dependent viral DNA polymerase, UL54, that is the target of antiviral drugs currently in use. Based on *in vitro* assays, UL54 exhibits activities predicted for a DNA polymerase including 3' → 5' exonuclease activity and sensitivity to deoxyribonucleoside analogs (Huang, 1975a; Huang, 1975b; Nishiyama *et al.*, 1983; Wahren *et al.*, 1985). The HCMV DNA polymerase associates with a double-stranded DNA binding protein, UL44, in a one-to-one ratio (Ertl and Powell, 1992). Biochemical assays have suggested that UL44 is required to bind double-stranded DNA and stimulate UL54 polymerase activity by increasing processivity of this enzyme (Weiland *et al.*, 1994). Human CMV UL44 is, therefore, the functional homolog of the HSV-1 polymerase processivity factor, UL42. The sequences of HCMV UL44 and HSV-1 UL42 diverge greatly at the amino acid level and direct comparisons between these two proteins cannot be made. Furthermore, UL44 may have different sequence requirements for activity than UL42. While the carboxy-terminus (C-terminus) of HSV-1 UL42 and its alphaherpesvirus homologs is divergent, the C-terminus of HCMV UL44 and other betaherpesvirus homologs is highly conserved. Thus, the functional domains of HCMV UL44 and the role(s) of this protein during HCMV infection remain to be determined.

Human CMV DNA synthesis takes place in distinct subnuclear regions within the infected cell. During infection, the HCMV replication functions, UL44 and UL57, follow an ordered progression of localization patterns ending in the formation of large, globular intranuclear structures that bear resemblance to HSV-1 replication compartments (shown in Fig. 1.2) (de Bruyn Kops and Knipe, 1988; Liptak *et al.*, 1996; Lukonis *et al.*, 1997; Penfold and Mocarski, 1997; Quinlan *et al.*, 1984; Rixon *et al.*, 1983; Uprichard and Knipe, 1997). These mature subnuclear domains are sites of HCMV DNA replication based on patterns of bromodeoxyuridine (BrdU) incorporation (Penfold and Mocarski, 1997). Interestingly, the products of UL112-113 localize into replication compartment precursors earlier than any of the known HCMV replication fork proteins, implicating the UL112-113 proteins in the seminal steps of replication compartment assembly.

As described above, herpesviruses deposit their DNA into preexisting sites in the nucleus called ND10. The finding that certain ND10-associated proteins are located in HSV-1 replication compartments has led to the suggestion that ND10 may be precursors of HSV-1 DNA replication compartments (Burkham *et al.*, 1998; Lukonis *et al.*, 1997; Puvion-Dutilleul *et al.*, 1995; Uprichard and Knipe, 1997). It is unclear whether HCMV replication compartments are formed from these same nuclear subdomains. However, the major HCMV transactivator proteins, IE1 and IE2, are known to disrupt ND10 and reorganize the protein composition of this compartment in a manner analogous to the restructuring of these nuclear bodies by the HSV-1 transactivator, ICP0 (Ahn and Hayward, 1997; Dyck *et al.*, 1994; Everett *et al.*, 1998; Everett and Maul, 1994; Kelly *et al.*, 1995; Korioth *et al.*, 1996; Maul and Everett, 1994).

The early precursors of herpesvirus replication compartments bear striking resemblance to the punctate nuclear structures in which eukaryotic DNA replication occurs (Laskey and Madine, 1996). Indeed, some of these early foci have been shown to be sites of cellular DNA synthesis in cells infected with HSV-1 (Lukonis *et al.*, 1997; Uprichard and Knipe, 1997). BrdU pulse-chase studies have demonstrated that a portion of these punctate foci are also sites of HCMV DNA synthesis (Penfold and Mocarski, 1997). Interestingly, low level HCMV DNA synthesis is still detected in the presence of viral DNA polymerase inhibitors (Morin *et al.*, 1996; Penfold and Mocarski, 1997). Thus, the localization of viral DNA replication to known sites of cellular DNA synthesis may represent an early phase of HCMV DNA amplification that is dependent on cellular replication factors and independent of viral replication proteins.

In addition to the core replication machinery, HCMV carries a set of enzymes involved in nucleotide metabolism and DNA repair. These include a ribonucleotide reductase, deoxyuridine triphosphatase, deoxyribonuclease and uracil DNA glycosylase (UDG). At least one of these open reading frames, the HCMV UDG, encoded by UL114 is required for efficient DNA replication of this virus (Prichard *et al.*, 1996).

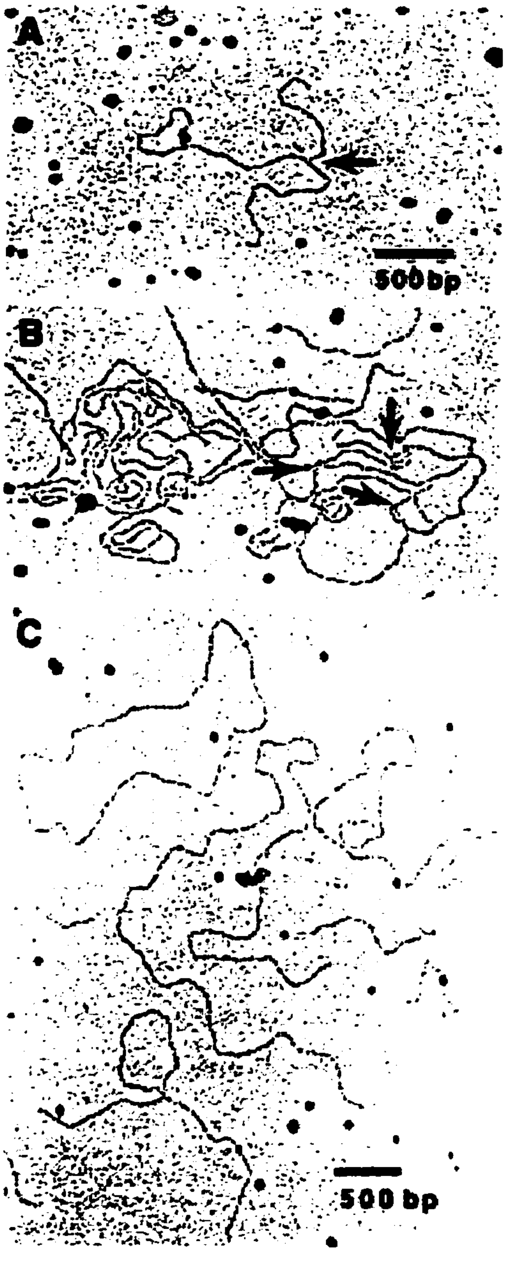
UL114 was not identified in the initial screen for replication proteins using transient replication assays, but a HCMV mutant in UL114 was found to have a prolonged growth cycle corresponding to a restriction prior to viral DNA synthesis (Prichard *et al.*, 1996). Two other examples of UDG requirement in viral DNA replication are known. First, an insertion mutant in HSV-1 UDG results in a recombinant virus that is attenuated in acute infection and latency in the mouse compared with wild-type (Pyles and Thompson, 1994), suggesting that UDG is required for efficient replication of

HSV-1 in animal hosts. Second, poxvirus mutants in UDG are nonviable and appear to be inhibited in DNA synthesis (Ellison *et al.*, 1996; Holzer and Falkner, 1997; Millns *et al.*, 1994; Stuart *et al.*, 1993). In all of these cases, the precise contribution of UDG activity to the process of DNA replication is unknown. There are some provocative observations that suggest that the uracil DNA glycosylase could act by directly recruiting the DNA replication machinery to sites of initiation. The human UDG has been shown to interact with DNA polymerase α (Seal and Sirover, 1986) and UDG activity is associated with replicating DNA (Krokan, 1981; Lee and Sirover, 1989). It may be that following binding to DNA, UDG forms a replication/repair complex that recruits replication proteins to these sites thus promoting DNA synthesis.

Numerous efforts to understand HCMV replication have focused on transient replication assays. These studies have identified the known core replication functions of HCMV and defined the origin of lytic replication, but have also suffered some limitations (Anders *et al.*, 1992; Anders and Punturieri, 1991; Hamzeh *et al.*, 1990; Masse *et al.*, 1992; Pari and Anders, 1993; Pari *et al.*, 1993). Some of the open reading frames described by these analyses have been found to play roles secondary to DNA replication (Goldmacher *et al.*, 1999; Greaves and Mocarski, 1998; Mocarski *et al.*, 1996); while still other open reading frames that seem to function in DNA synthesis based on mutagenesis studies have gone unidentified (Prichard *et al.*, 1996; Prichard *et al.*, 1999). Furthermore, these transient assays cannot reveal multiple functions in identified replication proteins.

In summary, the process by which HCMV initiates viral DNA synthesis is unknown and may involve viral and cellular replication fork proteins, cellular transcription factors or other HCMV genes. The mechanism by which HCMV switches from early to late phase DNA replication is unclear. Thus, the goal of this thesis was to understand the early events in viral DNA replication through the construction and characterization of HCMV mutants. To this end, I chose to study a mutant in UL114, the HCMV uracil DNA glycosylase, that was previously described to be restricted prior to viral DNA synthesis; and to derive a mutant in the HCMV core replication function, UL44.

Fig. 1.1. Electron microscopy of high-molecular-weight herpes simplex virus type 1 (HSV-1) replication intermediates isolated from two-dimensional (2D) gel electrophoresis. (A and B) HSV-1 DNA isolated fom representative branched regions of 2D gel. The arrows indicate branches in the viral DNA. (C) DNA isolated from linear regions of 2D gel. Reprinted with permission from (Severini *et al.*, 1996).


A
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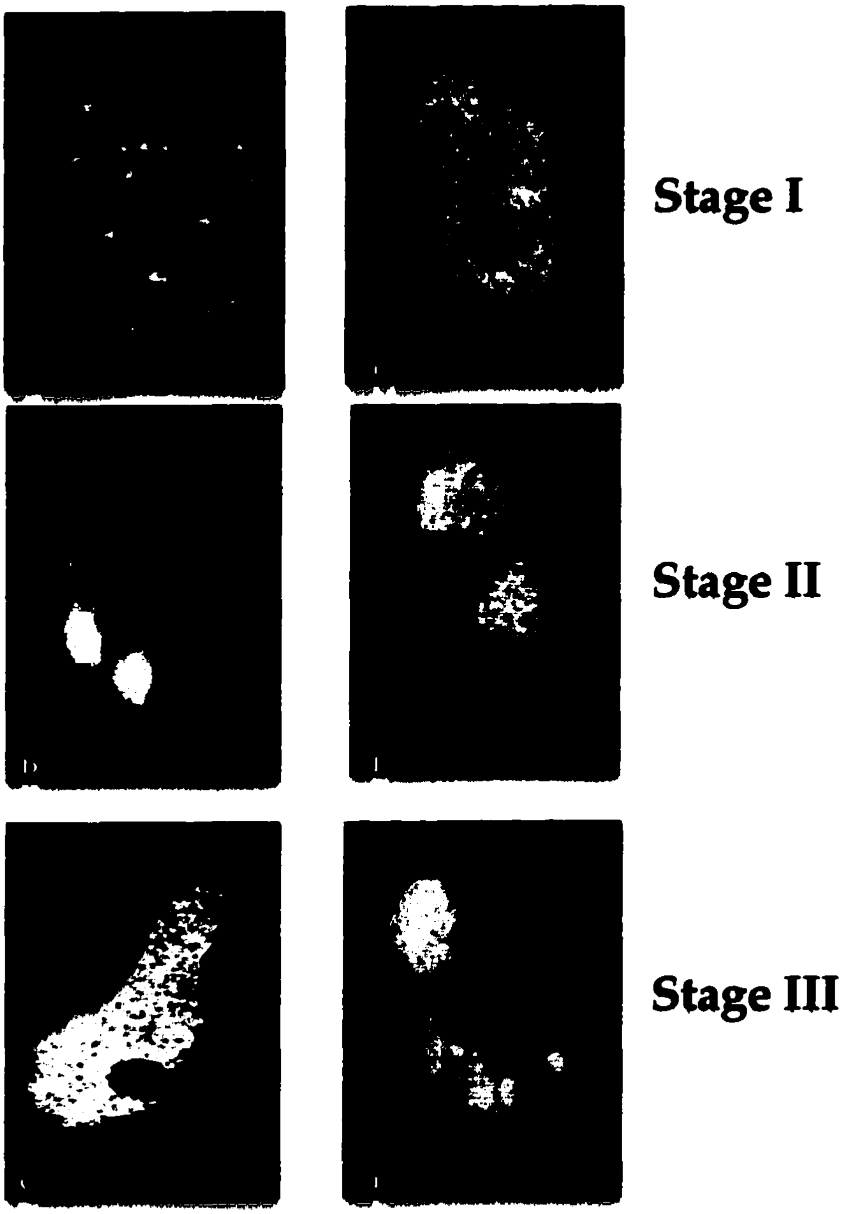
C
500 bp

Fig. 1.2. Localization of core HCMV replication proteins over the course of HCMV infection. Cells were grown on glass coverslips and infected with HCMV Towne strain at a multiplicity of infection of 3 pfu per cell. Following fixation at various times post-infection, cells were stained with (A, B and C) single-stranded DNA binding protein, UL57 and (D, E and F) viral DNA polymerase processivity factor, UL44. Each row represents viral antigen staining in cells at various stages of HCMV infection: stage I, early (up to 12 hpi); stage II, intermediate (24-48 hpi) and stage III, late (72 hpi and later).


Stage I
Stage II
Stage III

REFERENCES

Ahn, J.H. and Hayward, G.S. (1997) The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. *J Virol*, **71**, 4599-4613.

Akrigg, A., Wilkinson, G.W. and Oram, J.D. (1985) The structure of the major immediate early gene of human cytomegalovirus strain AD169. *Virus Res*, **2**, 107-121.

Albrecht, T., Boldogh, I., Fons, M., Lee, C.H., AbuBakar, S., Russell, J.M. and Au, W.W. (1989) Cell-activated responses to cytomegalovirus infection: Relationship to the phasing of CMV replication and to the induction of cellular damage. *Subcell. Biochem.*, **15**, 157-202.

Alford, C.A. and Britt, W.J. (1995) Cytomegalovirus. In Fields, B.N., Knipe, D.M. and Howley, P.M. (eds.), *Fields Virology*. Lippincott-Raven Publishers, New York, pp. 2493-2534.

Anders, D.G. and Gibson, W. (1988) Location, transcript analysis, and partial nucleotide sequence of the cytomegalovirus gene encoding an early DNA-binding protein with similarities to ICP8 of herpes simplex virus type 1. *J Virol*, **62**, 1364-1372.

Anders, D.G., Irmiere, A. and Gibson, W. (1986) Identification and characterization of a major early cytomegalovirus DNA-binding protein. *J Virol*, **58**, 253-262.

Anders, D.G., Kacica, M.A., Pari, G. and Punturieri, S.M. (1992) Boundaries and structure of human cytomegalovirus oriLyt, a complex origin for lytic-phase DNA replication. *J Virol*, **66**, 3373-3384.

Anders, D.G. and Punturieri, S.M. (1991) Multicomponent origin of cytomegalovirus lytic-phase DNA replication. *J. Virol.*, **65**, 931-937.

Ben-Porat, T. and Tokazewski, S.A. (1977) Replication of herpesvirus DNA. II. Sedimentation characteristics of newly synthesized DNA. *Virology*, **79**, 292-301.

Booy, F.P., Newcomb, W.W., Trus, B.L., Brown, J.C., Baker, T.S. and Steven, A.C. (1991) Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus. *Cell*, **64**, 1007-1015.

Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B. and Schaffner, W. (1985) A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell*, **41**, 521-530.

Bresnahan, W.A., Boldogh, I., Thompson, E.A. and Albrecht, T. (1996) Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1. *Virology*, **224**, 150-160.

Burkham, J., Coen, D.M. and Weller, S.K. (1998) ND10 protein PML is recruited to herpes simplex virus type 1 prereplicative sites and replication compartments in the presence of viral DNA polymerase. *J Virol*, **72**, 10100-10107.

Chee, M.S., Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerny, R., Horsnell, T., Hutchison, C.A.I., Kouzarides, T., Martignetti, J.A., Preddie, E., Satchwell, S.C., Tomlinson, P., Weston, K.M. and Barrell, B.G. (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol*, **154**, 125-170.

Chua, C.C., Carter, T.H. and St Jeor, S.C. (1981) Transcription of the human cytomegalovirus genome in productively infected cells. *J Gen Virol*, **56**, 1-11.

Coen, D.M. (1996) Viral DNA polymerases. In DePamphilis, M.L. (ed.) *DNA replication in eukaryotic cells*. Cold Spring Harbor Press, Cold Spring Harbor, pp. 495-523.

Compton, T., Nepomuceno, R.R. and Nowlin, D.M. (1992) Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. *Virology*, **191**, 387-395.

de Bruyn Kops, A. and Knipe, D.M. (1988) Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. *Cell*, **55**, 857-868.

DeMarchi, J.M. and Kaplan, A.S. (1976) Replication of human cytomegalovirus DNA: lack of dependence on cell DNA synthesis. *J Virol*, **18**, 1063-1070.

DeMarchi, J.M., Schmidt, C.A. and Kaplan, A.S. (1980) Patterns of transcription of human cytomegalovirus in permissively infected cells. *J Virol*, **35**, 277-286.

Dittmer, D. and Mocarski, E.S. (1997) Human cytomegalovirus infection inhibits G1/S transition. *J Virol*, **71**, 1629-1634.

Dutch, R.E., Bruckner, R.C., Mocarski, E.S. and Lehman, I.R. (1992) Herpes simplex virus type 1 recombination: Role of DNA replication and viral*a* sequences. *J Virol*, **66**, 277-285.

Dyck, J.A., Maul, G.G., Miller, W.H., Jr., Chen, J.D., Kakizuka, A. and Evans, R.M. (1994) A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell*, **76**, 333-343.

Efstathiou, S., Gompels, U.A., Craxton, M.A., Honess, R.W. and Ward, K. (1988) DNA homology between a novel human herpesvirus (HHV-6) and human cytomegalovirus [letter]. *Lancet*, **1**, 63-64.

Elias, P. and Lehman, I.R. (1988) Interaction of origin binding protein with an origin of replication of herpes simplex virus 1. *Proc Natl Acad Sci U S A*, **85**, 2959-2963.

Elias, P., O'Donnell, M.E., Mocarski, E.S. and Lehman, I.R. (1986) A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. *Proc Natl Acad Sci U S A*, **83**, 6322-6326.

Ellison, K.S., Peng, W. and McFadden, G. (1996) Mutations in active-site residues of the uracil-DNA glycosylase encoded by vaccinia virus are incompatible with virus viability. *J Virol*, **70**, 7965-7973.

Enquist, L.W. and Skalka, A. (1973) Replication of bacteriophage lambda DNA dependent on the function of host and viral genes. I. Interaction of red, gam and rec. *J Mol Biol*, **75**, 185-212.

Ertl, P.F. and Powell, K.L. (1992) Physical and functional interaction of human cytomegalovirus DNA polymerase and its accessory protein (ICP36) expressed in insect cells. *J Virol*, **66**, 4126-4133.

Estes, J.E. and Huang, E.S. (1977) Stimulation of cellular thymidine kinases by human cytomegalovirus. *J Virol*, **24**, 13-21.

Everett, R.D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M. and Parkinson, J. (1998) The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J Virol*, **72**, 6581-6591.

Everett, R.D. and Maul, G.G. (1994) HSV-1 IE protein Vmw110 causes redistribution of PML. *Embo J*, **13**, 5062-5069.

Frenkel, N., Schirmer, E.C., Wyatt, L.S., Katsafanas, G., Roffman, E., Danovich, R.M. and June, C.H. (1990) Isolation of a new herpesvirus from human CD4+ T cells [published erratum appears in Proc Natl Acad Sci U S A 1990 Oct;87(19):7797]. *Proc Natl Acad Sci U S A*, **87**, 748-752.

Fu, X.D. and Maniatis, T. (1990) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature*, **343**, 437-441.

Gold, E. and Nankervis, G.A. (1982) Cytomegalovirus. *Viral Infections of Humans: Epidemiology and Control.* Plenum Press, New York.

Goldmacher, V.S., Bartle, L.M., Skaletskaya, A., Dionne, C.A., Kedersha, N.L., Vater, C.A., Han, J., Lutz, R.J., Watanabe, S., McFarland, E.D., Kieff, E.D., Mocarski, E.S. and Chittenden, T. (1999) A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to bcl-2 [In Process Citation]. *Proc Natl Acad Sci U S A*, **96**, 12536-12541.

Goldstein, J.N. and Weller, S.K. (1998) In vitro processing of herpes simplex virus type 1 DNA replication intermediates by the viral alkaline nuclease, UL12. *J Virol*, **72**, 8772-8781.

Gompels, U.A., Nicholas, J., Lawrence, G., Jones, M., Thomson, B.J., Martin, M.E., Efstathiou, S., Craxton, M. and Macaulay, H.A. (1995) The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. *Virology*, **209**, 29-51.

Greaves, R.F. and Mocarski, E.S. (1998) Defective growth correlates with reduced accumulation of a viral DNA replication protein after low multiplicity infection by a human cytomegalovirus *ie1* mutant. *J. Virol.*, **72**, 366-379.

Hamzeh, F.M., Lietman, P.S., Gibson, W. and Hayward, G.S. (1990) Identification of the lytic origin of DNA replication in human cytomegalovirus by a novel approach utilizing ganciclovir-induced chain termination. *J. Virol.*, **64**, 6184-6195.

Hanshaw, J.B., Scheiner, A.P., Moxley, A.W., Gaev, L., Abel, V. and Scheiner, B. (1976) School failure and deafness after "silent" congenital cytomegalovirus infection. *N Engl J Med*, **295**, 468-470.

Holzer, G.W. and Falkner, F.G. (1997) Construction of a vaccinia virus deficient in the essential DNA repair enzyme uracil DNA glycosylase by a complementing cell line. *J Virol*, **71**, 4997-5002.

Huang, E.S. (1975a) Human cytomegalovirus. III. Virus-induced DNA polymerase. *J Virol*, **16**, 298-310.

Huang, E.S. (1975b) Human cytomegalovirus. IV. Specific inhibition of virus-induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. *J Virol*, **16**, 1560-1565.

Huang, L., Zhu, Y. and Anders, D.G. (1996) The variable 3' ends of a human cytomegalovirus *ori*Lyt transcript (SRT) overlap an essential, conserved replicator element. *J. Virol.*, **70**, 5272-5281.

Huberman, J.A. and Riggs, A.D. (1968) On the mechanism of DNA replication in mammalian chromosomes. *J Mol Biol*, **32**, 327-341.

Huberman, J.A. and Tsai, A. (1973) Direction of DNA replication in mammalian cells. *J Mol Biol*, **75**, 5-12.

Igarashi, K., Fawl, R., Roller, R.J. and Roizman, B. (1993) Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. *J Virol*, **67**, 2123-2132.

Ihara, S., Saito, S. and Watanabe, Y. (1980) Human cytomegalovirus-induced inhibition of exogenous thymidine uptake into cell DNA in HEL cells stimulated to proliferate with serum. *Tokai J Exp Clin Med*, **5**, 301-309.

Ishov, A.M. and Maul, G.G. (1996) The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. *J Cell Biol*, **134**, 815-826.
Ishov, A.M., Stenberg, R.M. and Maul, G.G. (1997) Human cytomegalovirus immediate early interaction with host nuclear structures: definition of an immediate transcript environment. *J Cell Biol*, **138**, 5-16.

Jacob, R.J., Morse, L.S. and Roizman, B. (1979) Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol*, **29**, 448-457.

Jault, F.M., Jault, J.M., Ruchti, F., Fortunato, E.A., Clark, C., Corbeil, J., Richman, D.D. and Spector, D.H. (1995) Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest. *J Virol*, **69**, 6697-6704.

Kelly, C., Van Driel, R. and Wilkinson, G.W. (1995) Disruption of PML-associated nuclear bodies during human cytomegalovirus infection. *J Gen Virol*, **76**, 2887-2893.

Kemble, G.W., McCormick, A.L., Pereira, L. and Mocarski, E.S. (1987) A cytomegalovirus protein with properties of herpes simplex virus ICP8: partial purification of the polypeptide and map position of the gene. *J Virol*, **61**, 3143-3151.

Kemble, G.W. and Mocarski, E.S. (1989) A host cell protein binds to a highly conserved sequence element (pac-2) within the cytomegalovirus *a* sequence. *J Virol*, **63**, 4715-4728.

Korioth, F., Maul, G.G., Plachter, B., Stamminger, T. and Frey, J. (1996) The nuclear domain 10 (ND10) is disrupted by the human cytomegalovirus gene product IE1. *Exp Cell Res*, **229**, 155-158.

Krokan, H. (1981) Preferential association of uracil-DNA glycosylase activity with replicating SV40 minichromosomes. *FEBS Lett*, **133**, 89-91.

LaFemina, R.L. and Hayward, G.S. (1983) Replicative forms of human cytomegalovirus DNA with joined termini are found in permissively infected human cells but not in non-permissive Balb/c-3T3 mouse cells. *J Gen Virol*, **64**, 373-389.

Laskey, R. and Madine, M. (1996) Roles of nuclear structure in DNA replication. *DNA Replication in Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, pp. 119-130.

Lawrence, G.L., Chee, M., Craxton, M.A., Gompels, U.A., Honess, R.W. and Barrell, B.G. (1990) Human herpesvirus 6 is closely related to human cytomegalovirus. *J Virol*, **64**, 287-299.

Lee, K.A. and Sirover, M.A. (1989) Physical association of base excision repair enzymes with parental and replicating DNA in BHK-21 cells. *Cancer Res*, **49**, 3037-3044.

Lehman, I.R. and Boehmer, P.E. (1999) Replication of herpes simplex virus DNA [In Process Citation]. *J Biol Chem*, **274**, 28059-28062.

Liptak, L.M., Uprichard, S.L. and Knipe, D.M. (1996) Functional order of assembly of herpes simplex virus DNA replication proteins into prereplicative site structures. *J Virol*, **70**, 1759-1767.

Littler, E., Lawrence, G., Liu, M.Y., Barrell, B.G. and Arrand, J.R. (1990) Identification, cloning, and expression of the major capsid protein gene of human herpesvirus 6. *J Virol*, **64**, 714-722.

Lu, M. and Shenk, T. (1996) Human cytomegalovirus infection inhibits cell cycle progression at multiple points, including the transition from G1 to S. *J Virol*, **70**, 8850-8857.

Lukonis, C.J., Burkham, J. and Weller, S.K. (1997) Herpes simplex virus type 1 prereplicative sites are a heterogeneous population: only a subset are likely to be precursors to replication compartments. *J Virol*, **71**, 4771-4781.

Martinez, R., Sarisky, R.T., Weber, P.C. and Weller, S.K. (1996) Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. *J Virol*, **70**, 2075-2085.

Masse, M.J., Karlin, S., Schachtel, G.A. and Mocarski, E.S. (1992) Human cytomegalovirus origin of DNA replication (oriLyt) resides within a highly complex repetitive region. *Proc Natl Acad Sci U S A*, **89**, 5246-5250.

Maul, G.G. and Everett, R.D. (1994) The nuclear location of PML, a cellular member of the C3HC4 zinc- binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. *J Gen Virol*, **75**, 1223-1233.

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

McDonough, S.H. and Spector, D.H. (1983) Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. *Virology*, **125**, 31-46.

McDonough, S.H., Staprans, S.I. and Spector, D.H. (1985) Analysis of the major transcripts encoded by the long repeat of human cytomegalovirus strain AD169. *J Virol*, **53**, 711-718.

McVoy, M.A. and Adler, S.P. (1994) Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. *J Virol*, **68**, 1040-1051.

Millns, A.K., Carpenter, M.S. and DeLange, A.M. (1994) The vaccinia virus-encoded uracil DNA glycosylase has an essential role in viral DNA replication. *Virology*, **198**, 504-513.

Mocarski, E.S. (1993) Cytomegalovirus biology and replication. In Roizman, B., Whitley, R. and Lopez, C. (eds.), *The Human Herpesviruses*. Raven Press, New York, pp. 173-226.

Mocarski, E.S. (1995) Cytomegaloviruses and their replication. In Fields, B.N., Knipe, D.M. and Howley, P.M. (eds.), *Fields Virology*. Lippincott-Raven Publishers, New York, pp. 2447-2492.

Mocarski, E.S., Kemble, G.W., Lyle, J.M. and Greaves, R.F. (1996) A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation. *Proc Natl Acad Sci U S A*, **93**, 11321-11326.

Mocarski, E.S., Liu, A.C. and Spaete, R.R. (1987) Structure and variability of the a sequence in the genome of human cytomegalovirus (Towne strain). *J Gen Virol*, **68**, 2223-2230.

Morin, J., Johann, S., O'Hara, B. and Gluzman, Y. (1996) Exogenous thymidine is preferentially incorporated into human cytomegalovirus DNA in infected human fibroblasts. *J Virol*, **70**, 6402-6404.

Mosig, G. (1998) Recombination and recombination-dependent DNA replication in bacteriophage T4. *Annu Rev Genet*, **32**, 379-413.

Mosig, G. and Colowick, N. (1995) DNA replication of bacteriophage T4 in vivo. *Methods Enzymol*, **262**, 587-604.

Muganda, P., Mendoza, O., Hernandez, J. and Qian, Q. (1994) Human cytomegalovirus elevates levels of the cellular protein p53 in infected fibroblasts. *J Virol*, **68**, 8028-8034.

Nicholas, J. (1996) Determination and analysis of the complete nucleotide sequence of human herpesvirus. *J Virol*, **70**, 5975-5989.

Nishiyama, Y., Maeno, K. and Yoshida, S. (1983) Characterization of human cytomegalovirus-induced DNA polymerase and the associated 3'-to-5' exonuclease. *Virology*, **124**, 221-231.

Olivo, P.D., Nelson, N.J. and Challberg, M.D. (1988) Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. *Proc Natl Acad Sci U S A*, **85**, 5414-5418.

Pari, G.S. and Anders, D.G. (1993) Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. *J Virol*, **67**, 6979-6988.

Pari, G.S., Kacica, M.A. and Anders, D.G. (1993) Open reading frames UL44, IRS1/TRS1, and UL36-38 are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA synthesis. *J Virol*, **67**, 2575-2582.

Penfold, M.E. and Mocarski, E.S. (1997) Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis. *Virology*, **239**, 46-61.

Prichard, M.N., Duke, G.M. and Mocarski, E.S. (1996) Human cytomegalovirus uracil DNA glycosylase is required for the normal temporal regulation of both DNA synthesis and viral replication. *J Virol*, **70**, 3018-3025.

Prichard, M.N., Gao, N., Jairath, S., Mulamba, G., Krosky, P., Coen, D.M., Parker, B.O. and Pari, G.S. (1999) A recombinant human cytomegalovirus with a large deletion in UL97 has a severe replication deficiency. *J Virol*, **73**, 5663-5670.

Prichard, M.N., Jairath, S., Penfold, M.E., St Jeor, S., Bohlman, M.C. and Pari, G.S. (1998) Identification of persistent RNA-DNA hybrid structures within the origin of replication of human cytomegalovirus. *J Virol*, **72**, 6997-7004.

Puvion-Dutilleul, F., Venturini, L., Guillemin, M.C., de The, H. and Puvion, E. (1995) Sequestration of PML and Sp100 proteins in an intranuclear viral structure during herpes simplex virus type 1 infection. *Exp Cell Res*, **221**, 448-461.

Pyles, R.B. and Thompson, R.L. (1994) Evidence that the herpes simplex virus type 1 uracil DNA glycosylase is required for efficient viral replication and latency in the murine nervous system. *J Virol*, **68**, 4963-4972.

Quinlan, M.P., Chen, L.B. and Knipe, D.M. (1984) The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell*, **36**, 857-868.

Rand, K.H., Pollard, R.B. and Merigan, T.C. (1978) Increased pulmonary superinfections in cardiac-transplant patients undergoing primary cytomegalovirus infection. *N Engl J Med*, **298**, 951-953.

Rasmussen, L. (1990) Immune response to human cytomegalovirus infection. *Curr Top Microbiol Immunol*, **154**, 221-254.

Reynolds, D.W., Stagno, S. and Alford, C.A. (1981) Chronic congenital and perinatal infection. *Neonatology Pathophysiology and Management of the Newborn*. Lippincott, Philadelphia, pp. 748-789.

Reynolds, D.W., Stagno, S., Hosty, T.S., Tiller, M. and Alford, C.J. (1973) Maternal cytomegalovirus excretion and perinatal infection. *N Engl J Med*, **289**, 1-5.

Riddell, S.R. and Greenberg, P.D. (1997) T cell therapy of human CMV and EBV infection in immunocompromised hosts. *Rev Med Virol*, **7**, 181-192.

Rixon, F.J., Atkinson, M.A. and Hay, J. (1983) Intranuclear distribution of herpes simplex virus type 2 DNA synthesis: examination by light and electron microscopy. *J Gen Virol*, **64**, 2087-2092.

Roizman, B., Borman, G.S. and Kamali-Rousta, M. (1965) Macromolecular synthesis in cells infected with herpes simplex virus. *Nature*, **206**, 1374-1375.

Roizman, B., Carmichael, L.E., Deinhardt, F., de The, G., Nahmias, W., Plowright, F., Rapp, F., Sheldrick, P., Takahashi, M. and Wolf, K. (1981) Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses. *Intervirology*, **16**, 201-217.

Roizman, B. and Sears, A.E. (1995) Herpes simplex viruses and their replication. In Fields, B.N., Knipe, D.M. and Howley, P.M. (eds.), *Fields Virology*. Lippincott-Raven Publishers, New York, pp. 2231-2296.

Sarisky, R.T. and Hayward, G.S. (1996) Evidence that the UL84 gene product of human cytomegalovirus is essential for promoting oriLyt-dependent DNA replication and formation of replication compartments in cotransfection assays. *J Virol*, **70**, 7398-7413.

Sarisky, R.T. and Weber, P.C. (1994) Requirement for double-strand breaks but not for specific DNA sequences in herpes simplex virus type 1 genome isomerization events. *J Virol*, **68**, 34-47.

Seal, G. and Sirover, M.A. (1986) Physical association of the human base-excision repair enzyme uracil DNA glycosylase with the 70,000-dalton catalytic subunit of DNA polymerase alpha. *Proc Natl Acad Sci U S A*, **83**, 7608-7612.

Severini, A., Morgan, A.R., Tovell, D.R. and Tyrrell, D.L. (1994) Study of the structure of replicative intermediates of HSV-1 DNA by pulsed-field gel electrophoresis. *Virology*, **200**, 428-435.

Severini, A., Scraba, D.G. and Tyrrell, D.L. (1996) Branched structures in the intracellular DNA of herpes simplex virus type 1. *J Virol*, **70**, 3169-3175.

Shlomai, J., Friedmann, A. and Becker, Y. (1976) Replication intermediates of herpes simplex virus DNA. *Virology*, **69**, 647-659.

Spaete, R.R. and Frenkel, N. (1985) The herpes simplex virus amplicon: analyses of cis-acting replication functions. *Proc Natl Acad Sci U S A*, **82**, 694-698.

Spaete, R.R. and Mocarski, E.S. (1985) The a sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. *J Virol*, **54**, 817-824.

Spector, D.L. (1990) Higher order nuclear organization: three-dimensional distribution of small nuclear ribonucleoprotein particles [published erratum appears in Proc Natl Acad Sci U S A 1990 Mar;87(6):2384]. *Proc Natl Acad Sci U S A*, **87**, 147-151.

Speir, E., Modali, R., Huang, E.S., Leon, M.B., Shawl, F., Finkel, T. and Epstein, S.E. (1994) Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. *Science*, **265**, 391-394.

St Jeor, S. and Hutt, R. (1977) Cell DNA replication as a function in the synthesis of human cytomegalovirus. *J Gen Virol*, **37**, 65-73.

Stagno, S., Pass, R.F., Cloud, G., Britt, W.J., Henderson, R.E., Walton, P.D., Veren, D.A., Page, F. and Alford, C.A. (1986) Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. *Jama*, **256**, 1904-1908.

Stagno, S., Pass, R.F., Dworsky, M.E. and Alford, C.A. (1983) Congenital and perinatal cytomegalovirus infections. *Seminars in Perinatology*, **7**, 31-42.

Stagno, S., Pass, R.F., Dworsky, M.E., Henderson, R.E., Moore, E.G., Walton, P.D. and Alford, C.A. (1982) Congenital cytomegalovirus infection: The relative importance of primary and recurrent maternal infection. *N Engl J Med*, **306**, 945-949.

Stagno, S., Reynolds, D.W., Pass, R.F. and Alford, C.A. (1980) Breast milk and the risk of cytomegalovirus infection. *N Engl J Med*, **302**, 1073-1076.

Stamminger, T. and Fleckenstein, B. (1990) Immediate-early transcription regulation of human cytomegalovirus. *Curr Top Microbiol Immunol*, **154**, 3-20.

Stasiak, P.C. and Mocarski, E.S. (1992) Transactivation of the cytomegalovirus ICP36 gene promoter requires the α gene product TRS1 in addition to IE1 and IE2. *J Virol*, **66**, 1050-1058.

Stenberg, R.M., Depto, A.S., Fortney, J. and Nelson, J.A. (1989) Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. *J Virol*, **63**, 2699-2708.

Stenberg, R.M. and Stinski, M.F. (1985) Autoregulation of the human cytomegalovirus major immediate-early gene. *J Virol*, **56**, 676-682.

Stenberg, R.M., Thomsen, D.R. and Stinski, M.F. (1984) Structural analysis of the major immediate early gene of human cytomegalovirus. *J Virol*, **49**, 190-199.

Stenberg, R.M., Witte, P.R. and Stinski, M.F. (1985) Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. *J Virol*, **56**, 665-675.

Stinski, M.F. (1978) Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. *J Virol*, **26**, 686-701.

Stuart, D.T., Upton, C., Higman, M.A., Niles, E.G. and McFadden, G. (1993) A poxvirus-encoded uracil DNA glycosylase is essential for virus viability. *J Virol*, **67**, 2503-2512.

Thomsen, D.R., Stenberg, R.M., Goins, W.F. and Stinski, M.F. (1984) Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. *Proc Natl Acad Sci U S A*, **81**, 659-663.

Uprichard, S.L. and Knipe, D.M. (1997) Assembly of herpes simplex virus replication proteins at two distinct intranuclear sites. *Virology*, **229**, 113-125.

Vierling, J.M. and Fennell, R.H., Jr. (1985) Histopathology of early and late human hepatic allograft rejection: evidence of progressive destruction of interlobular bile ducts. *Hepatology*, **5**, 1076-1082.

Wade, M., Kowalik, T.F., Mudryj, M., Huang, E.S. and Azizkhan, J.C. (1992) E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. *Mol Cell Biol*, **12**, 4364-4374.

Wahren, B., Ruden, U., Gadler, H., Oberg, B. and Eriksson, B. (1985) Activity of the cytomegalovirus genome in the presence of PPi analogs. *J Virol*, **56**, 996-1001.

Watanabe, S. and Yamaguchi, N. (1993) Deletion analysis of a replication origin of human cytomegalovirus by a novel assay system with a combination of microinjection and polymerase chain reaction. *Virology*, **192**, 332-335.

Wathen, M.W. and Stinski, M.F. (1982) Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. *J Virol*, **41**, 462-477.

Wathen, M.W., Thomsen, D.R. and Stinski, M.F. (1981) Temporal regulation of human cytomegalovirus transcription at immediate early and early times after infection. *J Virol*, **38**, 446-459.

Weiland, K.L., Oien, N.L., Homa, F. and Wathen, M.W. (1994) Functional analysis of human cytomegalovirus polymerase accessory protein. *Virus Res*, **34**, 191-206.

Weston, K. (1988) An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. *Virology*, **162**, 406-416.

Zhang, X., Efstathiou, S. and Simmons, A. (1994) Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. *Virology*, **202**, 530-539.

Chapter 2: A mutant in HCMV UL114 is restricted in a serum-dependent manner prior to the elongation phase of DNA synthesis.

ABSTRACT

A recombinant human cytomegalovirus (HCMV), RC2620, carrying a deletion in the viral uracil DNA glycosylase, UL114 was found to be delayed in the onset of viral DNA replication and virus production compared with wild-type AD169 virus (Prichard *et al.*, 1996). Delayed replication was observed despite the timely expression of viral immediate early and early gene products in this mutant. This observation led to the proposal that UL114, the gene encoding HCMV uracil DNA glycosylase, may be required for efficient viral DNA synthesis. The requirement for UL114 in replication is not absolute since the mutant is eventually able to grow to wild-type titers. The impact of UL114 was most dramatic in cells which were confluent or serum starved. The requirement for the UL114 gene product was completely supplanted when cells were actively growing at the time of infection. Transcript analysis revealed that high levels of human uracil DNA glycosylase are present in actively dividing cells, while expression remained undetectable in serum starved cells. These results suggest that human uracil DNA glycosylase compensates for the lack of viral enzyme. RC2620's defect in serum starved cells was found to occur at or before the elongation phase of viral DNA synthesis. Consistent with this idea, RNA transcript analysis showed that UL114 is normally expressed at early times of infection. Interestingly, we found that human uracil DNA glycosylase expression was induced by HCMV infection at times correlating with the onset of DNA synthesis in the mutant, further implicating the human enzyme in the complementation of this mutant virus. Taken together, these results suggest that uracil DNA glycosylase activity is required prior to DNA elongation and that the human enzyme is able to complement viral growth in RC2620.

INTRODUCTION

Cytomegalovirus (CMV) is a member of the β-herpesvirus family. Like other large DNA viruses, CMV encodes its own DNA replication machinery including a DNA polymerase, polymerase processivity factor, single-stranded DNA binding protein and a helicase-primase complex (Pari and Anders, 1993; Pari *et al.*, 1993). In addition to these proteins, CMV also encodes a homolog of uracil DNA glycosylase, dUTPase and ribonucleotide reductase (Chee *et al.*, 1990), genes that may function in maintainance of the CMV genome.

A recombinant HCMV containing a deletion in the viral UDG (approximately 80% deleted) and carrying an insertion of the *Escherichia coli gpt* gene had previously been isolated in our laboratory (Prichard *et al.*, 1996). This recombinant virus displayed all the hallmarks of a uracil DNA glycosylase mutant including increased incorporation of uracil residues into the viral genome and an acute sensitivity to the nucleoside analog 5-bromodeoxyuridine. This mutant virus was observed to have a lengthened replication cycle compared with wild-type virus despite appropriate timing of immediate early and early viral gene expression. Further analysis of this mutant demonstrated that this phenotype was associated with the delayed onset of viral DNA replication, although the exact function for this gene product in DNA synthesis is unknown.

Uracil DNA glycosylase (UDG) is a highly conserved repair enzyme that is found in all free-living organisms from bacteria such as *E. coli* through humans (Krokan *et al.*, 1983; Myrnes *et al.*, 1983; Percival *et al.*, 1989; Varshney *et al.*, 1988). This enzyme is also expressed in all large DNA viruses. UDG is involved in the first step of base excision repair of uracils arising from misincorporation of dUTP by DNA polymerase (Brynolf *et al.*, 1978; Tye and Lehman, 1977; Wist *et al.*, 1978) or from the spontaneous deamination of cytosine (Lindahl and Nyberg, 1974; Shapiro, 1980). Following cleavage of the glycosidic bond, the abasic site is recognized and processed by an apyrimidinic (AP) endonuclease to leave a gap in the DNA. The repair pathway is completed by the action of DNA polymerase and DNA ligase.

Two other examples of UDG requirement in DNA replication are known. First, an insertion mutant in herpes simplex virus 1 (HSV-1) UDG results in a recombinant virus that is attenuated in acute infection and latency in the mouse compared with wild-type virus (Pyles and Thompson, 1994a), suggesting that UDG is required for efficient replication of HSV-1. Second, poxvirus mutants in UDG are nonviable and appear to be inhibited in DNA synthesis (Ellison *et al.*, 1996; Holzer and Falkner, 1997; Millns *et al.*, 1994; Stuart *et al.*, 1993). In both cases, the exact role of UDG and the stage at which this protein is required is unclear. However, the defect in these systems may be related to the phenotype observed in the CMV UDG mutant.

UDG is important for DNA replication in large DNA viruses. Assessment of the role for this protein has been limited in HSV-1 as the mutant grows as well as wild-type virus in immortalized cell lines (Mullaney *et al.*, 1989; Pyles and Thompson, 1994a; Pyles and Thompson, 1994b) but is severely attenuated in animals (Pyles and Thompson, 1994a). Poxvirus UDG is also essential for virus viability (Ellison *et al.*, 1996; Holzer and Falkner, 1997; Millns *et al.*, 1994; Stuart *et al.*, 1993). Thus, the UDG mutant in HCMV affords one view into the role of uracil excision repair in viral DNA synthesis. The recombinant virus, RC2620, is delayed in the start of DNA replication but is still able to grow to wild-type titers in tissue culture (Prichard *et al.*, 1996). In this chapter, I identify the growth conditions that impact RC2620 replication and examine the point at which this mutant is restricted for DNA synthesis.

MATERIALS AND METHODS

Cells and virus. Primary human foreskin fibroblasts (HFFs) and human embryonic lung fibroblasts (HELs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% NuSerum I (Collaborative Research Inc.), 100 Units of penicillin G per ml, 100 μg of streptomycin sulfate per ml, 0.58 mg L-arginine per ml, 1.08 mg L-glutamine per ml, and 180 μg L-asparagine per ml.

Human CMV strains, AD169 and Towne, were obtained and cultured as previously described (Mocarski *et al.*, 1993; Spaete and Mocarski, 1985). The recombinant human CMV, RC2620, was described previously (Prichard *et al.*, 1996).

Plasmids. The plasmid, pON2619, was described previously (Prichard *et al.*, 1996). The plasmid, pGEM-3Zf/UDG1A (Muller-Weeks *et al.*, 1998), was a generous gift of Dr. Sal Caradonna.

Plasmid pON2260 was constructed by ligating a 7.36 kbp EcoRI-XhoI fragment from cosmid pCM1007 (Fleckenstein *et al.*, 1982), representing nucleotides 119499-126856 of the AD169 strain published sequence (Chee *et al.*, 1990), into the EcoRI/SalI sites of pGEM-3Zf+.

Plasmid pON2136 was constructed by ligating a 4.68 kbp PstI-AatII fragment from pCM1017, representing nucleotides 55369-60045 of the AD169 strain published sequence (Chee *et al.*, 1990), into the PstI/AatII sites of pGEM-3Zf+.

Transcript analysis. Total cellular RNA was purified from infected cell monolayers using Trizol reagent as recommended by the manufacturer (GibcoBRL.) When used, cycloheximide (Sigma; 50 µg/ml) was added to the culture medium beginning one hour prior to infection. Where indicated, sodium phosphonoformate (PFA, Sigma, 300 µg/ml) was added to the culture medium commencing at the time of infection. For RNA blot analysis, 10 µg RNA samples were separated by electrophoresis on denaturing formaldehyde-1% agarose gels, transferred to BrightStar-Plus nylon membrane (Ambion) and UV cross-linked. Hybridizations of filters to probes were carried out overnight at 65°C in NorthernMax Prehybridization/Hybridization Buffer (Ambion). Filters were washed at 68°C in High Stringency Wash Solution #2 (Ambion).

Cellular UDG was PCR amplified from pGEM3Zf/UDG1A using the primers UDG F1 5' ATGATCGGCCAGAAGACG and T7 UDG R1 5' TAATACGACTCACTATAGGGATGATATGGATCCTGTCC or UDG F2 5' CATGGACCTAATCAAGC and T7 UDG R2 5' TAATACGACTCACTATAGGGGCTCCTTCCAGTCAATGGG. PCR conditions were as follows: 1 cycle at 95°C, 5 min; 25 cycles at 95°C, 1 min, 56°C, 2 min, 72°C, 2 min and 1 cycle at 72°C, 10 min. Cellular UDG anti-sense riboprobes were generated from the resulting PCR products by *in vitro* transcription using T7 DNA polymerase and labeled with psoralen-biotin on modified CTP using a protocol suggested by the manufacturer (Ambion).

To generate the UL114-specific RNA probe, the first 330 bp of the UL114 ORF were PCR amplified from pON2619 using the primers 114F 5' ATGGCCCTCAAGCAGTGGATGCTC and T7-114R 5' TAATACGACTCACTATAGGGCGGTCGGCCCGCCAGCGTG using the amplification conditions described for cellular UDG. UL114 anti-sense riboprobe was generated as described for UDG riboprobes.

UL44 was PCR amplified from pON2136 using the primers UL44 F 5' GTGGTACCACTGGCGCTTTAAGGTCG and T7 UL44 R 5' TAATACGACTCACTATAGGGCAGGTACATGAAATTACC. UL44-specific anti-sense probes were generated as described above.

Bound biotin-labeled probes were detected with streptavidin conjugated with alkaline phosphatase and developed with a chemiluminescent substrate for the enzyme as recommended by the manufacturer (Ambion). Where indicated, membranes were stripped using a protocol recommended by the manufacturer (Ambion).

Infection under Serum Starvation Conditions. HFFs were seeded into 90 mm tissue culture plates at approximately 3×10^6 cells per plate. Monolayers were monitored daily until complete confluence was observed, usually after 4 days. Culture medium was then replaced with medium supplemented with 0.2% NuSerum and maintained under these conditions for 72 hours. Following this treatment, HFFs were infected with wild-type AD169 or mutant RC2620 at a multiplicity of infection (m.o.i.) of 5 in medium supplemented with 0.2% NuSerum. At 24, 48, 72, 96, 120, 144 and 168 hpi, infected monolayers were rinsed twice in PBS, collected by trypsinization and counted. The cell suspension was centrifuged at 1,000 x g in a table-top centrifuge for 5 min. Cell pellets were stored at -20°C for the duration of the time course.

PFA inhibition and release. HFFs were seeded and maintained as described above for serum starvation conditions. Following serum starvation, monolayers were infected with AD169 or RC2620 at a m.o.i. of 5 in medium supplemented with 0.2% NuSerum and 300 μg/ml PFA. Infected cell monolayers were maintained under PFA inhibition until 72 hpi. At 72 hpi, culture medium containing PFA was removed and monolayers were rinsed extensively with medium supplemented with 0.2% NuSerum followed by culture medium change to medium supplemented with 0.2% NuSerum. At 24, 48, 72, 96, 120, 144, 168 and 192 hpi, infected cell monolayers were collected as described for infection under serum starvation conditions above.

Viral DNA isolation. Infected cell pellets were resuspended in Tris-EDTA (TE) containing 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg of Proteinase K per ml and incubated at 55°C overnight. Viral DNA was purified by phenol and chloroform extractions with phase-lock gel (5 Prime → 3 Prime) and precipitated with ethanol.

DNA Blots and Quantification of DNA. Viral DNA from approximately 10^5 cells was digested to completion with restriction endonucleases, separated on a 0.7% agarose gel and visualized with ethidium bromide. Gels were denatured and viral DNA transferred to nitrocellulose membranes (and UV cross-linked.) Membranes were prehybridized in 6 × SSPE, 2 × Denhardt's, 0.5% SDS and 300 µg/ml salmon sperm DNA for one hour. Blots were hybridized with ^{32}P-radiolabelled DNA probes overnight at 65°C in 4 × SSPE, 3 × Denhardt's, 0.5% SDS, 15% formamide, 10% dextran sulfate and 400 µg/ml salmon sperm DNA. Filters were washed at 65°C in 0.1 × SSPE, 0.1% SDS. Membranes were exposed to a PhosphorScreen and results quantitated by densitometry using ImageQuant software (Molecular Dynamics.)

RESULTS

Viral UDG is required for efficient HCMV replication. A mutant in UL114, the HCMV UDG, was observed to be delayed in the start of viral DNA synthesis (Prichard *et al.*, 1996). To confirm this result, we isolated total cellular DNA from parental AD169 virus and mutant RC2620 virus-infected cells at 24, 48, 72, 96, 120, 144 and 168 hpi. DNA blot analysis of this resultant DNA probed for a HCMV-specific gene is shown in Fig. 2.1 (A,B). Relative DNA synthesis was expressed as a ratio of the density of each time point to the density at 24 hpi (Fig. 2.1B). In wild-type AD169-infected cells, viral DNA accumulation was observed by 48 hpi and increased to peak levels over the course of infection. In contrast, mutant RC2620 did not begin to accumulate wild-type levels of viral DNA until 120 hpi. This result confirmed a previous report (Prichard *et al.*, 1996) that UL114 was required for viral DNA replication and that the absence of this protein results in a delay of the onset of viral DNA synthesis.

Effect of Cell Confluency on HCMV replication. During the preparation of RC2620 virus stocks, we observed that this mutant had a prolonged growth cycle in confluent cells compared with subconfluent cells. This observation suggested that the requirement for HCMV UDG could be dependent on the growth phase of the infected cell. To test this possibility directly, we compared the rates of viral DNA accumulation for parental virus AD169 and mutant virus RC2620 in actively dividing cells and non-dividing, serum-starved HFF cells. To establish serum-starvation conditions, HFF cells were held for 4 days at 100% confluence and medium was then replaced with growth medium supplemented with 0.2% NuSerum. After 72 hours under low serum conditions, cells were infected with mutant RC2620 or wild-type AD169 under high m.o.i. (5 p.f.u./cell) conditions in low serum medium. DNA blot analyses of total viral DNA produced over time for each virus under these conditions are shown in Fig. 2.1 (A, C) and the corresponding densitometry analysis is presented in Fig. 2.1 (B, D). Wild-type AD169 DNA accumulation was unaffected by cell confluence and commenced by 48 hpi (Fig. 2.1 A, B). RC2620 showed a significant delay in the accumulation of viral DNA compared to wild-type AD169 in confluent cells. RC2620 was only able to reach wild-type levels of viral DNA synthesis at 120 hpi.

In contrast, when this analysis was repeated in actively dividing cells maintained under high serum conditions, RC2620 exhibited an increased growth capacity. Interestingly, the defect in mutant viral DNA replication was no longer detected (Fig. 2.1 C, D). Both mutant RC2620 virus and parental AD169 virus initiated viral DNA accumulation by 48 hpi and reached peak levels of DNA synthesis by 120 hpi (Fig. 2.1 C, D), suggesting that the mutant phenotype could be completely rescued in proliferating cells. This result was reproducible in a separate experiment. The differential replication of RC2620 on actively dividing versus confluent cells strongly demonstrates a serum-dependent phenotype for RC2620. Our data also suggests that a cellular factor present in actively replicating cells can compensate for the lack of viral UDG.

Cellular UDG levels in confluent, serum-starved cells. UL114 is highly homologous to other known UDGs, including the major human UDG (Fig. 2.2). HCMV UL114 shares approximately 40% identity with the human UDG at the amino acid level. Previous work had shown that human UDG is activated by the cell cycle (Haug *et al.*, 1998; Nagelhus *et al.*, 1995; Slupphaug *et* *al.*, 1991) and is expressed to high levels in tissues containing proliferating cells (Haug *et al.*, 1998). I hypothesized that the ability of RC2620 to grow in actively dividing cells may have been due to increased expression of cellular UDG. To determine whether cellular UDG expression was upregulated in proliferating cells maintained under our experimental conditions, we isolated RNA from cells that were actively dividing or serum-starved as described in Materials and Methods. RNA blot analysis of the resultant total cellular RNA probed for human UDG is shown in Fig. 2.3. UDG transcript was detected at high levels in subconfluent cells (lane 1) and was at levels below detection in confluent, serum-starved cells (lane 2). This result suggests that UDG is present at very low levels under our conditions of serum-starvation and is consistent with the idea that the human UDG enzyme may compensate for the lack of viral UDG in proliferating cells maintained under high serum conditions.

Kinetics of UL114 expression. These observation suggested that some form of uracil DNA glycosylase activity is required for the proper onset of viral DNA synthesis. I established a role for this protein during viral DNA replication by first determining when this gene is expressed in the viral life cycle. To determine whether this gene was regulated with temporal kinetics appropriate for a role in DNA replication, we isolated RNA at 4, 8, 12, 24, 48 and 72 hpi from HFF cells infected with wild-type Towne virus. A diagram of the HCMV genome and the probe used for UL114 transcript detection is presented in Fig. 2.4. The UL114 transcript was observed to accumulate by 4 hpi in the resultant total cell RNA. However, in cells treated for 9 hours (-1 to 8 hpi) with the protein synthesis inhibitor, cycloheximide, UL114 transcript was no longer present (Fig. 2.5A). Expression of this transcript increased over the course of viral infection and was resistant to treatment with the viral DNA replication inhibitor, phosphonoformate. The larger transcripts detected at late times of infection arise from readthrough transcription from genes upstream of UL114 as confirmed by a probe specific for UL115, an upstream ORF (data not shown). Our data demonstrate that UL114 transcription is dependent on *de novo* protein synthesis and is unaffected by PFA, an inhibitor of viral DNA polymerase. These results suggest that UL114 is expressed with early gene characteristics, consistent with a role for this ORF in viral DNA replication.

The same membrane was stripped and hybridized with a UL44 probe. Interestingly, UL44 transcript was not detected until 12 hpi — a full 8 hours after UL114 transcript was first detected (Fig. 2.5B). The expression of UL114 at an earlier time than a known HCMV DNA replication gene suggests that UL114 may act at a time preceding HCMV DNA elongation by the replication machinery.

UL114 acts prior to viral DNA elongation. The observation that UL114 was expressed early in the viral life cycle suggested that this ORF could be acting at either the initiation or elongation phase of DNA replication. To distinguish between these possibilities, we compared the rate of viral DNA accumulation of the parental AD169 virus with the UL114 insertion mutant, RC2620, under conditions of replication inhibition by PFA and following release from this drug block. PFA is a pyrophosphate analog that binds competitively with the viral DNA polymerase and thus inhibits the elongation step in DNA replication. HFF cells were maintained under serum starvation conditions as described in Materials and Methods. Following this treatment, cells were infected with RC2620 or AD169 at a high m.o.i. (5 p.f.u./cell) in the presence of PFA. At 72 hpi, PFA was removed from the culture medium by extensive medium changes and infection was allowed to proceed to 192 hpi. DNA blot analysis of the resultant total cellular DNA probed with a HCMV gene-specific probe is shown in Fig. 2.6 (A, B). Relative DNA synthesis was determined as described above. As predicted, DNA accumulation was not observed for both AD169 and RC2620 in the presence of PFA. Interestingly, RC2620 recovered DNA accumulation by 24 hours after PFA reversal and synthesized viral DNA with wild-type kinetics subsequent to release from this inhibitor. In contrast, the mutant did not reach wild-type levels of viral DNA synthesis until 120 hpi when cells were not first treated with PFA (Fig. 2.1 A, B). The ability of the mutant to replicate as well as wild-type AD169 following release from PFA demonstrates that UL114 is required for a step at or prior to the elongation phase of viral DNA synthesis.

Expression of human UDG in HCMV infected cells. Our data suggested that efficient viral DNA replication cannot begin in the absence of UDG activity. Yet following an initial delay in viral DNA replication, we observed that RC2620 was able to reach wild-type levels of viral DNA synthesized. Since this UDG activity could not be supplied by the mutant, we hypothesized that RC2620's recovery at late times could be due to expression of cellular UDG. To determine the effect of HCMV infection on cellular UDG transcript expression, we isolated RNA from HFF cells infected with wild-type AD169 virus at 0, 8 and 24 hpi. Similar levels of cellular UDG transcript were detected at 0 and 8 hpi (Fig. 2.7). A marked increase in the human UDG transcript was observed at 24 hpi and continued to 72 hpi (data not shown). A similar trend was also observed in RNA isolated from mutant virus-infected HFF cells (data not shown). The induction of cellular UDG only at 24 hpi compared with normal expression of UL114 by 4 hpi may explain the delay in RC2620 DNA synthesis in confluent cells. Our data demonstrates that HCMV infection induces cellular UDG expression and supports a role for human UDG in complementing the growth of a UL114 mutant.

DISCUSSION

Previous work from this laboratory had shown that a mutant HCMV deficient in UL114, the viral UDG homolog, was significantly delayed in the onset of viral replication (Prichard *et al.*, 1996). In this study, we report that the phenotype of a UL114 mutant HCMV is most dramatic in cells that have been serum starved prior to infection. Surprisingly, we found that UL114 was completely dispensible for viral growth when cells were actively dividing at the time of infection, suggesting that a cellular factor expressed in actively cycling cells may be able to substitute for the viral UDG in HCMV DNA replication.

We examined the human UDG enzyme as a possible candidate for the complementing activity for several reasons. First, uracil DNA glycosylase is a highly conserved repair enzyme found in many organisms (Arenaz and Sirover, 1983; Caradonna and Cheng, 1980; Sekiguchi *et al.*, 1976; Wist *et al.*, 1978; Wittwer *et al.*, 1989) and HCMV UL114 shares 40% sequence identity at the amino acid level with the human UDG, particularly in the active site residues of this enzyme (see Fig. 2.2). UDGs from several organisms have been shown to possess similar specificities for uracil excision from DNA (Arenaz and Sirover, 1983; Caradonna and Cheng, 1980; Ellison *et al.*, 1996; Focher *et al.*, 1993; Olsen *et al.*, 1991; Sekiguchi *et al.*, 1976; Winters and Williams, 1990; Wist *et al.*, 1978; Wittwer *et al.*, 1989) and it seems likely from the observed increase in uracil incorporation in RC2620 genomic DNA that UL114 is also involved in uracil excision. Furthermore, the expression of human UDG is known to be regulated by the cell cycle. Previous work has shown that human UDG is activated beginning late in the G_1 phase of the cell cycle and continuing into the early S phase with a half-life of 30 hours (Haug *et al.*, 1998; Nagelhus *et al.*, 1995; Slupphaug *et al.*, 1991). For these reasons, it seemed possible that if uracil DNA glycosylase activity was required for HCMV growth that the cellular enzyme could potentially substitute for deficient viral protein in proliferating cells. Consistent with this possibility, we found that high levels of cellular UDG transcript were present in actively dividing cells but transcript was not detectable in non-cycling, serum starved cells. We also observed that the human enzyme is induced at late times of viral infection, perhaps accounting for the ability of the mutant virus to synthesize DNA at later times. It would be interesting to know whether mutant virus can replicate at all in the absence of the human UDG. No human UDG mutants are currently available and attempts at human UDG knockouts have thus far been unsuccessful, suggesting that this activity is also essential for growth in human cells. The serum dependence of this mutant is, to our knowledge, the first observation that culture conditions and the replication state of the host cell can strongly influence the ability of HCMV to grow. Thus, these results suggest that our experimental cell culture conditions may be useful in the functional study and assessment of other recombinant mutant HCMV.

Our data suggest that the mutant in UL114 is not completely defective in DNA replication. Cell culture conditions in which high levels of human UDG are present appear to allow complementation of this defect, suggesting that UL114 is redundant for UDG activity and hence dispensible for HCMV replication. The situation in the host, however, may be different. HCMV associate with peripheral blood leukocytes during primary infection and these cells are believed to have roles in dissemination, pathogenesis and latency of the virus (Dankner *et al.*, 1990; Gerna *et al.*, 1992; Grundy *et al.*, 1998; Hahn *et al.*, 1998; Kondo *et al.*, 1994; Kondo and Mocarski, 1995; Revello *et al.*, 1992; Rice *et al.*, 1984; Saltzman *et al.*, 1988; Slobedman and Mocarski, 1999; Taylor-Wiedeman *et al.*, 1991; von Laer *et al.*, 1995). Similar to our observations in serum starved cells, previous studies have shown that human nuclear UDG transcript is below detectable levels in populations of peripheral blood leukocytes (Haug *et al.*, 1998). Therefore, while it appears that UL114 is a redundant activity in tissue culture, our results would suggest that this viral gene may be essential for HCMV replication *in vivo*. Indeed, different phenotypes have been reported for HSV-1 mutants in thymidine kinase and ribonucleotide reductase depending on viral growth conditions used (Daikoku *et al.*, 1991; Field and Wildy, 1978; Goldstein and Weller, 1988a; Goldstein and Weller, 1988b; Kit *et al.*, 1965; Preston *et al.*, 1984; Tenser *et al.*, 1979).

The observation that uracil DNA glycosylase may be essential for DNA replication is also suggested by observations in poxviruses and herpes simplex virus (Holzer and Falkner, 1997; Millns *et al.*, 1994; Pyles and Thompson, 1994a; Stuart *et al.*, 1993). A mutant of this gene in HSV-1 is attenuated in its ability to replicate in the mouse peripheral and central nervous system and does not reactivate from latency efficiently (Pyles and Thompson, 1994a). Members of the poxvirus family also encode a UDG which is essential for viral DNA replication (Holzer and Falkner, 1997; Millns *et al.*, 1994; Stuart *et al.*, 1993).

Based on the observations that RC2620 was delayed in the onset of viral DNA replication, Prichard and colleagues (Prichard *et al.*, 1996) proposed that UL114 function was required at an early step in the HCMV replication cycle. Consistent with this proposal, our results demonstrate that this gene is expressed with early gene kinetics and precedes the expression of UL44, a core component of the HCMV replication machinery. Furthermore, we show that the mutant is able to replicate as efficiently as wild-type virus following release from initial replication inhibition, suggesting that UL114 activity is likely required at initiation or early in elongation. We observed an induction of human UDG transcript following viral infection in RNA from HCMV-infected cells and suggest that this may complement the function of the UL114 protein in replication initiation. While these studies support the proposal that UL114 acts at initiation or early in elongation, additional experiments are required to elucidate the precise nature of this activity during DNA replication.

There are several ways that uracil DNA glycosylase activity could play a role in DNA replication. Some researchers have proposed that UDG may be needed to remove uracils from the viral genome and hence prepare the template for recognition by initiation factors. This view is supported by a study showing that the presence of uracil residues within the origin region of HSV-1 interferes with the ability of the origin binding protein to recognize and bind to this sequence (Focher *et al.,* 1992).

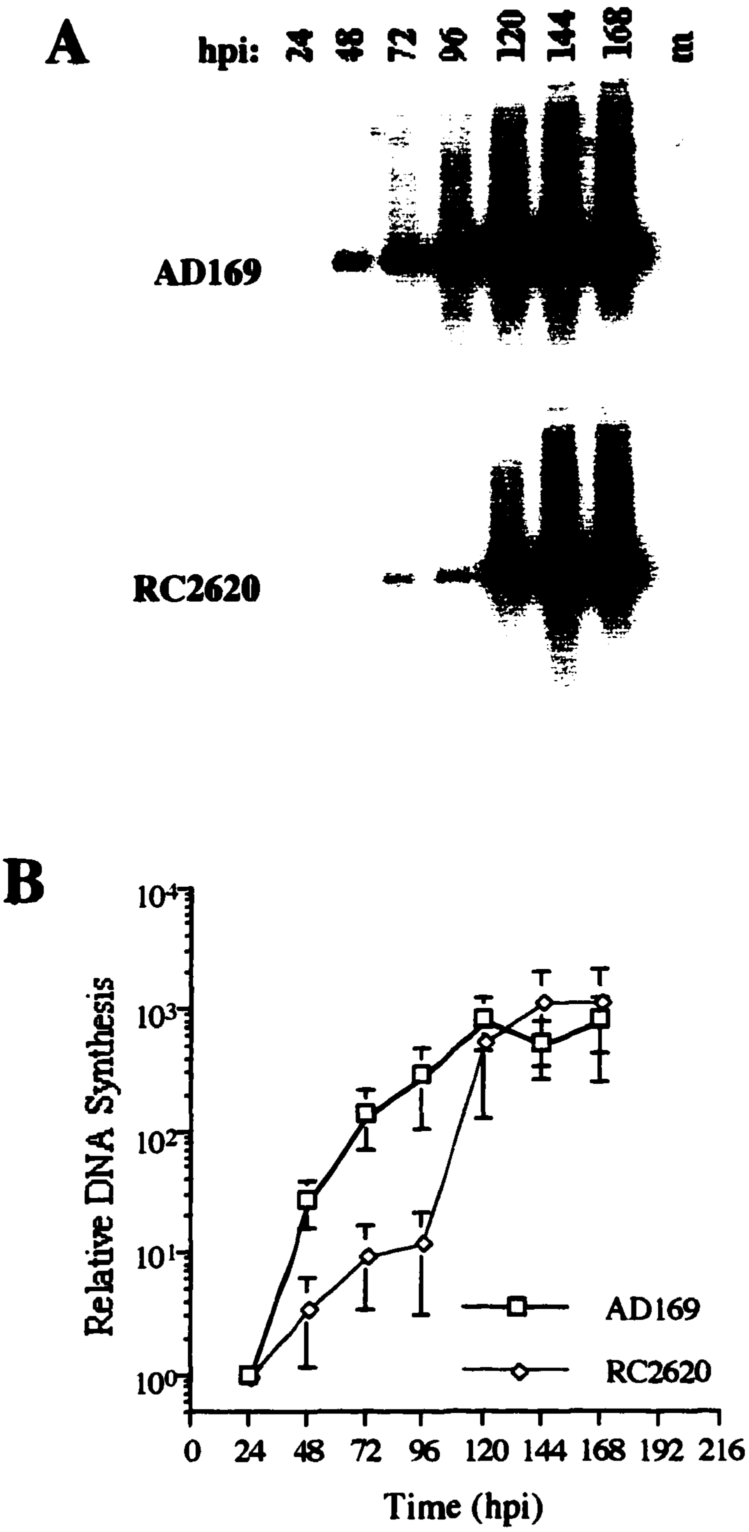
Other studies suggest that the uracil DNA glycosylase activity itself may play a more direct role in the replication process. The introduction of specific mutations at the active site residues of vaccinia virus UDG results in a loss of virus viability, despite the ability of these same mutated proteins to bind to DNA (Ellison *et al.,* 1996). Assuming that potential interactions between UDG and other proteins were not interrupted, this result would suggest that the critical function for this enzyme in vaccinia virus DNA replication is its ability to excise uracil. Previous work had shown that the RC2620 genome contained about threefold more uracils than the parental AD169 genome (Prichard *et al.,* 1996), consistent with the idea that UL114 provides UDG activity.

Alternatively, there are some provocative observations which suggest that the uracil DNA glycosylase could act by directly recruiting the DNA replication machinery to sites of initiation. The human UDG has been shown to interact with DNA polymerase α (Seal and Sirover, 1986) and UDG activity is associated with replicating DNA (Krokan, 1981; Lee and Sirover, 1989). Following binding to DNA, UDG may form a replication/repair complex that recruits replication machinery to these sites thus facilitating DNA synthesis. In support of this idea, the UL114 protein appears to associate with the HCMV DNA polymerase processivity factor, ppUL44 (Prichard, personal communication). However if the recruitment models are true, the mutagenesis studies in the poxvirus system would suggest that the excision event may also be required to start replication.

These latter possibilities are not mutually exclusive and are interesting to consider with respect to the highly recombinagenic nature of herpesvirus replication during lytic growth. Normal viral growth in HCMV and HSV is associated with high levels of DNA recombination with crossovers occurring throughout the viral genomes (McVoy and Adler, 1994; Sherman and Bachenheimer, 1987; Smiley *et al.,* 1981; Weber *et al.,* 1988). It has been proposed that these exchanges provide multiple sites for recombinational initiation of replication and serve as a mechanism for viral amplification. Given the association of pUL114 with the replication machinery, it is tempting to speculate that the nicks created by UDG activity may also serve as substrates for recombinational initiation. Such a role could also account for the observed delay in high level DNA replication in RC2620.

In the next chapter, we will examine the functional role of UL114 activity with special interest to the integrity of the viral genome during infection.

Fig. 2.1. Viral DNA accumulation in wild-type AD169 and mutant RC2620 infected confluent, serum starved cells (A, B) or subconfluent monolayers (C, D). (A, C) DNA blot hybridizations of viral DNA from 10^5 cells infected with AD169 or RC2620 isolated at the indicated times post-infection. The membrane was probed with ^{32}P-radiolabeled pON2260, specific for HCMV nucleotides 122699-124902. (B, D) Densitometry for each sample was determined using ImageQuant software (Molecular Dynamics) and expressed as a ratio of density at each time point to density at 24 hpi (or input DNA) for AD169 and RC2620. Error bars represent standard deviation of the geometric mean of three replicate samples.


A
hpi: 24 48 72 96 120 144 168 m
AD169
RC2620
B
Relative DNA Synthesis
10^4
10^3
10^2
10^1
10^0
0 24 48 72 96 120 144 168 192 216
Time (hpi)
AD169
RC2620

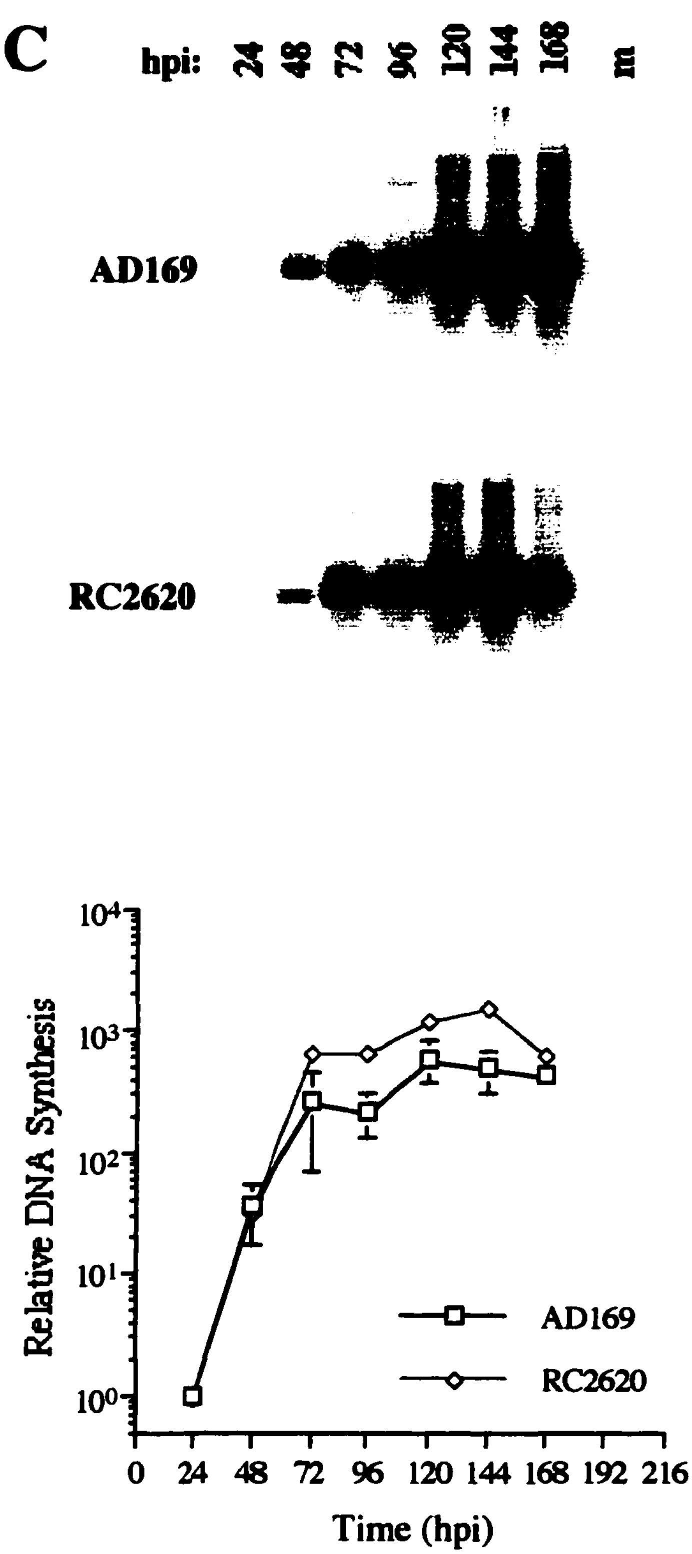
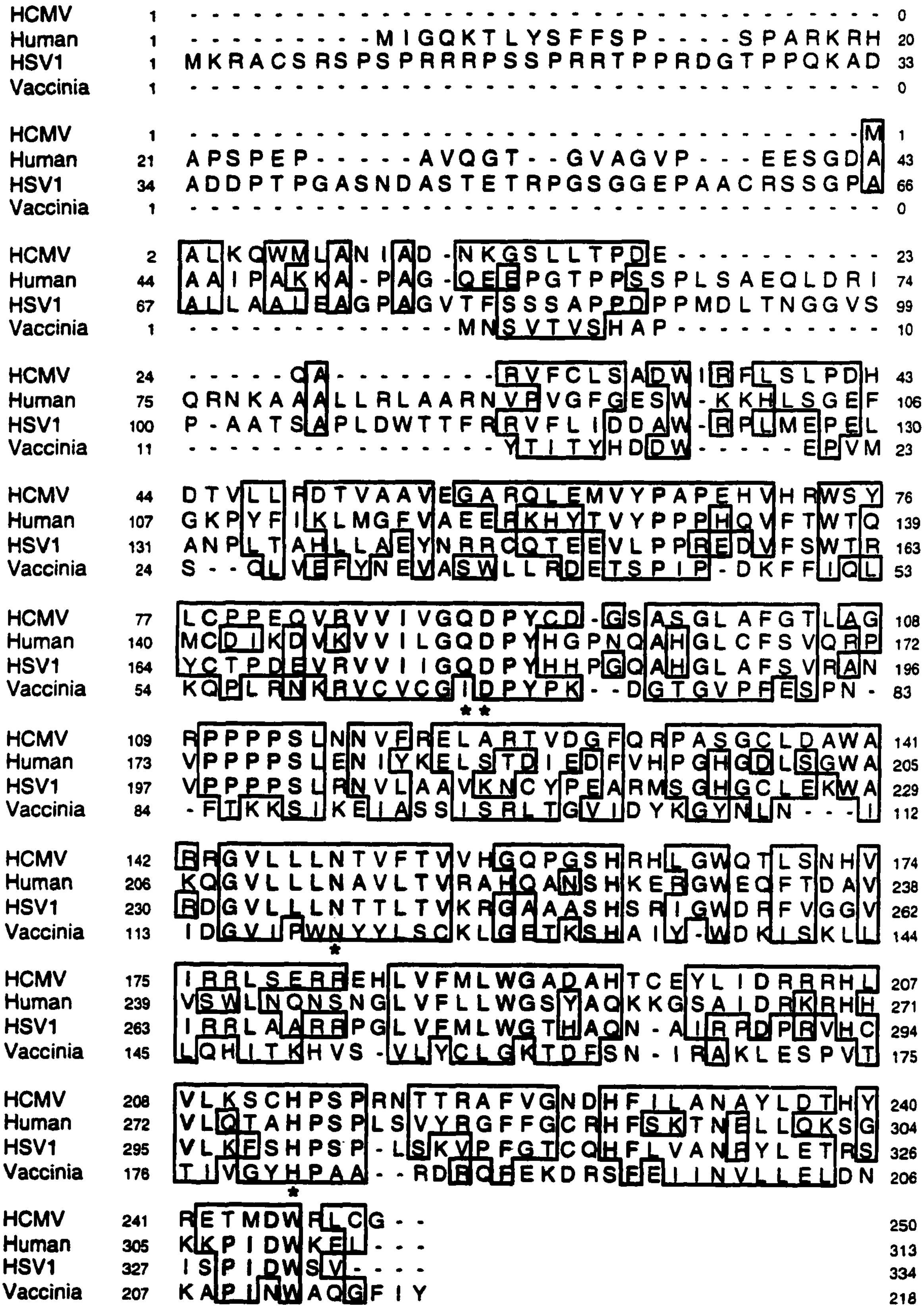

C
hpi:
24
48
72
96
120
144
168
m
AD169
RC2620
D
Relative DNA Synthesis
10⁴
10³
10²
10¹
10⁰
0
24
48
72
96
120
144
168
192
216
Time (hpi)
AD169
RC2620

Fig. 2.2. Sequence alignment for UDG proteins encoded by human CMV, *Homo sapiens*, HSV-1 and vaccinia virus using MCB Search Launcher program (Human Genome Center, Baylor College of Medicine) and assembled using SeqVu 1.0.1 (Garvan Institute of Medical Research, Sydney, Australia). The regions of identity are shaded; regions of homology are shown in blocks. Asterisks denote the residues which abrogate UDG activity in human UDG enzyme (Mol *et al.*, 1995).



```
HCMV        1  --------------------------------- 0
Human       1  ---------MIGQKTLYSFFSP----SPARKRH 20
HSV1        1  MKRACSRSPSPRRRPSSPRRTPPRDGTPPQKAD 33
Vaccinia    1  --------------------------------- 0

HCMV        1  --------------------------------M 1
Human      21  APSPEP-----AVQGT--GVAGVP---EESGDA 43
HSV1       34  ADDPTPGASNDASTETRPGSGGEPAACRSSGPA 66
Vaccinia    1  --------------------------------- 0

HCMV        2  ALKQWMLANIAD-NKGSLLTPDE---------- 23
Human      44  AAIPAKKA-PAG-QEEPGTPPSSPLSAEQLDRI 74
HSV1       67  ALLAALEAGPAGVTFSSSAPPDPPMDLTNGGVS 99
Vaccinia    1  -------------MNSVTVSHAP---------- 10

HCMV       24  -----QA--------RVFCLSADWIRFLSLPDH 43
Human      75  QRNKAAALLRLAARNVPVGFGESW-KKHLSGEF 106
HSV1      100  P-AATSAPLDWTTFRRVFLIDDAW-RPLMEPEL 130
Vaccinia   11  ---------------YTITYHDDW-----EPVM 23

HCMV       44  DTVLLRDTVAAVEGARQLEMVYPAPEHVHRWSY 76
Human     107  GKPYFIKLMGFVAEERKHYTVYPPPHQVFTWTQ 139
HSV1      131  ANPLTAHLLAEYNRRCQTEEVLPPREDVFSWTR 163
Vaccinia   24  S--QLVEFYNEVASWLLRDETSPIP-DKFFIQL 53

HCMV       77  LCPPEQVRVVIVGQDPYCD-GSASGLAFGTLAG 108
Human     140  MCDIKDVKVVILGQDPYHGPNQAHGLCFSVQRP 172
HSV1      164  YCTPDEVRVVIIGQDPYHHPGQAHGLAFSVRAN 196
Vaccinia   54  KQPLRNKRVCVCGIDPYPK--DGTGVPFESPN- 83
                            **

HCMV      109  RPPPPSLNNVFRELARTVDGFQRPASGCLDAWA 141
Human     173  VPPPPSLENIYKELSTDIEDFVHPGHGDLSGWA 205
HSV1      197  VPPPPSLRNVLAAVKNCYPEARMSGHGCLEKWA 229
Vaccinia   84  -FTKKSIKEIASSISRLTGVIDYKGYNLN---I 112

HCMV      142  RRGVLLLNTVFTVVHGQPGSHRHLGWQTLSNHV 174
Human     206  KQGVLLLNAVLTVRAHQANSHKERGWEQFTDAV 238
HSV1      230  RDGVLLLNTTLTVKRGAAASHSRIGWDRFVGGV 262
Vaccinia  113  IDGVIFWNYYLSCKLGETKSHAIY-WDKISKLL 144
                      *

HCMV      175  IRRLSERREHLVFMLWGADAHTCEYLIDRRRHL 207
Human     239  VSWLNQNSNGLVFLLWGSYAQKKGSAIDRKRHH 271
HSV1      263  IRRLAARRPGLVFMLWGTHAQN-AIRRDPRVHC 294
Vaccinia  145  LQHITKHVS-VLYCLGKTDFSN-IRAKLESPVT 175

HCMV      208  VLKSCHPSPRNTTRAFVGNDHFILANAYLDTHY 240
Human     272  VLQTAHPSPLSVYRGFFGCRHFSKTNELLQKSG 304
HSV1      295  VLKFSHPSP-LSKVPFGTCQHFLVANRYLETRS 326
Vaccinia  176  TIVGYHPAA--RDRQREKDRSFEIINVLLELDN 206
                    *

HCMV      241  RETMDWRLCG--                      250
Human     305  KKPIDWKEL---                      313
HSV1      327  ISPIDWSV----                      334
Vaccinia  207  KAPINWAQGFIY                      218
```

Fig. 2.3. RNA blot of total cellular RNA isolated from uninfected actively dividing cells (lane 1) and confluent, serum starved cells (lane 2), probed with biotin-psoralen labeled anti-sense riboprobe to human UDG and visualized with streptavidin-alkaline phosphatase as detailed in Materials and Methods. The arrow indicates the position of the UDG transcript; positions of the molecular weight markers are indicated on the left in kilobases.

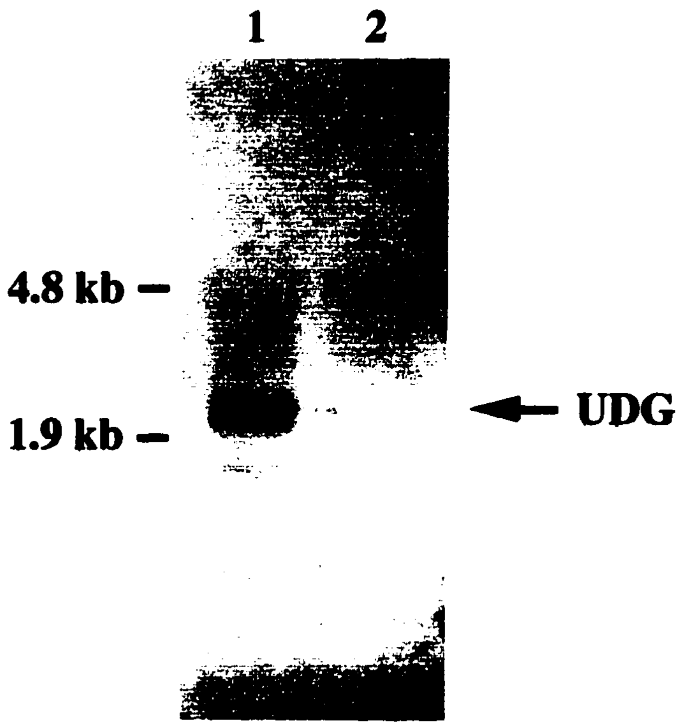

1
2
4.8 kb
1.9 kb
UDG

Fig. 2.4. Map of the UL114-119 region of the CMV genome. The top line represents a *Hin*dIII map of the AD169 genome, with the region containing UL114 (nucleotides 162973 to 168037) expanded below. The open-faced arrows denote the primers used to generate the UL114 anti-sense riboprobe. Predicted TATA boxes and polyadenylation signals are noted above putative transcripts. Previously mapped transcripts for the upstream UL115-119 ORFs are indicated in the bottom panel.

a (Leatham *et al.*, 1991).

b Transcripts that are sensitive to inhibitors of viral DNA synthesis (PFA, data not shown).

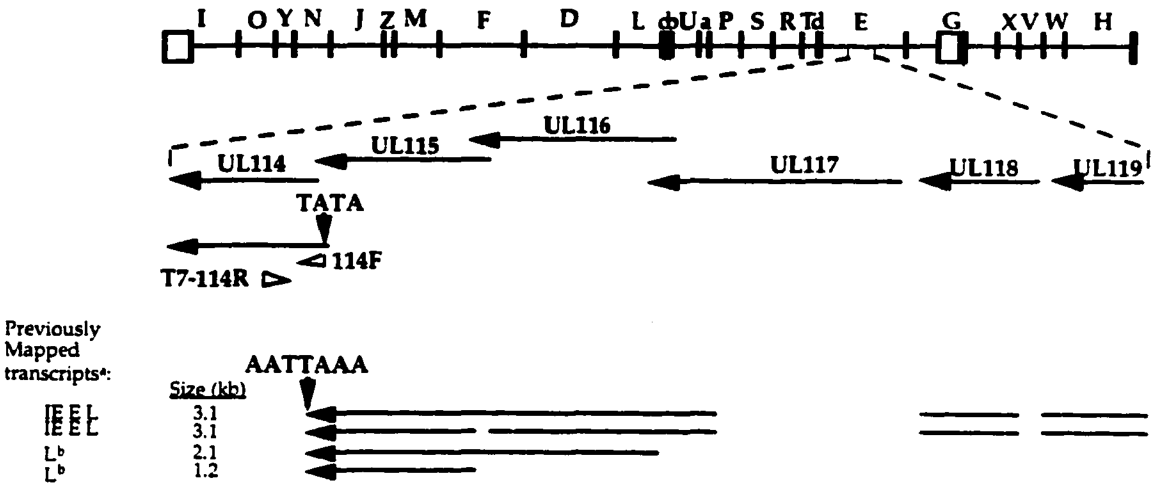

I O Y N J Z M F D L b U a P S R T d E G X V W H
UL116
UL115
UL114
UL117
UL118
UL119
TATA
114F
T7-114R
Previously Mapped transcripts^a:
AATTAAA
Size (kb)
IE E L 3.1
IE E L 3.1
L^b 2.1
L^b 1.2

Fig. 2.5. RNA blot analyses of total cellular RNA isolated from wild-type Towne-infected HFF cells at the indicated times post-infection. CH denotes cycloheximide treatment for 9 hours (-1 to 8 hpi); PFA denotes phosphonoformate treatment. (A) Membrane was hybridized with a biotin-psoralen labeled UL114-specific anti-sense riboprobe. Position of the UL114 transcript is indicated by the arrow. (B) Membrane shown in panel A was stripped and probed with UL44-specific anti-sense riboprobe. Position of the UL44 transcript is indicated by the arrow. Positions of the molecular weight markers are indicated on the left in kilobases. (C) Membrane shown in panels A and B was stripped and probed with 18S-specific riboprobe as a control for loading.

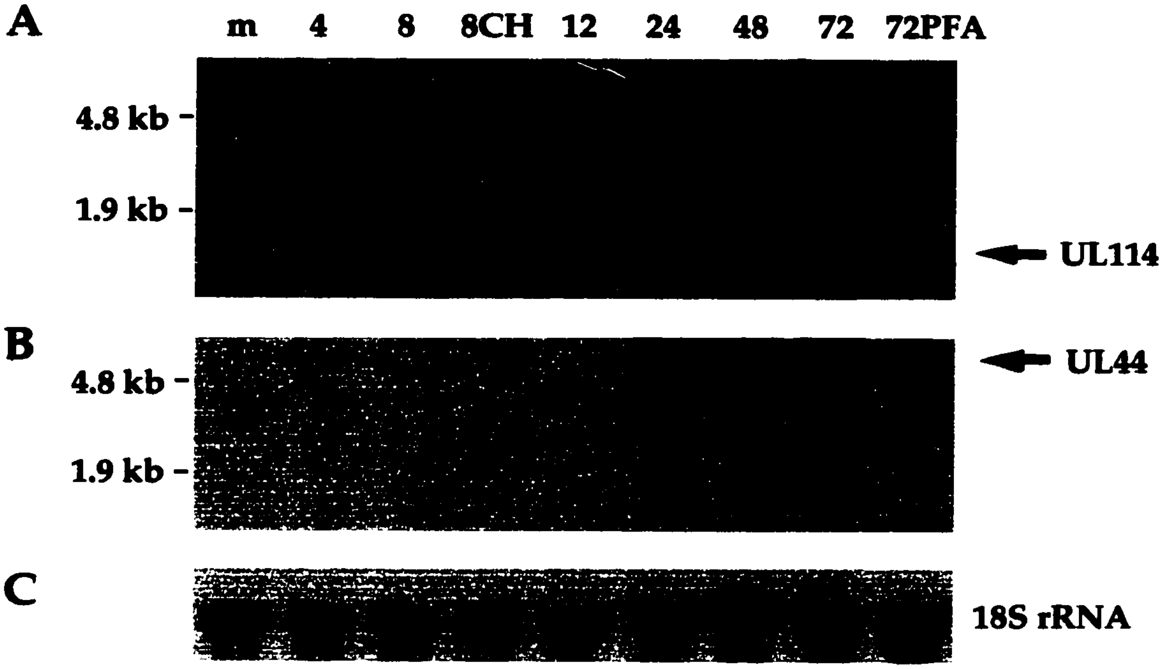

A
m
4
8
8CH
12
24
48
72
72PFA
4.8 kb
1.9 kb
UL114
B
4.8 kb
1.9 kb
UL44
C
18S rRNA

Fig. 2.6. Viral DNA accumulation in wild-type AD169 and mutant RC2620 infected confluent, serum starved cells treated with PFA and released from inhibition at 72 hpi. (A) DNA blot of total cellular DNA isolated from 10^5 cells infected with AD169 or RC2620 at the indicated times post-infection, separated on 0.7% agarose gel and transfered to nitrocellulose, membrane was probed with ^{32}P-radiolabeled pON2260 (nucleotides 122699-124902). (B) Densitometry for each sample determined using ImageQuant software (Molecular Dynamics) and expressed as a ratio of density at each time point to density at 24 hpi (or input DNA) for AD169 (□) and RC2620 (◊). Length of PFA treatment indicated by the open box. Error bars represent standard deviation of the geometric mean of three replicate samples.

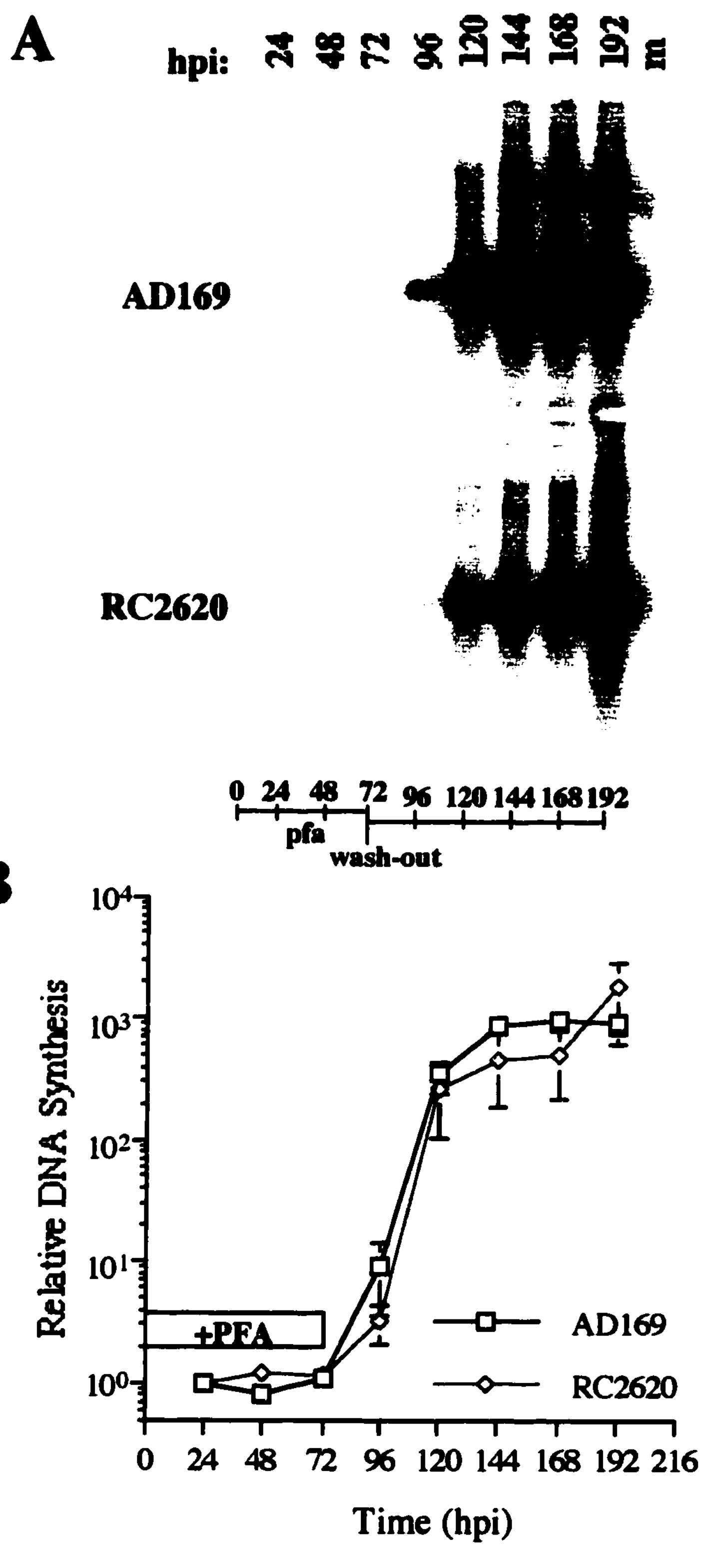
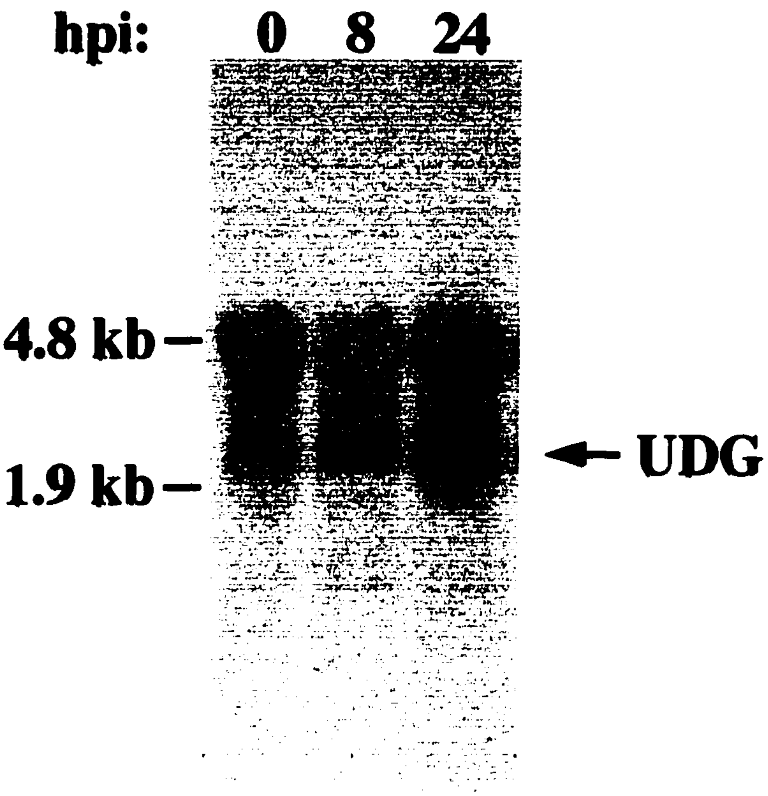

A
hpi:
24
48
72
96
120
144
168
192
m
AD169
RC2620
0
24
48
72
96
120
144
168
192
pfa
wash-out
B
Relative DNA Synthesis
10^4
10^3
10^2
10^1
10^0
+PFA
AD169
RC2620
0
24
48
72
96
120
144
168
192
216
Time (hpi)

Fig. 2.7. RNA blot of total cellular RNA isolated from uninfected (0 hpi) and wild-type Towne virus infected HFF cells at 8 and 24 hpi. Membrane was hybridized with anti-sense riboprobe to human UDG; the arrow indicates the position of the cellular UDG transcript. Positions of the molecular weight markers are indicated on the left in kilobases.


hpi:
0
8
24
4.8 kb
1.9 kb
UDG

REFERENCES

Arenaz, P. and Sirover, M.A. (1983) Isolation and characterization of monoclonal antibodies directed against the DNA repair enzyme uracil DNA glycosylase from human placenta. *Proc Natl Acad Sci U S A*, **80**, 5822-5826.

Brynolf, K., Eliasson, R. and Reichard, P. (1978) Formation of Okazaki fragments in polyoma DNA synthesis caused by misincorporation of uracil. *Cell*, **13**, 573-580.

Caradonna, S.J. and Cheng, Y.C. (1980) Uracil DNA-glycosylase. Purification and properties of this enzyme isolated from blast cells of acute myelocytic leukemia patients. *J Biol Chem*, **255**, 2293-2300.

Chee, M.S., Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerny, R., Horsnell, T., Hutchison, C.A.I., Kouzarides, T., Martignetti, J.A., Preddie, E., Satchwell, S.C., Tomlinson, P., Weston, K.M. and Barrell, B.G. (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol*, **154**, 125-170.

Daikoku, T., Yamamoto, N., Maeno, K. and Nishiyama, Y. (1991) Role of viral ribonucleotide reductase in the increase of dTTP pool size in herpes simplex virus-infected Vero cells. *J Gen Virol*, **72**, 1441-1444.

Dankner, W.M., McCutchan, J.A., Richman, D.D., Hirata, K. and Spector, S.A. (1990) Localization of human cytomegalovirus in peripheral blood leukocytes by in situ hybridization. *J Infect Dis*, **161**, 31-36.

Ellison, K.S., Peng, W. and McFadden, G. (1996) Mutations in active-site residues of the uracil-DNA glycosylase encoded by vaccinia virus are incompatible with virus viability. *J Virol*, **70**, 7965-7973.

Field, H.J. and Wildy, P. (1978) The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice. *J Hyg (Lond)*, **81**, 267-277.

Fleckenstein, B., Muller, I. and Collins, J. (1982) Cloning of the complete human cytomegalovirus genome in cosmids. *Gene*, **18**, 39-46.

Focher, F., Verri, A., Spadari, S., Manservigi, R., Gambino, J. and Wright, G.E. (1993) Herpes simplex virus type 1 uracil-DNA glycosylase: isolation and selective inhibition by novel uracil derivatives. *Biochem J*, **292**, 883-889.

Focher, F., Verri, A., Verzeletti, S., Mazzarello, P. and Spadari, S. (1992) Uracil in OriS of herpes simplex 1 alters its specific recognition by origin binding protein (OBP): does virus induced uracil-DNA glycosylase play a key role in viral reactivation and replication? *Chromosoma*, **102**, S67-71.

Gerna, G., Zipeto, D., Percivalle, E., Parea, M., Revello, M.G., Maccario, R., Peri, G. and Milanesi, G. (1992) Human cytomegalovirus infection of the major leukocyte subpopulations and evidence for initial viral replication in polymorphonuclear leukocytes from viremic patients. *J Infect Dis*, **166**, 1236-1244.

Goldstein, D.J. and Weller, S.K. (1988a) Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. *Virology*, **166**, 41-51.

Goldstein, D.J. and Weller, S.K. (1988b) Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. *J Virol*, **62**, 196-205.

Grundy, J.E., Lawson, K.M., MacCormac, L.P., Fletcher, J.M. and Yong, K.L. (1998) Cytomegalovirus-infected endothelial cells recruit neutrophils by the secretion of C-X-C chemokines and transmit virus by direct neutrophil-endothelial cell contact and during neutrophil transendothelial migration. *J Infect Dis*, **177**, 1465-1474.

Hahn, G., Jores, R. and Mocarski, E.S. (1998) Cytomegalovirus remains latent in a common progenitor of dendritic and myeloid cells. *Proc. Natl. Acad. Sci. USA*, (in press).

Haug, T., Skorpen, F., Aas, P.A., Malm, V., Skjelbred, C. and Krokan, H.E. (1998) Regulation of expression of nuclear and mitochondrial forms of human uracil-DNA glycosylase. *Nucleic Acids Res*, **26**, 1449-1457.

Holzer, G.W. and Falkner, F.G. (1997) Construction of a vaccinia virus deficient in the essential DNA repair enzyme uracil DNA glycosylase by a complementing cell line. *J Virol*, **71**, 4997-5002.

Kit, S., Dubbs, D.R., DeTorres, R.A. and Melnick, J.L. (1965) Enhanced thymidine kinase activity following infection of green monkey kidney cells by simian adenoviruses, simian papovavirus SV40, and an adenovirus-SV40 "hybrid". *Virology*, **27**, 453-457.

Kondo, K., Kaneshima, H. and Mocarski, E.S. (1994) Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. *Proc Natl Acad Sci U S A*, **91**, 11879-11883.

Kondo, K. and Mocarski, E.S. (1995) Cytomegalovirus latency and latency-specific transcription in hematopoietic progenitors. *Scand J Infect Dis Suppl*, **99**, 63-67.

Krokan, H. (1981) Preferential association of uracil-DNA glycosylase activity with replicating SV40 minichromosomes. *FEBS Lett*, **133**, 89-91.

Krokan, H., Haugen, A., Myrnes, B. and Guddal, P.H. (1983) Repair of premutagenic DNA lesions in human fetal tissues: evidence for low levels of O6-methylguanine-DNA methyltransferase and uracil-DNA glycosylase activity in some tissues. *Carcinogenesis*, **4**, 1559-1564.

Leatham, M.P., Witte, P.R. and Stinski, M.F. (1991) Alternate promoter selection within a human cytomegalovirus immediate-early and early transcription unit (UL119-115) defines true late transcripts containing open reading frames for putative viral glycoproteins. *J Virol*, **65**, 6144-6153.

Lee, K.A. and Sirover, M.A. (1989) Physical association of base excision repair enzymes with parental and replicating DNA in BHK-21 cells. *Cancer Res*, **49**, 3037-3044.

Lindahl, T. and Nyberg, B. (1974) Heat-induced deamination of cytosine residues in deoxyribonucleic acid. *Biochemistry*, **13**, 3405-3410.

McVoy, M.A. and Adler, S.P. (1994) Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. *J Virol*, **68**, 1040-1051.

Millns, A.K., Carpenter, M.S. and DeLange, A.M. (1994) The vaccinia virus-encoded uracil DNA glycosylase has an essential role in viral DNA replication. *Virology*, **198**, 504-513.

Mocarski, E.S., Bonyhadi, M., Salimi, S., McCune, J.M. and Kaneshima, H. (1993) Human cytomegalovirus in a SCID-hu mouse: thymic epithelial cells are prominent targets of viral replication. *Proc Natl Acad Sci U S A*, **90**, 104-108.

Mol, C.D., Arvai, A.S., Slupphaug, G., Kavli, B., Alseth, I., Krokan, H.E. and Tainer, J.A. (1995) Crystal structure and mutational analysis of human uracil-DNA glycosylase: structural basis for specificity and catalysis [see comments]. *Cell*, **80**, 869-878.

Mullaney, J., Moss, H.W. and McGeoch, D.J. (1989) Gene UL2 of herpes simplex virus type 1 encodes a uracil-DNA glycosylase. *J Gen Virol*, **70**, 449-454.

Muller-Weeks, S., Mastran, B. and Caradonna, S. (1998) The nuclear isoform of the highly conserved human uracil-DNA glycosylase is an Mr 36,000 phosphoprotein. *J Biol Chem*, **273**, 21909-21917.

Myrnes, B., Giercksky, K.E. and Krokan, H. (1983) Interindividual variation in the activity of O6-methyl guanine-DNA methyltransferase and uracil-DNA glycosylase in human organs. *Carcinogenesis*, **4**, 1565-1568.

Nagelhus, T.A., Slupphaug, G., Lindmo, T. and Krokan, H.E. (1995) Cell cycle regulation and subcellular localization of the major human uracil-DNA glycosylase. *Exp Cell Res*, **220**, 292-297.

Olsen, L.C., Aasland, R., Krokan, H.E. and Helland, D.E. (1991) Human uracil-DNA glycosylase complements E. coli ung mutants. *Nucleic Acids Res*, **19**, 4473-4478.

Pari, G.S. and Anders, D.G. (1993) Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. *J Virol*, **67**, 6979-6988.

Pari, G.S., Kacica, M.A. and Anders, D.G. (1993) Open reading frames UL44, IRS1/TRS1, and UL36-38 are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA synthesis. *J Virol*, **67**, 2575-2582.

Percival, K.J., Klein, M.B. and Burgers, P.M. (1989) Molecular cloning and primary structure of the uracil-DNA-glycosylase gene from Saccharomyces cerevisiae. *J Biol Chem*, **264**, 2593-2598.

Preston, V.G., Palfreyman, J.W. and Dutia, B.M. (1984) Identification of a herpes simplex virus type 1 polypeptide which is a component of the virus-induced ribonucleotide reductase. *J Gen Virol*, **65**, 1457-1466.

Prichard, M.N., Duke, G.M. and Mocarski, E.S. (1996) Human cytomegalovirus uracil DNA glycosylase is required for the normal temporal regulation of both DNA synthesis and viral replication. *J Virol*, **70**, 3018-3025.

Pyles, R.B. and Thompson, R.L. (1994a) Evidence that the herpes simplex virus type 1 uracil DNA glycosylase is required for efficient viral replication and latency in the murine nervous system. *J Virol*, **68**, 4963-4972.

Pyles, R.B. and Thompson, R.L. (1994b) Mutations in accessory DNA replicating functions alter the relative mutation frequency of herpes simplex virus type 1 strains in cultured murine cells. *J Virol*, **68**, 4514-4524.

Revello, M.G., Percivalle, E., Di, M.A., Morini, F. and Gerna, G. (1992) Nuclear expression of the lower matrix protein of human cytomegalovirus in peripheral blood leukocytes of immunocompromised viraemic patients. *J Gen Virol.*

Rice, G.P., Schrier, R.D. and Oldstone, M.B. (1984) Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. *Proc Natl Acad Sci U S A*, **81**, 6134-6138.

Saltzman, R.L., Quirk, M.R. and Jordan, M.C. (1988) Disseminated cytomegalovirus infection. Molecular analysis of virus and leukocyte interactions in viremia. *J Clin Invest*, **81**, 75-81.

Seal, G. and Sirover, M.A. (1986) Physical association of the human base-excision repair enzyme uracil DNA glycosylase with the 70,000-dalton catalytic subunit of DNA polymerase alpha. *Proc Natl Acad Sci U S A*, **83**, 7608-7612.

Sekiguchi, M., Hayakawa, H., Makino, F., Tanaka, K. and Okada, Y. (1976) A human enzyme that liberates uracil from DNA. *Biochem Biophys Res Commun*, **73**, 293-299.

Shapiro, R. (1980) In Kleppe, E.S.a.K. (ed.) *Chromosome Damage and Repair.* Plenum Press, New York, pp. 3-18.

Sherman, G. and Bachenheimer, S.L. (1987) DNA processing in temperature-sensitive morphogenic mutants of HSV-1. *Virology*, **158**, 427-430.

Slobedman, B. and Mocarski, E.S. (1999) Quantitative analysis of latent human cytomegalovirus. *J Virol*, **73**, 4806-4812.

Slupphaug, G., Olsen, L.C., Helland, D., Aasland, R. and Krokan, H.E. (1991) Cell cycle regulation and in vitro hybrid arrest analysis of the major human uracil-DNA glycosylase. *Nucleic Acids Res*, **19**, 5131-5137.

Smiley, J.R., Fong, B.S. and Leung, W.C. (1981) Construction of a double-jointed herpes simplex viral DNA molecule: inverted repeats are required for segment inversion, and direct repeats promote deletions. *Virology*, **113**, 345-362.

Spaete, R.R. and Mocarski, E.S. (1985) Regulation of cytomegalovirus gene expression: α and β promoters are trans activated by viral functions in permissive human fibroblasts. *J Virol*, **56**, 135-143.

Stuart, D.T., Upton, C., Higman, M.A., Niles, E.G. and McFadden, G. (1993) A poxvirus-encoded uracil DNA glycosylase is essential for virus viability. *J Virol*, **67**, 2503-2512.

Taylor-Wiedeman, J., Sissons, J.G., Borysiewicz, L.K. and Sinclair, J.H. (1991) Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J Gen Virol*, **72**, 2059-2064.

Tenser, R.B., Miller, R.L. and Rapp, F. (1979) Trigeminal ganglion infection by thymidine kinase-negative mutants of herpes simplex virus. *Science*, **205**, 915-917.

Tye, B.K. and Lehman, I.R. (1977) Excision repair of uracil incorporated in DNA as a result of a defect in dUTPase. *J Mol Biol*, **117**, 293-306.

Varshney, U., Hutcheon, T. and van de Sande, J.H. (1988) Sequence analysis, expression, and conservation of Escherichia coli uracil DNA glycosylase and its gene (ung). *J Biol Chem*, **263**, 7776-7784.

von Laer, D., Meyer-Koenig, U., Serr, A., Finke, J., Kanz, L., Fauser, A.A., Neumann-Haefelin, D., Brugger, W. and Hufert, F.T. (1995) Detection of cytomegalovirus DNA in CD34+ cells from blood and bone marrow. *Blood*, **86**, 4086-4090.

Weber, P.C., Challberg, M.D., Nelson, N.J., Levine, M. and Glorioso, J.C. (1988) Inversion events in the HSV-1 genome are directly mediated by the viral DNA replication machinery and lack sequence specificity. *Cell*, **54**, 369-381.

Winters, T.A. and Williams, M.V. (1990) Use of the PBS2 uracil-DNA glycosylase inhibitor to differentiate the uracil-DNA glycosylase activities encoded by herpes simplex virus types 1 and 2. *J Virol Methods*, **29**, 233-242.

Wist, E., Unhjem, O. and Krokan, H. (1978) Accumulation of small fragments of DNA in isolated HeLa cell nuclei due to transient incorporation of dUMP. *Biochim Biophys Acta*, **520**, 253-270.

Wittwer, C.U., Bauw, G. and Krokan, H.E. (1989) Purification and determination of the NH2-terminal amino acid sequence of uracil-DNA glycosylase from human placenta. *Biochemistry*, **28**, 780-784.

Chapter 3: Uracil incorporation into HCMV DNA and its consequences.

ABSTRACT

The uracil DNA glycosylase, made from the UL114 gene, of human cytomegalovirus (HCMV) is required for efficient viral DNA replication. A recombinant HCMV, RC2620, carrying a large deletion in this gene fails to replicate in a timely manner and exhibits a prolonged growth cycle compared with wild-type virus (Prichard *et al.*, 1996). Although the function of this repair enzyme in viral DNA replication is not well understood, uracil excision may play some role since human uracil DNA glycosylase appears to substitute for UL114 (Chapter 2). In trying to understand how this activity might contribute to DNA replication, we examined the uracil content and genomic integrity of HCMV during infection. Interestingly, we found that wild-type HCMV strain AD169 incorporates uracils into its genome at the start of high level DNA replication and during viral DNA amplification. The incorporated uracil was removed prior to packaging of the wild-type viral genome and was below limits of detection in virus particles, suggesting that the uracil DNA glycosylase activity of UL114 may act at the transition to or during late phase DNA replication. Consistent with this idea, we found that mutant virus particles "cured" of uracils through passage in a UL114-complementing cell line still exhibited a delay in the onset of high level viral DNA synthesis compared with wild-type virus. In addition, we found that the frequency of uracil incorporation into mutant virus particles was similar to that observed for wild-type virus particles, further suggesting that uracil DNA glycosylase activity is not required prior to initiation of DNA replication but at a later step. Based on these observations, we propose a model in which UL114 creates substrates for initiation of late phase DNA amplification through excision of uracils incorporated in the early rounds of DNA replication.

INTRODUCTION

Uracil incorporation into DNA can arise through the misincorporation of dUTP by DNA polymerase (Brynolf *et al.*, 1978; Tye and Lehman, 1977; Wist *et al.*, 1978) or from spontaneous deamination of cytosine (Lindahl and Nyberg, 1974; Shapiro, 1980). The latter reaction is potentially mutagenic if left unrepaired before the next round of DNA replication as it results in a GC to AT transition. To avoid such genetic damage, free-living organisms — such as *E. coli*, yeast and humans — encode the DNA repair enzyme uracil DNA glycosylase (UDG) (Krokan *et al.*, 1983; Myrnes *et al.*, 1983; Percival *et al.*, 1989; Varshney *et al.*, 1988) for excision of this errant base from DNA.

Interestingly, this repair enzyme is also encoded by all large DNA viruses, including the herpesvirus family, and appears to play a critical role in the replication of these viruses. Like other members of the herpesvirus family, cytomegalovirus (CMV) encodes a homolog of the UDG enzyme which shares approximately 40% sequence identity at the amino acid level with the human protein (see Fig. 2.2). A recombinant HCMV, RC2620, carrying a large deletion in UL114, the viral UDG gene, had previously been isolated in our laboratory (Prichard *et al.*, 1996). Analysis of this mutant demonstrated that RC2620 had a prolonged replication cycle corresponding to restriction in a step prior to or at the elongation phase of viral DNA synthesis (see Chapter 2).

While it is clear that the HCMV UDG plays an important role in the replication of this virus, the precise nature of this activity remains thus far unknown. The presence of uracil in DNA templates has not been found to inhibit any of the DNA polymerases tested so far, suggesting that the defect in RC2620 is not due to a direct block of DNA replication fork progression (Trower, 1994). The inability of uracils in DNA to inhibit replication, notwithstanding, several studies have suggested that the ability of UDG to excise uracils is required for replication to proceed. Active site mutations in the vaccinia virus UDG prevent viral DNA replication despite normal expression of early viral genes (Ellison *et al.*, 1996). Furthermore, our previous studies using the HCMV UDG mutant have correlated the timely onset of viral DNA replication with expression of the human UDG, suggesting that this uracil excision activity can complement the defect in RC2620 (Chapter 2).

Some researchers have proposed that UDG is required for excision of uracil prior to recognition by initiation factors in replication. In this scenario, extremely high uracil loads in the viral genome inhibit replication initiation from occuring. Consistent with this possibilty, Focher and colleagues found that uracil residues in the origin sequence of HSV-1 alter the recognition and binding potential of the viral origin binding protein (Focher *et al.*, 1992). Also consistent with this idea is our previous finding that RC2620 accumulates more uracils in its genome than wild-type virus (Prichard *et al.*, 1996).

Alternatively, UDG activity may play a more direct role in DNA replication than a simple repair function. Some studies have uncovered direct interactions between UDG and replication factors, suggesting a role for this protein in recruitment of the replication machinery to sites of initiation. Human UDG is known to interact with DNA polymerase α (Seal and Sirover, 1986) and UDG activity is closely associated with replicating DNA (Krokan, 1981; Lee and Sirover, 1989). It may be that UDG binding to DNA results in the formation of a replication/repair complex which then allows initiation of DNA synthesis at these sites. UL114 has been shown to physically associate with the HCMV DNA polymerase accessory protein (Prichard, personal communication), lending creedence to such a role for this gene.

To distinguish between these models and to better understand the functional role of uracil DNA glycosylase in HCMV DNA replication, we have characterized the uracil content and genomic integrity of wild-type and mutant viruses during infection.

MATERIALS AND METHODS

Cells and virus. Primary human foreskin fibroblasts (HFFs) and human embryonic lung fibroblasts (HELs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% NuSerum I (Collaborative Research Inc.), 100 units of penicillin G per ml, 100 μg of streptomycin sulfate per ml, 0.58 mg L-arginine per ml, 1.08 mg L-glutamine per ml, and 180 μg L-asparagine per ml. PA317 cells (Halbert *et al.*, 1991) were a kind gift of Denise Galloway, and were maintained in medium with 10% fetal calf serum.

Human CMV strain, AD169 was obtained and cultured as previously described (Mocarski *et al.*, 1993; Spaete and Mocarski, 1985). The recombinant human CMV, RC2620, was described previously (Prichard *et al.*, 1996).

Plasmids. Plasmid pON2260 was constructed by ligating a 7.36 kbp EcoRI-XhoI fragment from cosmid pCM1007 (Fleckenstein *et al.*, 1982), representing nucleotides 119499-126856 of the published AD169 strain sequence (Chee *et al.*, 1990), into the EcoRI/SalI sites of pGEM-3Zf+.

The plasmid, pON2159, was constructed by cloning a 1.78 kbp EcoRI fragment (nucleotides 163071 to 164853 of the AD169 genome) containing the viral UDG open reading frame (ORF) into the MfeI site of pWZLNeo (Morgenstern and Land, 1990), a kind gift of Dr. Garry Nolan.

Infection under serum starvation conditions. HFFs were seeded into 90 mm tissue culture plates at approximately 3 × 10^6 cells per plate. Monolayers were monitored daily until complete confluence was observed, usually after 4 days. Culture medium was then replaced with medium supplemented with 0.2% NuSerum and maintained under these conditions for 72 hours. Following this treatment, HFFs were infected with AD169 or RC2620 at a m.o.i. of 5 p.f.u./cell in medium supplemented with 0.2% NuSerum. Where indicated, the virus preparations used were isolated following propagation on HL114 cells. At 24, 48, 72, 96 and 120 hpi, infected monolayers were rinsed twice in PBS, collected by trypsinization and counted. The cell suspension was centrifuged at 1,000 × g in a table-top centrifuge for 5 min. Cell pellets were stored at -20°C for the duration of the time course.

Viral DNA isolation. Infected cell pellets were resuspended in TE containing 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg of Proteinase K per ml and incubated at 55°C overnight. Viral DNA was purified by phenol and chloroform extractions with phase-lock gel (5 Prime → 3 Prime) and precipitated with ethanol.

DNA blots and quantification of DNA. Viral DNA was digested to completion with restriction endonucleases, separated on a 0.7% agarose gel and visualized with ethidium bromide. Gels were denatured and viral DNA transferred to nitrocellulose membranes (and UV cross-linked). Membranes were prehybridized in 6 × SSPE, 2 × Denhardt's, 0.5% SDS and 300 µg/ml salmon sperm DNA for one hour. Blots were hybridized with ^{32}P-radiolabelled DNA probes overnight at 65°C in 4 × SSPE, 3 × Denhardt's, 0.5% SDS, 15% formamide, 10% dextran sulfate and 400 µg/ml salmon sperm

DNA. Filters were washed at 65°C in 0.1 × SSPE, 0.1% SDS. Membranes were exposed to a PhosphorScreen and results quantitated by densitometry using ImageQuant software (Molecular Dynamics.)

HL114 cell construction. PA317 cells were transfected with pON2159 by the calcium phosphate method (Chen and Okayama, 1987). The transiently produced defective retrovirus was used to transduce UL114 expression in low passage primary HELs (Mocarski *et al.*, 1996). Infected cell cultures were selected with 400 µg/ml Geneticin (G418, Gibco BRL) commencing at 24 hpi and continuing for 10 days.

HCMV virion isolation. HFF cells or HL114 cells were infected with AD169 or RC2620 at a m.o.i. of 0.01 p.f.u./cell. Four days after the cells exhibited 100% cytopathic effect (CPE), infected cell supernatants were harvested and cleared of cell debris by centrifugation at 3,300 rpm for 30 min at 4°C. The resulting supernatant was subjected to high speed ultracentrifugation at 28,000 rpm for one hour at 4°C to isolate virion particles. Virus particles were resuspended in medium without serum and stored at -80°C.

Uracil content assessment on alkaline denaturing gels. Viral DNA was isolated from AD169 or RC2620 infected cells under serum starvation conditions at 4, 8, 12, 24, 48, 72 and 96 hpi. To assess the viral genome for uracils, parallel 2 µg samples of viral DNA were either treated with 4U uracil DNA glycosylase (New England Biolabs) or mock treated for two hours prior to the addition of 0.1M NaOH to cleave alkali-labile, apyrimidinic sites created by uracil excision. The samples were then separated on a 0.5% alkaline denaturing agarose gel and transferred to a nylon membrane. To control for our limits of detection, viral DNA samples were also UV-irradiated for either 60 sec or 120 sec using a 15 watt germicidal lamp (254 nm, 0.67 $J/m^2/sec$ at the sample position). These doses have been previously characterized to produce pyrimidine dimers at frequencies of one dimer per 6 kb and one dimer per 3 kb, respectively (Spivak and Hanawalt, 1995; Courcelle and Ganesan, personal communication). The irradiated DNA was then processed as described above, except that T4 endonuclease V which specifically cleaves at sites of DNA pyrimidine dimers, was used in place of the uracil DNA glycosylase. Membranes were hybridized overnight with ^{32}P-radiolabelled HCMV-specific probe and exposed to a PhosphorScreen. Results were quantitated by densitometry using ImageQuant software (Molecular Dynamics) and the average size of treated and mock treated viral DNA fragments were then compared.

RESULTS

Effect of uracil residues in viral DNA. RC2620, the HCMV UL114 insertion mutant, was previously observed to have a prolonged replication cycle corresponding to a defect prior to or at the elongation phase of viral DNA replication (see Chapter 2). We had also found that RC2620 had incorporated threefold more uracil in packaged viral DNA than wild-type virus (Prichard *et al.*, 1996). One possible explanation for the lengthened growth cycle of mutant RC2620 virus is that uracils present in virion DNA need to be removed before replication can proceed. Such a role is supported by observations from Focher and workers who report altered binding of the HSV-1 origin-binding protein to origin sequences containing uracils (Focher *et al.*, 1992).

A primary prediction of this origin repair model is that uracils present at the time of infection prevent initiation of viral DNA replication. To test this possibility, we first passaged stocks of mutant RC2620 virus on a complementing, UL114-expressing cell line, HL114. A previous study had shown that a single round of propagation on HL114 cells produced mutant and wild-type HCMV DNA with similar uracil content (Prichard, personal communication). Thus if uracils incorporated into RC2620 mutant viral genomes were responsible for preventing initiation of DNA replication, mutant grown on the complementing cell line would be expected to grow better than mutant propagated on noncomplementing cells. We therefore compared relative DNA synthesis rates of mutant and wild-type HCMV in non-dividing HFF cells following passage on either HL114 cells or non-expressing HFF cells.

DNA blot analysis of the total viral DNA produced over time for mutant virus propagated on HL114 and HFF cells is shown in Fig. 3.1. As expected, viral DNA accumulation proceeded in a timely manner for wild-type virus regardless of the cell type used to propagate viral stocks (data not shown). Interestingly, we were unable to detect any differences in the rates of replication between mutant RC2620 virus propagated on either complementing or noncomplementing cells (Fig. 3.1), although consistent with our previous observations, this virus still displayed a delay in the onset of viral DNA accumulation compared to wild-type AD169 virus. Thus, the delay in growth is independent of uracil load in the input viral genome.

Uracil content in HCMV virion DNA. Our observations would suggest that higher levels of uracil in the DNA of mutant virus particles do not impede replication. However, this does not address the possibility that uracils present in the DNA of infecting virus particles are normally excised by UDG following entry into the nucleus but before replication proceeds. We therefore examined the frequency of uracil incorporation into HCMV DNA isolated from wild-type and mutant virus particles released into culture fluid. To assess uracil load in HCMV DNA, we treated total virion DNA with *E. coli* UDG and alkali to nick the DNA backbone at sites of uracil incorporation. The average size and intensity of DNA fragments arising from UDG treatment were then compared to untreated DNA samples on alkaline denaturing agarose gels. The results of this assay are shown in Fig. 3.2 (lanes 1 to 8). Surprisingly, we did not observe any detectable differences in size or intensity of wild-type and mutant viral DNA regardless of the cell type used to propagate virus. The majority of UDG-treated viral DNA migrated close to the wells with sizes greater than 10 kb. In contrast, when viral DNA samples containing cyclobutane pyrimidine dimers at known frequencies were analyzed in a similar manner using dimer-specific T4 endonuclease V (TEV), smears centering around the expected sizes of 3 kb or 6 kb were observed (Fig. 3.2, lanes 9 to 12). Comparison of the UDG-treated lanes with the UV-irradiated lanes, suggests that the frequency of uracil incorporation into virion DNA is low. Based on this analysis, infecting viral DNA contains less than one uracil per 10 kilobases of genomic DNA. The low level of uracil present in mutant DNA strongly argues against the proposal that UDG is strictly required in repair of uracils incorporated into viral genomes. The general lack of uracils in infecting virus particles would also suggest that if UDG itself is required for replication to occur, it is not required in a preparative step before initiation.

Uracil incorporation and excision during HCMV infection. Our results suggested that RC2620 viral DNA synthesis was not impeded by uracils in input viral DNA. Yet, based on our observation that cellular UDG enzyme can complement mutant RC2620 virus it seemed reasonable to predict that

UL114 may be required for uracil excision prior to the start of viral DNA replication (see Chapter 2). To determine when this gene might impact on the early phases of HCMV DNA replication, we compared the patterns of uracil incorporation and excision during wild-type AD169 and mutant RC2620 virus infection. Total viral DNA was isolated from non-dividing, serum starved cells infected with wild-type AD169 virus or mutant RC2620 virus at 4, 8, 12, 24, 48, 72 and 96 hpi. Equal amounts of the isolated DNA were run on an alkaline denaturing agarose gel and probed with total viral genomic DNA to determine the size and quantity of HCMV DNA over the course of the viral replication cycle. The analysis was performed with and without UDG treatment to assess when uracil glycosylase activity may be functioning to modify uracils incorporated into viral DNA. DNA blot analyses of a typical experiment is shown in Fig. 3.3 and Fig. 3.4.

Consistent with our previous experiment, no difference was observed in wild-type genomic DNA before and after UDG treatment confirming that the uracil content of infecting virus is low (Fig. 3.3A, lane 1). Following infection, we saw a gradual loss in the amount of viral DNA up to 24 hpi (Fig. 3.3A, lanes 3 to 6). Although the total amount of viral DNA present was quite low at these times, no uracil incorporation was apparent during these early times. Interestingly however, we observed high levels of uracil incorporation in AD169 virus infection at times of robust DNA amplification (72 and 96 hpi) (Fig. 3.3A, lanes 8 and 9, Fig. 3.3B). Thus, these results demonstrate that although no uracil is present in the genome at the time of infection, HCMV incorporates uracils into its DNA beginning at times of rapid DNA amplification.

We next examined mutant virus DNA for uracil incorporation over time in a similar manner. No significant difference was observed in mutant virion DNA that was mock treated or treated with exogenous UDG (Fig. 3.4A, lane 1). Similar to our observations for wild-type virus infection, we detected a gradual loss in the amount of infecting viral DNA up to 24 hpi (Fig. 3.3B, lanes 3 to 6). At the time period corresponding to the initiation of viral DNA replication in wild-type virus, we observed that mutant virus also appeared to accumulate viral DNA as seen by the increase of signal between 24 hpi and later time points (Fig. 3.4B). However, the DNA replication in mutant RC2620 virus-infected cells was not as robust as DNA accumulation in wild-type virus-infected cells (10-fold increase in mutant DNA signal between 24 hpi and 96 hpi compared with 100-fold in wild-type). Also in contrast to AD169 infection, we did not detect uracil incorporation in the small amount of mutant DNA that was replicated at these late time points (Fig. 3.4A. lanes 8 and 9). Taken together, these results suggest that uracil incorporation into viral DNA occurs during the late phase of DNA amplification. Our results also demonstrate that while RC2620 replication appears to begin at the appropriate time, this mutant which lacks HCMV uracil DNA glycosylase is unable to amplify its genome to levels seen in wild-type virus infection.

DISCUSSION

We had previously found that a mutant in UL114, the HCMV uracil DNA glycosylase, was restricted at a step prior to or early in the elongation phase of viral DNA synthesis (see Chapter 2). In this chapter, we report that mutant and wild-type virus particles contain similar amounts of uracils and that mutant appears to begin DNA replication at the same time as wild-type virus. Interestingly, we found that wild-type HCMV incorporates large amounts of uracil into its genome at times correlating with intense DNA synthesis, suggesting a role for uracil turnover in the transition from early to late phase HCMV DNA replication. In support of this idea, we demonstrated that while replication initiates in a timely manner in the HCMV UDG mutant, it immediately "stalls out" at times corresponding to the start of high level viral DNA amplification.

Early *in vitro* binding studies by Focher and colleagues had suggested that UDG activity is required to remove uracils from the HSV-1 origin and hence allow recognition of origin sequences by the initiator protein (Focher *et al.*, 1992). Contrary to this hypothesis, we found that neither wild-type nor mutant HCMV contained significant levels of uracil in their genomes at the time of infection. Using our assay, the uracil incorporation frequency of both wild-type and mutant viruses is less than one uracil residue per 10 kb. On the other hand, the origin region of HCMV has been reported to be approximately 2.5 kb (Anders *et al.*, 1992; Anders and Punturieri, 1991; Hamzeh *et al.*, 1990; Masse *et al.*, 1992). Assuming that HCMV does not encode a specialized pathway for site-specific incorporation of uracil, our results predict that each HCMV origin contains approximately 0.25 uracils. Thus, the lack of detectable uracil in infecting viral genomes strongly argues against the idea that uracils present in mutant virus genomes inhibit timely DNA synthesis. Virion DNA isolated from mutant RC2620 passaged on non-complementing cells was observed to contain small DNA species (approximately 0.5 kb) following mock treatment or UDG treatment, it is unclear whether these fragments arise as a result of site-specific cleavage or what their origin is. This question can be resolved with the use of site-specific probes. Finally, we found that even after we "cured" our mutant in HCMV UDG of uracils in its genome, RC2620 was still restricted in DNA replication. Taken together, our results suggest that UL114 is required during a single infectious cycle and argues against a simple repair function for this protein in viral DNA replication. Instead, these data would imply that UL114 activity is required after the initiation events in HCMV DNA replication.

When we examined the genomic integrity and uracil content of wild-type and mutant viral DNA, we found little to no uracil incorporation in the DNA of infecting virus particles continuing out to 48 hpi. Interestingly, we observed a transient increase in uracil incorporation into wild-type HCMV DNA corresponding to the start of rapid viral DNA replication. In constrast while the UL114 mutant was able to initiate early rounds of DNA replication at the appropriate time, we were unable to detect late phase, rapid DNA amplification in RC2620 for the time points assessed or a similar increase in the presence of uracil in mutant virus DNA. Though correlative, the observation that the UL114 mutant is restricted at the precise point when uracil is incorporated into wild-type virus DNA strongly suggests that UL114 function is likely to be required for amplification of the viral genome to occur.

It is thought that herpesvirus DNA replication occurs as a biphasic process (Igarashi *et al.*, 1993; Lehman and Boehmer, 1999; Roizman *et al.*, 1965; St Jeor and Hutt, 1977; Stinski, 1978) involving early *theta* form replication and proceeding to late rolling circle form replication (Ben-Porat and Tokazewski, 1977; Jacob *et al.*, 1979) during which the bulk of viral DNA is synthesized (Igarashi *et al.*, 1993). Much of the information on replication forms during herpesvirus infection has been derived from studies of HSV-1, although HCMV is believed to employ similar replication mechanisms as it shares biological properties with this virus (LaFemina and Hayward, 1983; McVoy and Adler, 1994; Pari and Anders, 1993; Sarisky and Hayward, 1996).

That the switch to late phase DNA replication occurs is clear, however the mechanism involved in this transition is as yet unknown. An intriguing possibility for how UL114 acts in the transition to late phase DNA amplification is that uracils excised by UL114 create sites that serve as substrates for initiation of recombination-dependent replication (Figure 3.5). It has been suggested that recombination is intimately associated with herpesvirus replication (Dutch *et al.*, 1992; Sarisky and Weber, 1994; Zhang *et al.*, 1994). Such strand exchanges are thought to initiate multiple rounds of origin-independent DNA replication at random sites throughout the genome of bacteriophage T4 and lambda, thus allowing mass DNA replication in these phages (Enquist and Skalka, 1973; Mosig, 1998). A similar form of replication may occur for herpesviruses as well.

During the early phase of replication, DNA synthesis initiates in a bidirectional manner from the origin of replication (Fig. 3.5A). The lack of detectable uracils in the genome of infecting virus particles implies that uracil is not incorporated before the initial rounds of DNA synthesis. At the start of late phase DNA replication, large numbers of incorporated uracils are detected in the viral genome which would become substrates for the uracil excision activity of UL114. This activity is expected to create 3'-OH in the DNA template following cleavage of the abasic sites by apurinic/apyrimidinic (AP) endonucleases. In one model, the induced nicks can occur throughout the genome thus allowing the start of late rolling circle form of DNA replication (Fig. 3.5B) in a manner similar to lambda bacteriophage (Enquist and Skalka, 1973).

The UL114 generated nicks may also serve as substrates for recombination (Fig. 3.5C). There is much evidence available from HSV-1 studies to suggest that recombination and replication are linked in herpesvirus replication. First, replication and recombination occur at similar times during the viral life cycle (Dutch *et al.*, 1992; Zhang *et al.*, 1994). Second, there is a strong association between the replication machinery of HSV-1 and genome inversion (Sarisky and Weber, 1994). Third, HSV-1 replication intermediates are found in a complex branched DNA structure which is thought to arise from frequent recombination (Severini *et al.*, 1996; Shlomai *et al.*, 1976). Regions of single-stranded DNA (ssDNA) generated by the activities of nucleases or helicases in bacteriophage T4 are thought to promote pathways of recombination-dependent replication that are important for efficient late phase DNA replication in this phage (Mosig, 1998). Strand invasion by these ssDNA tails primes DNA synthesis from 3'-OHs and supplants the need for T4 primase (Mosig, 1998). By analogy to this system, nicks induced by UL114 may result in strand invasion and recombination-dependent replication thus facilitating initiation throughout the viral genome and allowing the transition to late phase amplification of the HCMV genome. In support of a nicking model of initiation, newly synthesized HSV-1 DNA is known to contain a greater number of fragments than mature virion DNA arising from single-stranded DNA breaks and multiple initiation sites (Frenkel and Roizman, 1972; Wilkie, 1973). Taken together, these results suggest that nicks and breaks in viral DNA may serve a functional role in herpesvirus DNA replication.

Consistent with the possibility that UL114 is involved in the switch to late phase replication, we found previously that mutant RC2620 virus was restricted to low levels of DNA synthesis (approximately 10% of wild-type) under sub-optimal culture conditions; robust levels of viral DNA replication were only seen following induction of cellular UDG (see Chapter 2). Interestingly, bacteriophage λ *red* mutants replicate DNA at an abnormally low rate and exhibit a decrease in the total amount of phage DNA synthesized (Enquist and Skalka, 1973). The similarity in phenotypes between λ recombination mutants and RC2620 suggests that UL114 may somehow link recombination and DNA replication in HCMV.

Similar mechanisms of late phase DNA amplification may be utilized by other DNA viruses. In animal studies, a mutant in HSV-1 UDG exhibits a 100-fold decrease in virus titers compared with wild-type virus, suggesting a requirement for UDG activity for efficient replication of this virus (Pyles and Thompson, 1994). More strikingly, a vaccinia virus UDG mutant is able to replicate DNA at approximately 2% of wild-type but is restricted in the transition to high level, late phase DNA amplification (Millns *et al.*, 1994). These studies strongly suggest that UDG may act as a switch from early to late phase DNA replication during HCMV infection. Future studies using neutral two-dimensional (2D) gel electrophoretic analysis of the structure of replication intermediates in wild-type AD169 and mutant RC2620 virus may provide the link between UDG and recombination facilitated replication. A prediction from the models proposed here is that replicating mutant RC2620

DNA will show decreased presence of X and Y branches compared to wild-type AD169 replicating DNA.

Fig. 3.1. Viral DNA accumulation in mutant RC2620 following passage on UL114-expressing cells or HFF cells. DNA blots of viral DNA from 10^5 cells infected with mutant RC2620 virus isolated at the indicated times post-infection, separated on a 0.7% agarose gel and transfered to nitrocellulose membrane. DNA from HFF cells infected with HL114-propagated virus is shown in the top panel and indicated by HL114 on the right; HFF cells infected with virus passaged on non-complementing cells is shown in the bottom panel and indicated by HFF on the right. The membrane was probed with ^{32}P-radiolabelled pON2260, specific for HCMV nucleotides 122699-124902.

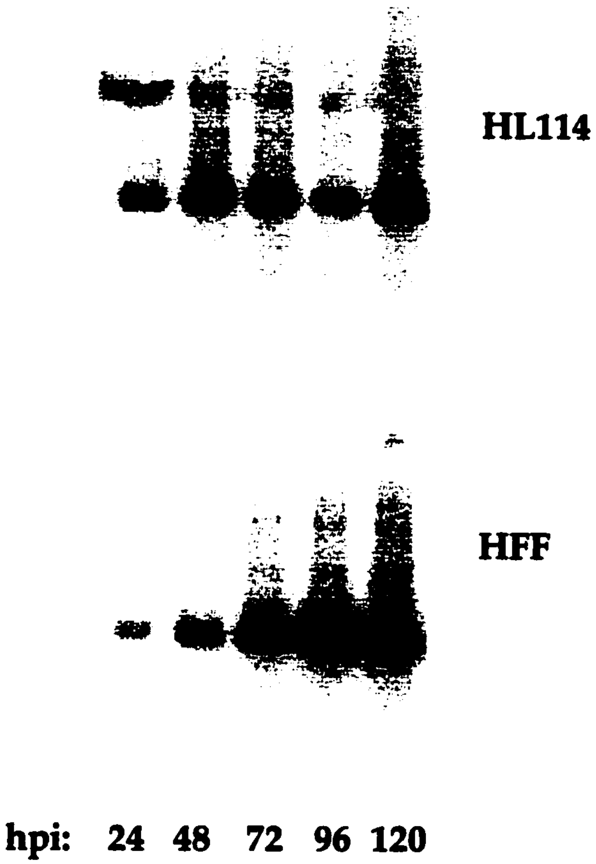

HL114
HFF
hpi: 24 48 72 96 120

Fig. 3.2. Uracil load in wild-type AD169 and mutant RC2620 virus particles. DNA was isolated from AD169 (lanes 1, 2, 5 and 6) and RC2620 (lanes 3, 4, 7 and 8) virions following passage on HFF cells (lanes 1 to 4) or UL114-expressing cells, HL114 (lanes 5 to 8). Half of the viral DNA from each sample was treated with *E. coli* uracil DNA glycosylase to determine uracil levels (lanes 2, 4, 6 and 8). AD169 virion DNA was also treated with UV and T4 Endonuclease V (TEV) as follows: untreated AD169 (lane 9); AD169 UV-irradiated at 80 kJ/m^2 (lane 10); AD169 UV-irradiated at 40 kJ/m^2 and incubated with TEV (lane 11) and AD169 UV-irradiated at 80 kJ/m^2 and incubated with TEV. Reaction products were subjected to alkaline denaturing agarose gel electrophoresis, transfered to nylon membrane and probed with ^{32}P-radiolabelled viral DNA. Blot is shown overexposed to reveal any faint bands. Positions of the molecular weight markers are indicated on the left in kilobases.

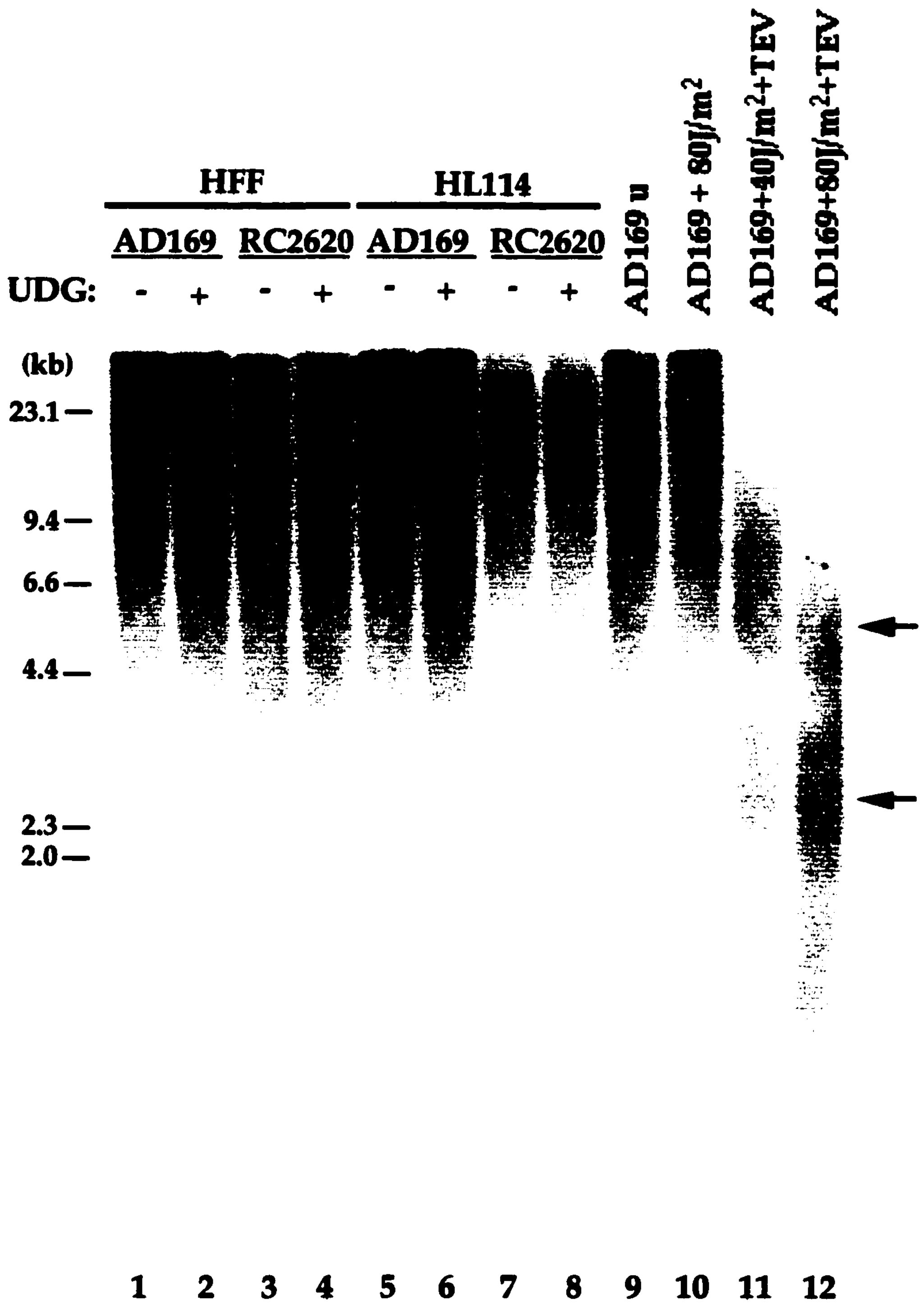

HFF
HL114
AD169
RC2620
AD169
RC2620
UDG: - + - + - + - +
AD169 u
AD169 + 80J/m2
AD169+40J/m2+TEV
AD169+80J/m2+TEV
(kb)
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 3.3. Uracil incorporation during wild-type AD169 virus infection. (A) DNA blot analysis of viral DNA isolated from AD169 infected HFF cells at the indicated times post-infection. Parallel 2 µg DNA samples from each time point was treated with *E. coli* UDG (bottom panel) or left untreated (top panel). Reactions were subjected to alkaline denaturing agarose gel electrophoresis, transfered to nylon membrane and probed with ^{32}P-radiolabelled viral DNA. The blot is shown overexposed to show any faint bands. Mock infected cellular DNA is shown in lane 2. Positions of the molecular weight markers are indicated on the left in kilobases. (B) Densitometry for selected samples plotted as a function of time in hours post-infection.


A
1 2 3 4 5 6 7 8 9
(kb)
23.1
9.4
6.6
4.4
2.3
2.0
- UDG
23.1
9.4
6.6
4.4
2.3
2.0
+ UDG
hpi: 0 m 4 8 12 24 48 72 96

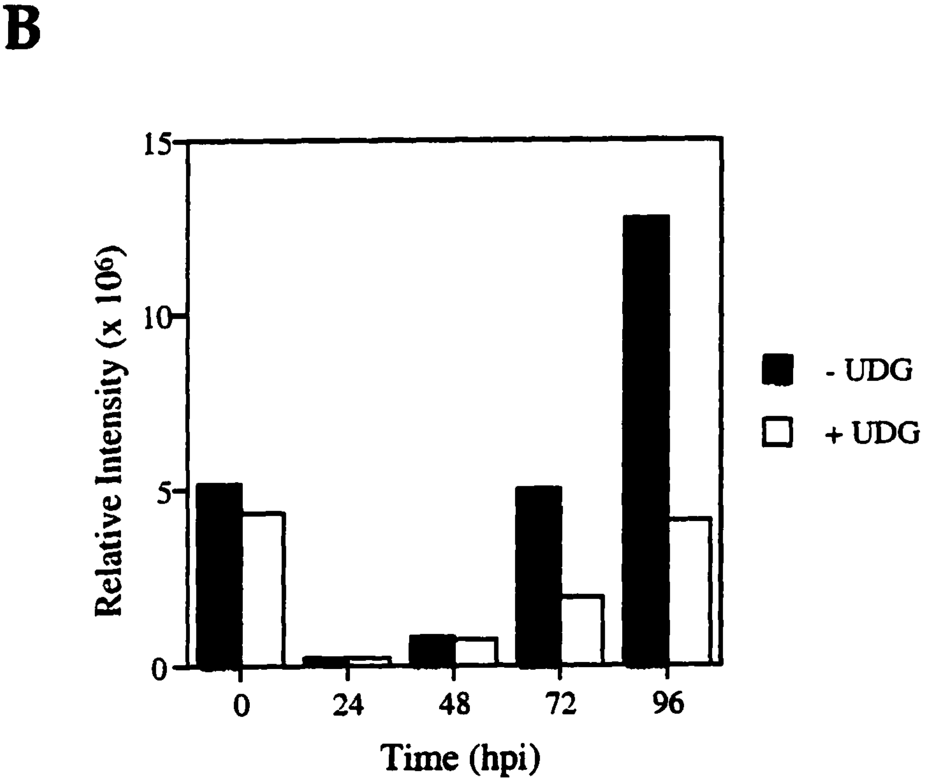
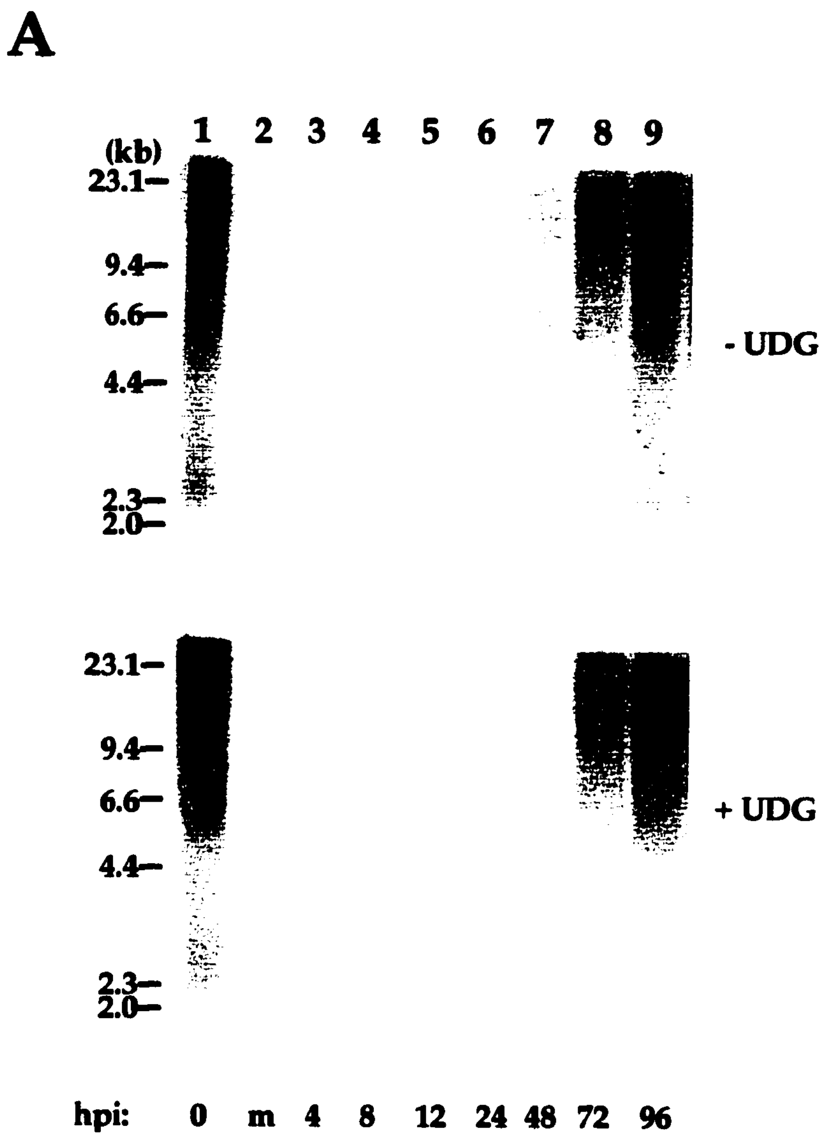

B
15
10
5
0
Relative Intensity (x 10^6)
0
24
48
72
96
Time (hpi)
■ - UDG
□ + UDG

Fig. 3.4. Uracil incorporation during mutant RC2620 virus infection. (A) DNA blot analysis of viral DNA isolated from RC2620 infected HFF cells at the indicated times post-infection. Parallel 2 µg DNA samples from each time point was treated with *E. coli* UDG (bottom panel) or left untreated (top panel). Reactions were subjected to alkaline denaturing agarose gel electrophoresis and transfered to nylon membrane and probed with ^{32}P-radiolabelled viral DNA. Blot is shown overexposed to show any faint bands. Mock infected cellular DNA is shown in lane 2. Positions of the molecular weight markers are indicated on the left in kilobases. (B) Densitometry for selected samples plotted as a function of time in hours post-infection.


A
(kb)
1 2 3 4 5 6 7 8 9
23.1
9.4
6.6
4.4
2.3
2.0
- UDG
23.1
9.4
6.6
4.4
2.3
2.0
+ UDG
hpi: 0 m 4 8 12 24 48 72 96

B

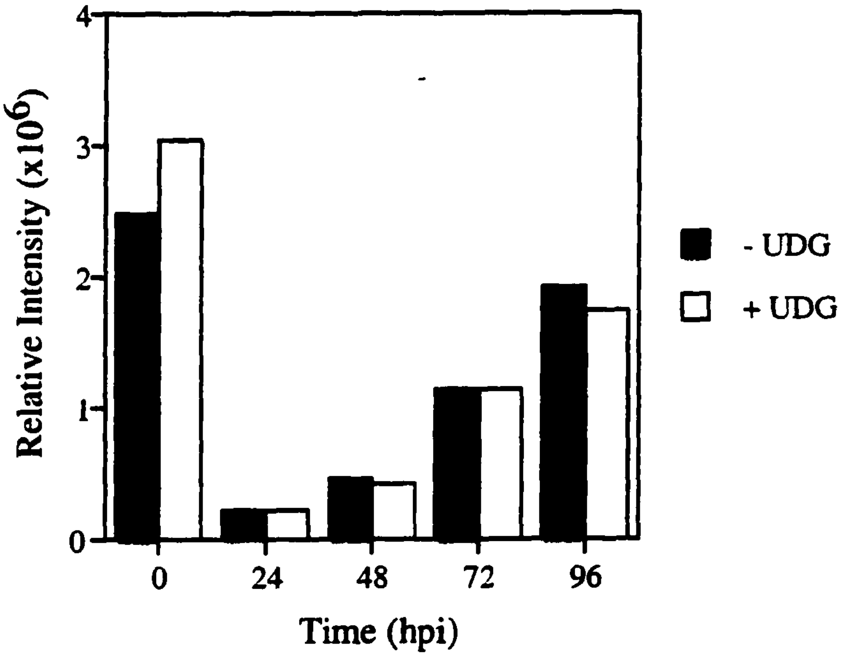
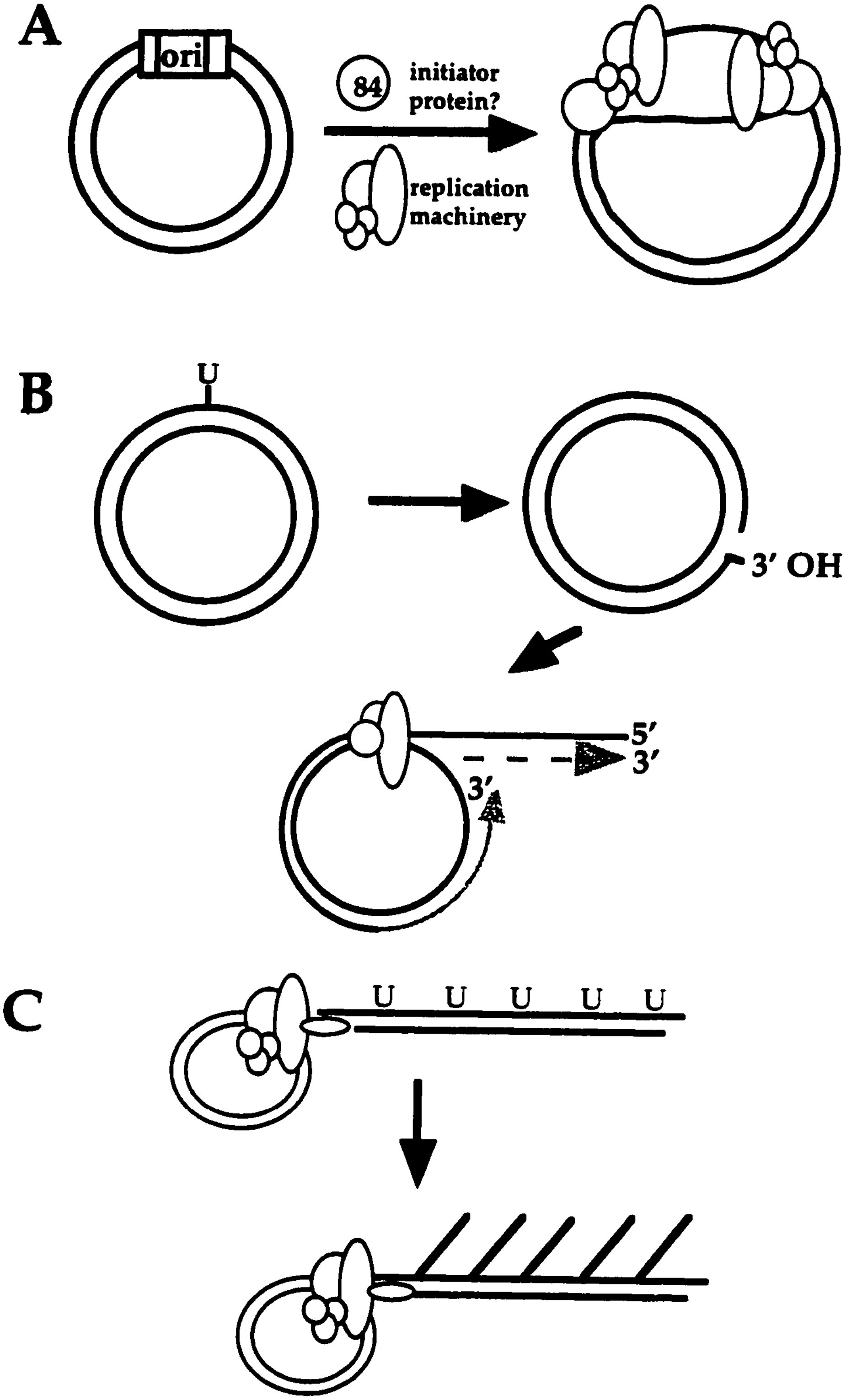


Fig. 3.5. Models for how UL114 could be required in the transition to late phase HCMV DNA replication. (A) Early phase HCMV replication using origin-dependent *theta* mechanism of DNA synthesis. (B) Excision of uracil from newly synthesized viral DNA templates results in random nicking of the viral genome and conversion to rolling circle replication. A single nick is shown for simplicity. (C) Excision of uracil from viral DNA templates leads to nicks in the genome and the start of recombination-dependent replication.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REFERENCES

Anders, D.G., Kacica, M.A., Pari, G. and Punturieri, S.M. (1992) Boundaries and structure of human cytomegalovirus oriLyt, a complex origin for lytic-phase DNA replication. *J Virol*, **66**, 3373-3384.

Anders, D.G. and Punturieri, S.M. (1991) Multicomponent origin of cytomegalovirus lytic-phase DNA replication. *J. Virol.*, **65**, 931-937.

Ben-Porat, T. and Tokazewski, S.A. (1977) Replication of herpesvirus DNA. II. Sedimentation characteristics of newly synthesized DNA. *Virology*, **79**, 292-301.

Brynolf, K., Eliasson, R. and Reichard, P. (1978) Formation of Okazaki fragments in polyoma DNA synthesis caused by misincorporation of uracil. *Cell*, **13**, 573-580.

Chee, M.S., Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerny, R., Horsnell, T., Hutchison, C.A.I., Kouzarides, T., Martignetti, J.A., Preddie, E., Satchwell, S.C., Tomlinson, P., Weston, K.M. and Barrell, B.G. (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol*, **154**, 125-170.

Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol*, **7**, 2745-2752.

Dutch, R.E., Bruckner, R.C., Mocarski, E.S. and Lehman, I.R. (1992) Herpes simplex virus type 1 recombination: Role of DNA replication and viral*a* sequences. *J Virol*, **66**, 277-285.

Ellison, K.S., Peng, W. and McFadden, G. (1996) Mutations in active-site residues of the uracil-DNA glycosylase encoded by vaccinia virus are incompatible with virus viability. *J Virol*, **70**, 7965-7973.

Enquist, L.W. and Skalka, A. (1973) Replication of bacteriophage lambda DNA dependent on the function of host and viral genes. I. Interaction of red, gam and rec. *J Mol Biol*, **75**, 185-212.

Fleckenstein, B., Muller, I. and Collins, J. (1982) Cloning of the complete human cytomegalovirus genome in cosmids. *Gene*, **18**, 39-46.

Focher, F., Verri, A., Verzeletti, S., Mazzarello, P. and Spadari, S. (1992) Uracil in OriS of herpes simplex 1 alters its specific recognition by origin binding protein (OBP): does virus induced uracil-DNA glycosylase play a key role in viral reactivation and replication? *Chromosoma*, **102**, S67-71.

Frenkel, N. and Roizman, B. (1972) Separation of the herpesvirus deoxyribonucleic acid duplex into unique fragments and intact strand on sedimentation in alkaline gradients. *J Virol*, **10**, 565-572.

Halbert, C.L., Demers, G.W. and Galloway, D.A. (1991) The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J Virol*, **65**, 473-478.

Hamzeh, F.M., Lietman, P.S., Gibson, W. and Hayward, G.S. (1990) Identification of the lytic origin of DNA replication in human cytomegalovirus by a novel approach utilizing ganciclovir-induced chain termination. *J. Virol.*, **64**, 6184-6195.

Igarashi, K., Fawl, R., Roller, R.J. and Roizman, B. (1993) Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. *J Virol*, **67**, 2123-2132.

Jacob, R.J., Morse, L.S. and Roizman, B. (1979) Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol*, **29**, 448-457.

Krokan, H. (1981) Preferential association of uracil-DNA glycosylase activity with replicating SV40 minichromosomes. *FEBS Lett*, **133**, 89-91.

Krokan, H., Haugen, A., Myrnes, B. and Guddal, P.H. (1983) Repair of premutagenic DNA lesions in human fetal tissues: evidence for low levels of O6-methylguanine-DNA methyltransferase and uracil-DNA glycosylase activity in some tissues. *Carcinogenesis*, **4**, 1559-1564.

LaFemina, R.L. and Hayward, G.S. (1983) Replicative forms of human cytomegalovirus DNA with joined termini are found in permissively infected human cells but not in non-permissive Balb/c-3T3 mouse cells. *J Gen Virol*, **64**, 373-389.

Lee, K.A. and Sirover, M.A. (1989) Physical association of base excision repair enzymes with parental and replicating DNA in BHK-21 cells. *Cancer Res*, **49**, 3037-3044.

Lehman, I.R. and Boehmer, P.E. (1999) Replication of herpes simplex virus DNA [In Process Citation]. *J Biol Chem*, **274**, 28059-28062.

Lindahl, T. and Nyberg, B. (1974) Heat-induced deamination of cytosine residues in deoxyribonucleic acid. *Biochemistry*, **13**, 3405-3410.

Masse, M.J., Karlin, S., Schachtel, G.A. and Mocarski, E.S. (1992) Human cytomegalovirus origin of DNA replication (oriLyt) resides within a highly complex repetitive region. *Proc Natl Acad Sci U S A*, **89**, 5246-5250.

McVoy, M.A. and Adler, S.P. (1994) Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. *J Virol*, **68**, 1040-1051.

Millns, A.K., Carpenter, M.S. and DeLange, A.M. (1994) The vaccinia virus-encoded uracil DNA glycosylase has an essential role in viral DNA replication. *Virology*, **198**, 504-513.

Mocarski, E.S., Bonyhadi, M., Salimi, S., McCune, J.M. and Kaneshima, H. (1993) Human cytomegalovirus in a SCID-hu mouse: thymic epithelial cells are prominent targets of viral replication. *Proc Natl Acad Sci U S A*, **90**, 104-108.

Mocarski, E.S., Kemble, G.W., Lyle, J.M. and Greaves, R.F. (1996) A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation. *Proc Natl Acad Sci U S A*, **93**, 11321-11326.

Morgenstern, J.P. and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res*, **18**, 3587-3596.

Mosig, G. (1998) Recombination and recombination-dependent DNA replication in bacteriophage T4. *Annu Rev Genet*, **32**, 379-413.

Myrnes, B., Giercksky, K.E. and Krokan, H. (1983) Interindividual variation in the activity of O6-methyl guanine-DNA methyltransferase and uracil-DNA glycosylase in human organs. *Carcinogenesis*, **4**, 1565-1568.

Pari, G.S. and Anders, D.G. (1993) Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. *J Virol*, **67**, 6979-6988.

Percival, K.J., Klein, M.B. and Burgers, P.M. (1989) Molecular cloning and primary structure of the uracil-DNA-glycosylase gene from Saccharomyces cerevisiae. *J Biol Chem*, **264**, 2593-2598.

Prichard, M.N., Duke, G.M. and Mocarski, E.S. (1996) Human cytomegalovirus uracil DNA glycosylase is required for the normal temporal regulation of both DNA synthesis and viral replication. *J Virol*, **70**, 3018-3025.

Pyles, R.B. and Thompson, R.L. (1994) Evidence that the herpes simplex virus type 1 uracil DNA glycosylase is required for efficient viral replication and latency in the murine nervous system. *J Virol*, **68**, 4963-4972.

Roizman, B., Borman, G.S. and Kamali-Rousta, M. (1965) Macromolecular synthesis in cells infected with herpes simplex virus. *Nature*, **206**, 1374-1375.

Sarisky, R.T. and Hayward, G.S. (1996) Evidence that the UL84 gene product of human cytomegalovirus is essential for promoting oriLyt-dependent DNA replication and formation of replication compartments in cotransfection assays. *J Virol*, **70**, 7398-7413.

Sarisky, R.T. and Weber, P.C. (1994) Requirement for double-strand breaks but not for specific DNA sequences in herpes simplex virus type 1 genome isomerization events. *J Virol*, **68**, 34-47.

Seal, G. and Sirover, M.A. (1986) Physical association of the human base-excision repair enzyme uracil DNA glycosylase with the 70,000-dalton catalytic subunit of DNA polymerase alpha. *Proc Natl Acad Sci U S A*, **83**, 7608-7612.

Severini, A., Scraba, D.G. and Tyrrell, D.L. (1996) Branched structures in the intracellular DNA of herpes simplex virus type 1. *J Virol*, **70**, 3169-3175.

Shapiro, R. (1980) In Kleppe, E.S.a.K. (ed.) *Chromosome Damage and Repair.* Plenum Press, New York, pp. 3-18.

Shlomai, J., Friedmann, A. and Becker, Y. (1976) Replication intermediates of herpes simplex virus DNA. *Virology*, **69**, 647-659.

Spaete, R.R. and Mocarski, E.S. (1985) Regulation of cytomegalovirus gene expression: a and b promoters are trans activated by viral functions in permissive human fibroblasts. *J Virol*, **56**, 135-143.

Spivak, G. and Hanawalt, P.C. (1995) Determination of Damage and Repair in Specific DNA sequences. *METHODS: A companion to Methods in Enzymology*. Academic Press, Inc., Vol. 7, pp. 147-161.

St Jeor, S. and Hutt, R. (1977) Cell DNA replication as a function in the synthesis of human cytomegalovirus. *J Gen Virol*, **37**, 65-73.

Stinski, M.F. (1978) Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. *J Virol*, **26**, 686-701.

Trower, M.K. (1994) Site-directed mutagenesis using a uracil-containing phagemid template. *Methods Mol Biol*, **31**, 67-77.

Tye, B.K. and Lehman, I.R. (1977) Excision repair of uracil incorporated in DNA as a result of a defect in dUTPase. *J Mol Biol*, **117**, 293-306.

Varshney, U., Hutcheon, T. and van de Sande, J.H. (1988) Sequence analysis, expression, and conservation of Escherichia coli uracil DNA glycosylase and its gene (ung). *J Biol Chem*, **263**, 7776-7784.

Wilkie, N.M. (1973) The synthesis and substructure of herpesvirus DNA: the distribution of alkali-labile single strand interruptions in HSV-1 DNA. *J Gen Virol*, **21**, 453-467.

Wist, E., Unhjem, O. and Krokan, H. (1978) Accumulation of small fragments of DNA in isolated HeLa cell nuclei due to transient incorporation of dUMP. *Biochim Biophys Acta*, **520**, 253-270.

Zhang, X., Efstathiou, S. and Simmons, A. (1994) Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. *Virology*, **202**, 530-539.

Chapter 4: Construction and Characterization of a Cell Line expressing the HCMV UL44 gene and Generation of a UL44 null HCMV.

ABSTRACT

Human cytomegalovirus encodes six herpesvirus conserved replication genes — the viral DNA polymerase (ppUL54), polymerase associated processivity factor (ppUL44), the single-stranded DNA binding protein (ppUL57) and a helicase-primase complex (UL70, UL102, UL105) — that are required for its replication (Coen, 1996; Mocarski, 1995). The study of the role of ppUL44 in HCMV replication has been limited to transient assays due to the heretofore lack of immortalized cell lines capable of complementing this gene function and supporting full viral replication. While these assays have been useful for the identification of the putative herpesvirus conserved core and auxiliary replication proteins and defining the origin of replication, they provide limited insight into the role of UL44 *in vivo*. Thus, the goal of this study was to construct and characterize a UL44 knock-out HCMV. To this end, we generated a cell line that expressed UL44 consitutively and in the nucleus as in natural infection. This cell line, IHF2280, was able to support full replication of wild-type HCMV Towne strain and titers of progeny virus were similar to that obtained from primary fibroblasts. Using this cell line, we were able to obtain a recombinant HCMV mutagenized at UL44 by the insertion of the *E. coli gpt* gene. Attempts at purifying this recombinant away from contaminating wild-type virus on IHF2280 cells were unsuccessful despite expression of UL44 by this cell line. This result suggests that the IHF2280 cell line cannot fully complement this gene activity.

INTRODUCTION

Cytomegalovirus (CMV) is the prototype member of the betaherpesvirus subfamily which also includes the herpesviruses, HHV-6 and HHV-7. CMV infects 50-80% of the population and is an important cause of disease in transplant recipients and AIDS patients (Mocarski, 1995). As with other members of the herpesvirus family, human CMV (HCMV) encodes six herpesvirus conserved replication proteins — the viral DNA polymerase (ppUL54), polymerase associated processivity factor (ppUL44), the single-stranded DNA binding protein (ppUL57) and a helicase-primase complex (UL70, UL102, UL105) — that together serve as the core replication complex (Coen, 1996; Mocarski, 1995). These and five other open reading frames (ORFs) are required for HCMV lytic origin dependent replication as assessed by transient assays (Pari and Anders, 1993; Pari *et al.*, 1993).

The product of the UL44 ORF, ppUL44, is essential for viral replication in transient replication assays (Pari and Anders, 1993; Pari *et al.*, 1993; Ripalti *et al.*, 1995) and is associated with the viral DNA polymerase in a one-to-one ratio (Ertl and Powell, 1992). The UL44 gene is transcriptionally active at early times of infection, however only small amounts of protein are expressed at this time (Geballe *et al.*, 1986). The protein product of UL44 accumulates to high levels at late times, with maximal expression following the onset of DNA synthesis (Geballe *et al.*, 1986). At late times of infection, the amount of ppUL44 present is in excess of viral DNA polymerase- polymerase accessory protein complexes. A similar overexpression of processivity factors is seen during *E. coli* and human DNA replication.

The first 309 amino acids of ppUL44 have been determined to bind to double-stranded DNA and to stimulate viral DNA polymerase activity in biochemical assays (Weiland *et al.*, 1994). The UL44 homolog in herpes simplex virus (HSV), UL42 shares a similar requirement for the first two-thirds of its protein (Digard *et al.*, 1993; Johnson *et al.*, 1991; Reddig *et al.*, 1994). More information has been collected on the functional domains in HSV UL42 compared to HCMV UL44 (Digard *et al.*, 1993; Gao *et al.*, 1993; Johnson *et al.*, 1991; Reddig *et al.*, 1994), however direct sequence comparison between UL44 and HSV UL42 cannot be made as the two proteins differ greatly at the amino acid level. Furthermore, UL44 may have different sequence requirements for activity than HSV UL42. While the carboxy-terminus (C-terminus) of HSV-1 UL42 and its alphaherpesvirus homologs is divergent, the C-terminus of HCMV UL44 and other betaherpesvirus homologs is highly conserved. This sequence conservation points to a possible requirement for this domain in betaherpesvirus infection. This would be distinct from HSV where the C-terminus of UL42 has been shown to be dispensible in tissue culture (Gao *et al.*, 1993).

During herpesvirus infection, replication proteins — including HCMV UL44 and HSV UL42 — can be found in subnuclear regions of infected cell nuclei as assessed by immunofluorescence assays using antibodies to viral antigens (de Bruyn Kops and Knipe, 1988; Penfold and Mocarski, 1997; Quinlan *et al.*, 1984; Rixon *et al.*, 1983). Cellular proteins involved in replication and cell cycle control can also be found in these nuclear compartments of HCMV and HSV (Penfold and Mocarski, 1997; Wilcock and Lane, 1991). Interestingly, while UL44, HSV UL42 and human proliferating cell nuclear antigen (PCNA) share similar functions, UL44 localization differs from HSV UL42 and PCNA localization patterns at late times of infection. Like HSV UL42 and PCNA, the majority of HCMV UL44 remains in nuclear compartments, however some ppUL44 can also be found in the periphery of the nucleus (Penfold and Mocarski, 1997; Plachter *et al.*, 1992). This observation implicates additional roles for this protein during HCMV infection.

While many parallels can be drawn between HCMV ppUL44 and HSV UL42, it is clear that ppUL44 may bear functions specific to its role in HCMV replication. The localization pattern of ppUL44, the kinetics of ppUL44 expression and the conservation of the C-terminus of this protein among betaherpesvirus members all suggest that ppUL44 may have multiple activities during HCMV replication. Finally, many of the interactions observed between human replication proteins, cell cycle proteins and repair enzymes are conserved in HCMV making this virus an ideal model to study cellular DNA replication.

To date, emphasis has been placed on transient replication assays to study the role of ppUL44 in HCMV replication. These studies have provided limited information on the function(s) of UL44 during viral infection, thus we set out to construct a UL44 mutant in HCMV for use in functional analysis of this important viral protein.

MATERIALS AND METHODS

Cells and virus. Primary human foreskin fibroblasts (HFFs) and human embryonic lung fibroblasts (HELs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% NuSerum I (Collaborative Research Inc.), 100 Units of penicillin G per ml, 100 µg of streptomycin sulfate per ml, 0.58 mg L-arginine per ml, 1.08 mg L-glutamine per ml, and 180 µg L-asparagine per ml. The immortalized cell line, IHFie1.3, was maintained in DMEM supplemented with 10% NuSerum as previously described (Greaves and Mocarski, 1998; Mocarski *et al.*, 1996). The packaging cell line, ϕNX-A (Achacoso and Nolan,), was maintained in the same medium but supplemented with 10% fetal bovine serum (FBS) and was a generous gift of Garry P. Nolan. The LXSN16E6E7 amphotropic retroviral packaging cell line (Halbert *et al.*, 1991) was maintained in culture medium supplemented with 10% FBS and was a kind gift of Denise Galloway.

Human CMV strains, AD169 and Towne, were obtained and cultured as previously described (Mocarski *et al.*, 1993; Spaete and Mocarski, 1985). For viral growth curves, approximately 5×10^5 HFF cells were seeded into each well of six-well tissue culture dishes. After one day, the cells were infected at a multiplicity of infection (m.o.i.) of 5 p.f.u./cell. The input inocula were titered by plaque assay at the beginning of the experiment, and this was used as the zero time point. At 1, 2, 3, 5 and 7 days post-infection, the infected cells and 1/3rd of the supernatant were harvested into an equal volume of sterilized, reconstituted non-fat milk, and stored at -80°C for the duration of the experiment. All frozen samples were thawed, sonicated and titered using plaque assay.

Plasmids. The retroviral vector pWZL-Neo has been previously described (Morgenstern and Land, 1990) and was a generous gift of Garry P. Nolan. The plasmid pON2275 was cloned by ligating a 9.23 kb *Xba*I fragment from the Towne cosmid TN23 into the *Spe*I site of pGEM-3Zf+. Plasmid pON2278 was constructed by ligating a 2.95 kb *Kpn*I fragment from pON2275, representing nucleotides 53754-56701 of the published AD169 strain sequence (Chee *et al.*, 1990), into the *Kpn*I site of pGEM-3Zf+. The plasmid pON2280 was constructed by ligating a 2.5 kb *Bam*HI fragment from pON2278 into the *Bgl*II site of pWZL-Neo. The orientation of the UL44 open reading frame (ORF) within pON2280 and with respect to the retroviral packaging signal was confirmed using *Bam*HI/*Bgl*II digestion.

The plasmid pON2136 was constructed by ligating a 4.68 kb *Pst*I-*Aat*II fragment from pCM1017, representing nucleotides 55369-60045 of the published sequence (Chee *et al.*, 1990) into the *Pst*I/*Aat*II sites of pGEM-3Zf+. Plasmid pON2284 was constructed by ligating a 1.07 kb *Bam*HI-*Bgl*II fragment containing the *E. coli gpt* gene under control of the HSV *tk* promoter from the plasmid pON1101 (Greaves *et al.*, 1995) into the *Bgl*II site of pON2136. The *gpt* cassette was inserted in the same orientation as the UL44 ORF as confirmed by restriction endonuclease digestion with *Kpn*I.

IHF2280 cell line construction. To construct the ppUL44 expressing cell line, five to ten μg of pON2280 DNA was transfected by the calcium phosphate method (Chen and Okayama, 1987) into a T25 flask containing approximately 2.0×10^6 ϕNX-A cells. Supernatant from transfected ϕNX-A cells was collected at 48 h post-transfection, filtered through a 0.45 μm filter and transfered to a T25 flask containing approximately 1.0×10^6 HEL cells. At 24 h post-infection (hpi), medium was changed to growth medium supplemented with 400 μg of Geneticin (G418) per ml. Cells were maintained under G418 selection for two weeks. The resulting G418-resistant colonies were immortalized with human papillomavirus 16 E6/E7 genes produced from the retroviral packaging cell line LXSN16E6E7 as described previously (Greaves *et al.*, 1995).

Immunoblot analysis. HFF cells were infected with HCMV Towne at a multiplicity of infection (m.o.i.) of 5 PFU per cell or mock-infected in parallel. To obtain proteins for immunoblot analysis, IHF2280 cells, mock-infected HFF cells and AD169-infected HFF cells were resuspended in a buffer of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 100 μg of phenylmethylsulfonyl fluoride per ml and 1 μg of aprotinin per ml and lysed on ice for 30 min. Insoluble material was removed by high speed centrifugation. The resulting supernatant was stored at -20°C until use. A total of 3.0×10^5 cell equivalents per sample were denatured and separated on 10% SDS-polyacrylamide gel. Separated proteins were transfered to nitrocellulose membrane and subjected to immunoblot analysis using a mouse monoclonal antibody against ppUL44 (Goodwin Institute of Cancer Research, 1202) as described previously (Leach and Mocarski, 1989).

Immunofluorescence assays and confocal microscopy. HFF cells and IHF2280 cells were seeded onto glass cover slips in 24-well tissue culture plates at a density of 1.0×10^5 cells per well. Twenty-four hours later, HFF cells were infected with HCMV AD169 virus at a m.o.i. of 5 p.f.u./cell or mock-infected in parallel. IHF2280 cells, mock-infected HFF cells and AD169-infected HFF cells were fixed with methanol:acetic acid (3:1) and subjected to indirect immunofluorescence assay using mouse monoclonal antibody against ppUL44 as described previously (Penfold and Mocarski, 1997). Fluorescence was visualized on a confocal microscope (Molecular Dynamics) and images collected using Molecular Dynamics Image Space software as described previously (Penfold and Mocarski, 1997).

Construction of Recombinant Virus. To construct recombinant viruses, 8 μg of plasmid DNA was transfected by the calcium phosphate method (Chen and Okayama, 1987) into a well of a six-well dish containing approximately 2.5×10^5 IHF2280 cells. Twelve hours post-transfection, the monolayers were rinsed with medium and the cells were infected with HCMV Towne virus at a m.o.i. of 5 p.f.u./cell. The resultant progeny virus was harvested at 5 days post-infection from the supernatant and transfered to fresh HFF cells. After absorption for 4 hours, medium was replaced with medium supplemented with 100 μM mycophenolic acid and 25 μM xanthine. Virus progeny was harvested 5 days after the culture reached 100% CPE. After two additional rounds of such enrichment, the resulting recombinant viruses were further purified by limiting dilution.

Viral DNA isolation and DNA blot analysis. Infected cell pellets were resuspended in TE containing 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg of Proteinase K per ml and incubated at 55°C overnight. Viral DNA was purified by phenol and chloroform extractions with phase-lock gel (5 Prime → 3 Prime) and precipitated with ethanol.

Viral DNA from approximately 10^5 cells was digested to completion with restriction endonucleases, separated on a 0.7% agarose gel and visualized with ethidium bromide. Gels were denatured and viral DNA transferred to nitrocellulose membranes (and UV cross-linked.) Membranes were prehybridized in 6 × SSPE, 2 × Denhardt's, 0.5% SDS and 300 µg/ml salmon sperm DNA for one hour. Blots were hybridized with ^{32}P-radiolabelled DNA probes overnight at 65°C in 4 × SSPE, 3 × Denhardt's, 0.5% SDS, 15% formamide, 10% dextran sulfate and 400 µg/ml salmon sperm DNA. Filters were washed at 65°C in 0.1 × SSPE, 0.1% SDS. Membranes were exposed to a PhosphorScreen and results visualized using ImageQuant software (Molecular Dynamics).

RESULTS

Construction of a cell line expressing ppUL44. We anticipated based on *in vitro* studies that UL44 was essential for HCMV replication as the viral DNA polymerase accessory factor (Ertl and Powell, 1992; Reddig *et al.*, 1994; Weiland *et al.*, 1994). Therefore, we set out to construct a complementing cell line for the isolation of a UL44 mutant HCMV. We generated a clone, pON2280, carrying the complete coding sequence for UL44 including its minimal promoter region and contained within a defective amphotropic retroviral vector bearing the neomycin resistance gene under translational control of the encephalomyocarditis virus internal ribosomal entry site (EMCV IRES; Fig. 4.1). To construct the UL44 expressing cell line, we transfected pON2280 DNA into ϕNX-A packaging cells and harvested the transiently produced retrovirus 2280. Typical retroviral titers produced were on the order of 10^5 to 10^6 focus forming units per ml as assessed on NIH3T3 cells. The resultant retrovirus was used to infect low passage human embryonic lung fibroblasts. At 24 hpi, the drug Geneticin (G418) was added to the culture medium and the cells were maintained under G418 selection for two weeks. Three independent G418-resistant cell lines were obtained using this method and these were subsequently immortalized with the human papillomavirus 16 E6/E7 genes produced from LXSN16E6E7 cells (Halbert *et al.*, 1991) as previously described (Greaves *et al.*, 1995). With continued culture passage under G418 selection, only two of the three immortalized cell lines survived. The remaining two cell lines possessed doubling properties similar to other cell lines used in our laboratory for growth of HCMV. These cell lines were pooled to increase culture stability.

Expression of UL44 protein in IHF2280 cells. The clone pON2280 was designed to allow expression of the UL44 ORF from transcripts initiating within the retroviral LTR and in the absence of HCMV infection. This same transcript was also predicted to encode the neomycin resistance gene under translational control of the EMCV IRES. Based on our ability to isolate G418-resistant clones, we concluded that the IHF2280 cell line produced transcripts containing the UL44 coding sequence and hypothesized that UL44 protein product was expressed in these cells. We first assessed the ability of IHF2280 cells to produce UL44 protein (ppUL44) by immunoblot analysis. Cell lysates were prepared from IHF2280 cells, mock-infected HFF cells, IHFie1.3 cells and HCMV Towne virus-infected HFF cells at 48 hpi following the protocol described in the Materials and Methods section. Immunoblot analysis of the resultant total cell protein using mouse monoclonal antibody against UL44 protein is shown in Fig. 4.2. Consistent with previous work (Geballe *et al.*, 1986; Kemble *et al.*, 1987; Leach and Mocarski, 1989), the family of UL44 proteins was observed in HCMV Towne virus-infected cells. The IHF2280 cells, on the other hand, produced a single immunoreactive protein that comigrated with the largest and most abundant species of the UL44 family suggesting constitutive expression of ppUL44 in this cell line. In contrast, mock-infected cells and an immortalized cell line expressing the HCMV *ie1* protein product (IHFie1.3) were negative for UL44 expression.

Our results demonstrated that ppUL44 was expressed by the IHF2280 cell line, however, it was unclear whether the expressed protein localized to the nucleus as observed during infection. To determine the fate of ppUL44 within IHF2280 cells, we compared the localization pattern of ppUL44 expressed in this cell line with that expressed during HCMV infection. IHF2280 cells, mock-infected HFF cells, and HCMV AD169 virus-infected HFF cells at 72 hpi were fixed onto glass coverslips and the presence of UL44 was detected using indirect immunofluorescence assay as shown in Fig. 4.3. Consistent with our immunoblot results, IHF2280 cells were observed to express ppUL44 (Fig. 4.3 A, B). The UL44 protein expressed by this cell line localized to the nucleus in a diffuse pattern similar to this protein's localization during the early stages of HCMV infection (Penfold and Mocarski, 1997). In addition, greater than 90% of the IHF2280 cell population stained positive for UL44 suggesting efficient expression of ppUL44 within this cell line. In contrast, mock-infected HFF cells did not express ppUL44 (Fig. 4.3D).

ppUL44 was observed in replication compartments within the nucleus and at the periphery of the nuclear membrane in HCMV-infected HFF cells at 72 hpi (Fig. 4.3C), consistent with previous observations (Penfold and Mocarski, 1997). This pattern was never observed in IHF2280 cells suggesting that other HCMV proteins are required for the reorganization of ppUL44 into replication compartments. This idea is consistent with results from an *in vitro* study suggesting that UL44, along with other core and auxiliary replication factors, and the HCMV origin of replication are required concurrently for the formation of replication compartments within transfected cells (Sarisky and Hayward, 1996).

Ability of IHF2280 cells to support HCMV replication. Our observations demonstrated that the IHF2280 cell line was able to produce ppUL44 and that the expressed protein localized to the nucleus as in natural HCMV infection. To determine whether constitutive expression of ppUL44 by IHF2280 cells inhibited the ability of HCMV to replicate, we compared the growth kinetics of wild-type HCMV Towne virus in IHF2280 cells with growth in primary HFF cells. IHF2280 and HFF cells were infected with HCMV Towne at a high m.o.i. (5 p.f.u./cell) and viral progeny was harvested at 1, 2, 3, 5 and 7 days post-infection. Growth kinetics of HCMV Towne virus in each of these cell types was determined using plaque assay and expressed as a function of progeny virus yield over time (Fig. 4.4). Viral plaque formation was as efficient in IHF2280 cells as in HFF cells. IHF2280 cells produced similar titers of progeny virus as HFF cells and with the same temporal kinetics. This result demonstrates that IHF2280 cells are able to support HCMV growth and suggests that this cell line is an appropriate candidate for propagation of UL44 viral mutants.

Construction of a HCMV mutant in UL44. Extensive sequence identity exists among UL44 homologs from the betaherpesvirus family and in particular in the amino-terminal two-thirds of these proteins (Fig. 4.5). Biochemical assays using *E. coli* expression systems had also previously detemined that the amino-terminal 309 amino acids contained the functional domain of HCMV UL44 (Weiland *et al.*, 1994). Therefore, we designed a mutation to disrupt the

UL44 ORF within this region. A 1.1 kb fragment containing the *E. coli gpt* gene under control of the HSV *tk* promoter was inserted into the *Bgl*II site in the UL44 sequence. This insertion interrupts the UL44 ORF after the first 115 amino acids, disrupting the remaining 72% of the UL44 gene. To construct a recombinant HCMV with this disruption in UL44, we transfected IHF2280 cells with the plasmid pON2284 using the calcium phosphate method (Chen and Okayama, 1987), superinfected these cells with HCMV Towne virus and enriched for mutant virus using mycophenolic acid and xanthine. The predicted genome structures of parental Towne virus and recombinant RC2284 mutant virus are shown in Fig. 4.6. Following three rounds of selection on IHF2280 cells, we assessed pools of progeny virus for the presence of the recombinant HCMV RC2284 using DNA blot hybridization with a probe specific for *gpt*. By DNA blot hybridization, a 4.1 kb *Hin*DIII fragment containing the *gpt* insert as well as a portion of UL44 and UL42-UL43 sequence was identified in a single pool of virus (Fig. 4.7). This result suggested that we had obtained RC2284 recombinant HCMV and that the *gpt* gene was incorporated into the desired location of the viral genome. Further analysis of additional digests of viral DNA using UL44 probes suggested that approximately 25% of this pool was recombinant in nature (data not shown). Subsequent attempts to purify RC2284 away from wild-type virus were unsuccessful as were attempts to obtain other independent pools of RC2284. This result suggests that IHF2280 cells are unable to complement HCMV mutants in UL44 despite constitutive expression of ppUL44 by this cell line.

DISCUSSION

In this chapter, we set out to construct a recombinant HCMV mutated in the UL44 ORF of this virus. Since *in vitro* studies had identified UL44 as the HCMV DNA polymerase accessory protein (Ertl and Powell, 1992; Weiland *et al.*, 1994) and transient replication assays had identified this ORF as required for lytic origin dependent DNA synthesis (Pari and Anders, 1993; Pari *et al.*, 1993), a cell line expressing UL44 protein was constructed for the isolation of a UL44 null HCMV.

We chose to generate a ppUL44-expressing cell line through use of a defective amphotropic retrovirus bearing the UL44 gene, the UL44 minimal promoter and a selectable neomycin phosphotransferase (*neo*) gene marker.

This design was selected to allow the highest efficiency of transduction into primary HEL cells and to provide expression of the UL44 product from the retroviral LTR in the absence of viral infection. Using this method, we isolated three independent G418-resistant cell lines, IHF2280, that were immortalized with the human papillomavirus 16 E6/E7 genes. Two of the three cell lines were subsequently pooled for increased passage stability.

IHF2280 cells expressed ppUL44 constitutively and the protein was found localized to the nucleus as observed during natural HCMV infection. Constitutive expression of UL44 had no effect on the doubling properties of IHF2280 cells and this cell line was found to support HCMV replication to levels seen in primary human fibroblasts. Based on these results, we believed that the IHF2280 cell line was an appropriate substrate for the isolation and purification of a knock-out in HCMV UL44.

Previous studies of UL44 function have employed *in vitro* assays conducted in the absence of natural HCMV infection and using minimal HCMV origin sequence as artificial templates for DNA synthesis (Ertl and Powell, 1992; Pari and Anders, 1993; Pari *et al.*, 1993; Weiland *et al.*, 1994). While this approach has identified the boundaries of the functional domain in UL44 important for its activity as the HCMV DNA polymerase accessory protein, it provides limited insight into the role(s) of this viral protein during the course of the HCMV life cycle. We sought to understand the contribution of UL44 to HCMV DNA replication during the early stages of infection and to define the functional domains within UL44 through the construction of a null mutant in UL44.

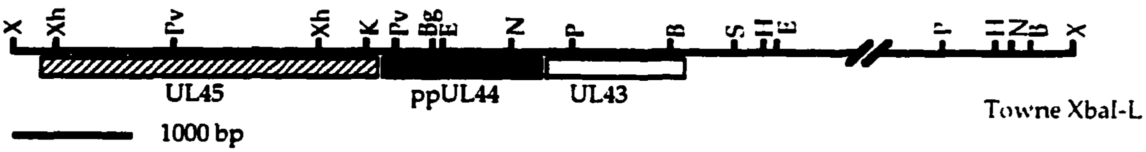
A recombinant HCMV disrupted in the UL44 gene by insertion of the *E. coli gpt* gene was obtained on IHF2280 cells following selection with mycophenolic acid and xanthine. The site of insertion into the HCMV genome was predicted to result in expression of a truncated UL44 protein that is abrogated for UL44 activity as the DNA polymerase processivity factor. We were unable to purify this recombinant HCMV out of a mixed population of wild-type and mutant viruses using standard methods of limiting dilution or plaque purification. This result leads us to believe that the IHF2280 cells do not complement growth of a HCMV UL44 knock-out.

It is unclear why the IHF2280 cells were unable to support isolation of a UL44 null HCMV. One reason may be that the cell line does not produce adequate quantities of UL44 protein or that UL44 expression in IHF2280 cells does not occur with appropriate temporal kinetics. Indeed, we were just able to detect the predominant species of ppUL44 in IHF2280 cell lysates by immunoblot analysis suggesting that only small amounts of UL44 protein were being made in the absence of HCMV infection. We had attempted to address the issue of expression levels and kinetics in this cell line during HCMV infection by including the previously identified UL44 minimal promoter (Leach and Mocarski, 1989) in our retroviral construct. This minimal promoter sequence had been determined to produce levels of expression comparable to the intact UL44 promoter and with kinetics appropriate for an HCMV early gene using transient transfection/infection assays with *E. coli lacZ* gene as indicator (Leach and Mocarski, 1989). Nonetheless, we are unable to determine if UL44 expression in IHF2280 cells is in fact activated by HCMV infection as there is nothing to distinguish between protein expressed from IHF2280 cells and that expressed from the HCMV genome during infection.

Another explanation for our inability to purify the UL44 knock-out HCMV may be due to the type of mutation we engineered. UL44 is located in a very transcriptionally active region of the HCMV genome. Several transcripts initiate immediately downstream of this gene at immediate early, early and late times of infection (Kouzarides *et al.*, 1988; Leach, 1990; Wilkinson *et al.*, 1984). There is also readthrough transcription from genes upstream of UL44 (Geballe *et al.*, 1986) at late times of infection. Moreover, we have identified one other transcript of approximately 1.9 kb that is expressed at immediate early times and has not been characterized (Tan and Mocarski, unpublished observations). Our laboratory has had previous success with insertion of the *E. coli gpt* gene into transcriptionally complex regions of the HCMV genome (Greaves *et al.*, 1995; Prichard *et al.*, 1996). However, it is possible that insertion of this sizeable cassette into UL44 sequence may have disrupted the expression of other genes in the region. In such a case, more subtle mutations such as frameshift mutations may allow isolation of a UL44 null mutant on the existing IHF2280 cell line.

Finally, it is formally possible that the mutation we have engineered is unstable due to recombination between the mutation in the virus and the UL44 gene carried by the cell line. The cell line contains 530 bp of homology on the 5' end of the UL44 mutation and 1.6 kb of homology on the 3' end. Mutations in the viral copy of UL44 designed to limit the possibility of recombination may allow successful derivation of UL44 knock-out viruses using the UL44-expressing cell line generated here.

Fig. 4.1. Structure of the HCMV UL44 region and defective amphotropic retroviral vector carrying UL44. The top line shows the restriction map of the UL44 region with the open reading frames of UL44 and flanking genes, UL43 and UL45, depicted below (boxes). Complete *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*DIII (H), *Nco*I (N), *Sal*I (S), *Xba*I (X) and *Xho*I (Xh) maps and partial *Kpn*I (K), *Pst*I (P) and *Pvu*II (Pv) restriction sites are shown. The bottom line of the figure represents the retroviral clone generated to contain the HCMV UL44 gene and its minimal promoter (beginning at the *Kpn*I site). This vector contains the neomycin phosphotransferase gene under translational control of the encephalomyocarditis virus internal ribosomal entry site (EMCV IRES-neo) and allows for selection with Geneticin (G418).


X
Xh
Pv
Xh
K
Pv
Bg
E
N
P
B
S
H
H
E
P
H
H
N
B
X
UL45
ppUL44
UL43
Towne XbaI-L
1000 bp

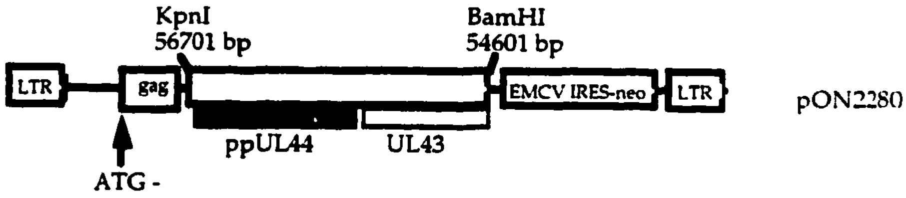

KpnI
56701 bp
BamHI
54601 bp
LTR
gag
EMCV IRES-neo
LTR
pON2280
ppUL44
UL43
ATG -

Fig. 4.2. Expression of ppUL44 by IHF2280 cells. Cell lysates were prepared from mock-infected HFF cells (mock inf.), HCMV Towne virus-infected HFF cells at 48 hpi (Towne inf.), IHF2280 cells and IHFie1.3 cells, separated by SDS-PAGE on a 10% gel and transfered to nitrocellulose membrane. ppUL44 was detected using mouse monoclonal antibody against ppUL44 (1202). The largest and most abundant species of the ppUL44 family of proteins is indicated with an arrow. The positions of the molecular weight markers are indicated on the right in kilodaltons.

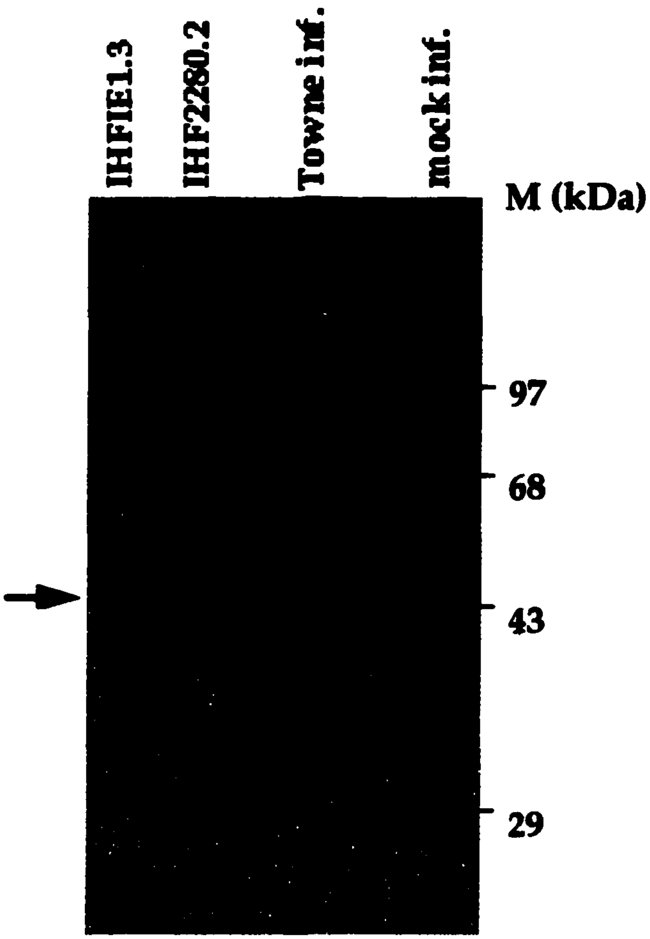

IHFIE1.3
IHF2280.2
Towne inf.
mock inf.
M (kDa)
97
68
43
29

Fig. 4.3. Localization of ppUL44 in IHF2280 cells. IHF2280 cells, HCMV Towne virus-infected HFF cell at 72 hpi and mock-infected cells were fixed on glass coverslips and stained with mouse monoclonal antibody against ppUL44 (1202), followed by FITC horse anti-mouse immunoglobulin antibody. Confocal images of IHF2280 cells (A, B), HCMV infected cells (C) and mock-infected cells (D).

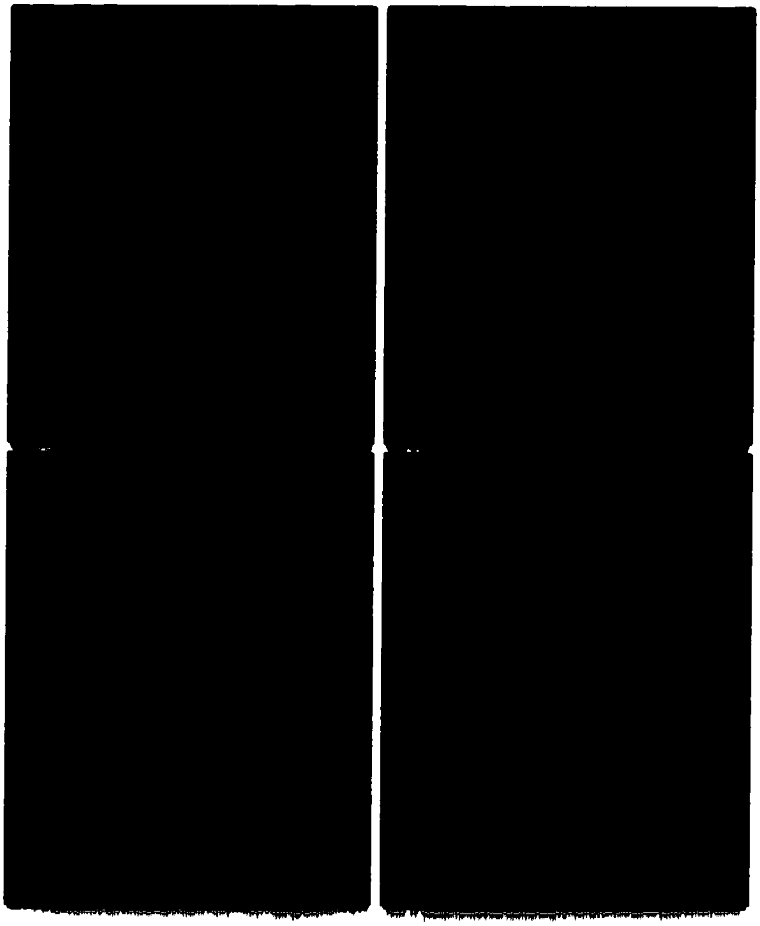


Fig. 4.4. Growth of HCMV Towne virus on IHF2280 cells. IHF2280 cells and HFF cells were infected with HCMV Towne strain at a m.o.i. of 5 p.f.u./cell and titers of the resulting progeny virus were determined at the indicated times post-infection. Titers of HCMV Towne virus on IHF2280 cells (●) and HFF cells (□) are shown. The time point at 0 hpi represents the titer of the input virus.

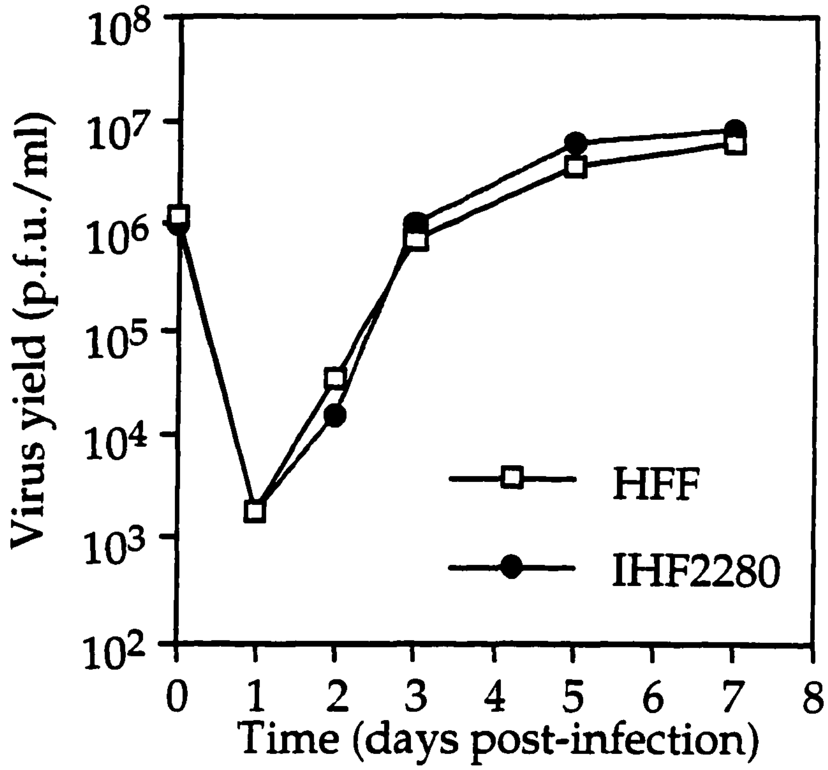
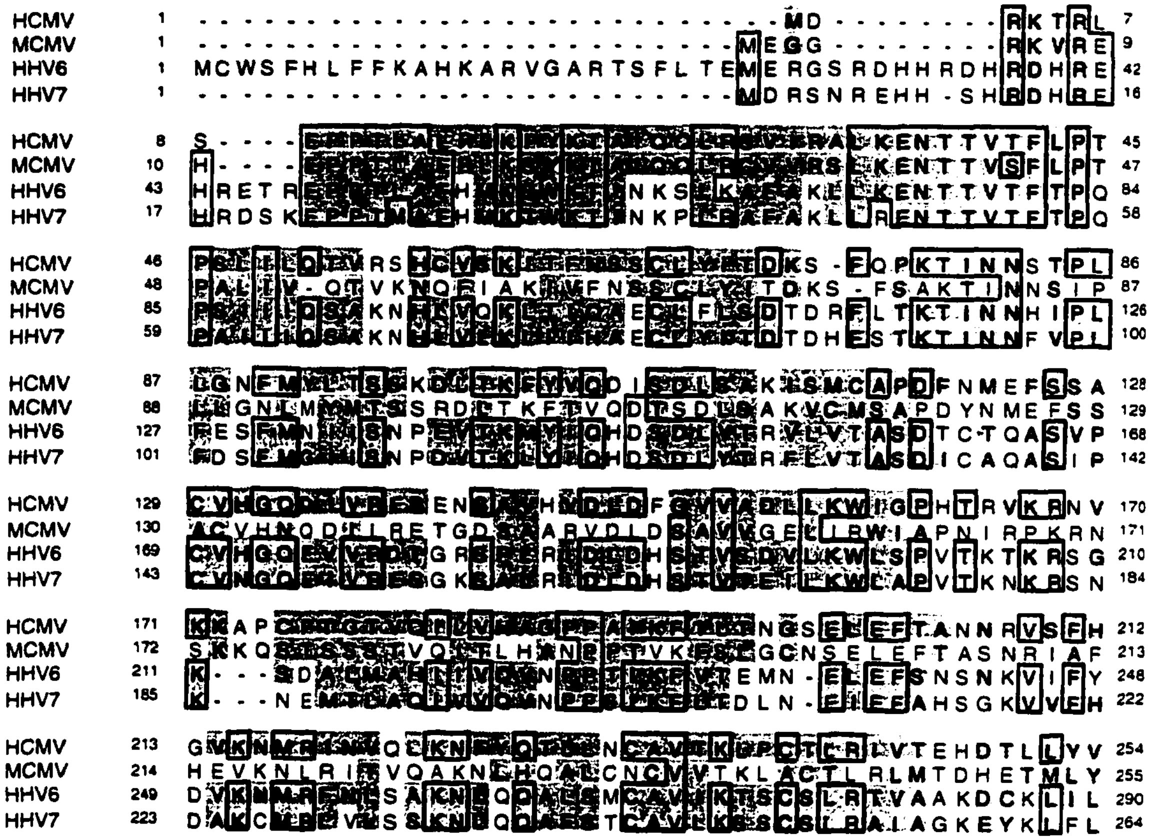

10^8
10^7
10^6
10^5
10^4
10^3
10^2
Virus yield (p.f.u./ml)
0 1 2 3 4 5 6 7 8
Time (days post-infection)
HFF
IHF2280

Fig. 4.5. Sequence alignment for UL44 homologs from human CMV, murine CMV, HHV-6 and HHV-7 using MCB Search Launcher program (Human Genome Center, Baylor College of Medicine) and assembled using SeqVu 1.0.1 (Garvan Institute of Medical Research, Sydney, Australia). The regions of identity are shown in blocks; regions of homology are shaded.


HCMV
MCMV
HHV6
HHV7

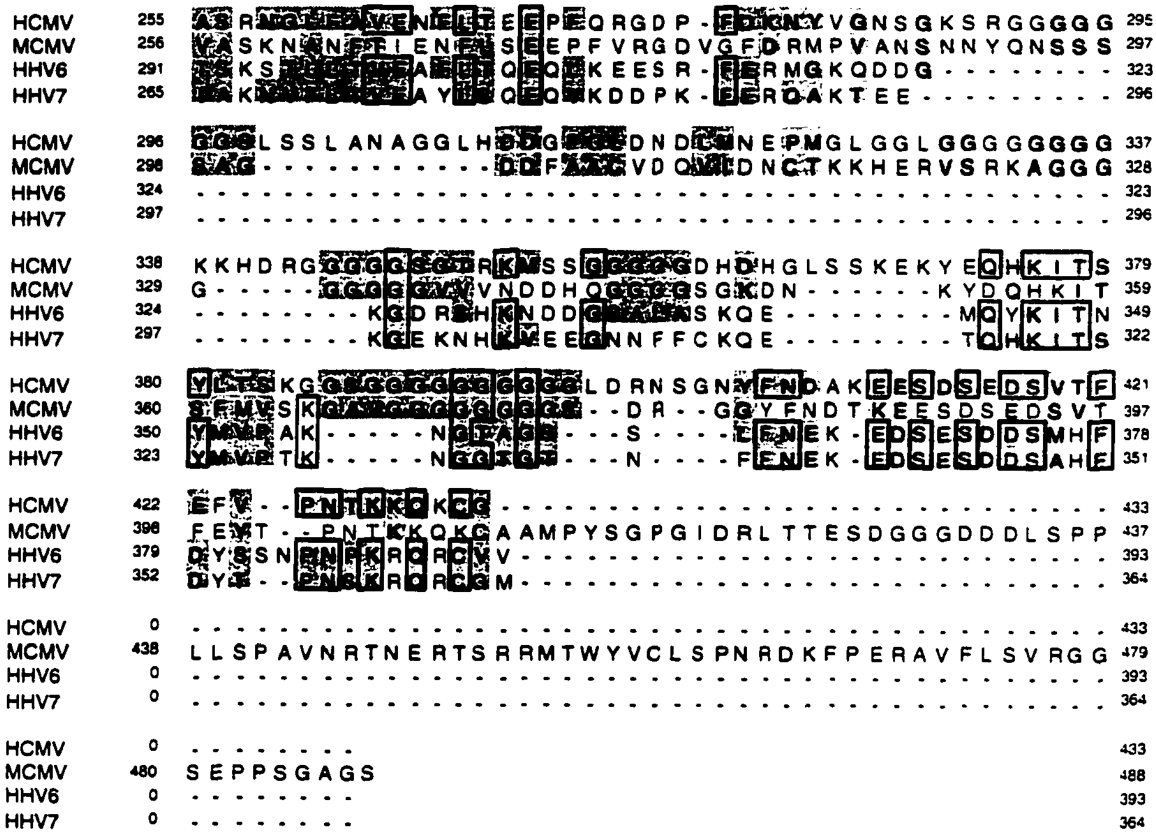
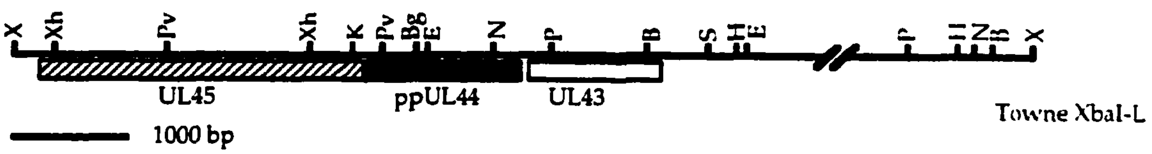

HCMV 255 AS R [illegible] V E N [illegible] E E P E Q R G D P - F [illegible] V G N S G K S R G G G G G 295
MCMV 256 V A S K N G N F T I E N F N S E E P F V R G D V G F D R M P V A N S N N Y Q N S S S 297
HHV6 291 T S K S [illegible] A [illegible] Q E Q [illegible] K E E S R - F E R M G K Q D D G - - - - - - - - 323
HHV7 265 [illegible] K [illegible] A Y [illegible] Q E Q [illegible] K D D P K - F E R Q A K T E E - - - - - - - - - 296
HCMV 296 G G G L S S L A N A G G L H D D G [illegible] D N D [illegible] N E P M G L G G L G G G G G G G G 337
MCMV 298 S A G - - - - - - - - - - - - - D D F A A G V D Q [illegible] D N C T K K H E R V S R K A G G G 328
HHV6 324 323
HHV7 297 296
HCMV 338 K K H D R G G G G G S G G D R K M S S G G G G G G D H D H G L S S K E K Y E Q H K I T S 379
MCMV 329 G - - - - - G G G G G V V V N D D H Q G G G G G S G K D N - - - - - - K Y D Q H K I T 359
HHV6 324 - - - - - - - - - K G D R S H K N D D G [illegible] S K Q E - - - - - - - - - M Q Y K I T N 349
HHV7 297 - - - - - - - - - K G E K N H K M E E G N N F F C K Q E - - - - - - - - - T Q H K I T S 322
HCMV 380 Y L T S K G [illegible] L D R N S G N Y F N D A K E E S D S E D S V T F 421
MCMV 360 S F M V S K [illegible] - - D R - - G G Y F N D T K E E S D S E D S V T 397
HHV6 350 Y [illegible] A K - - - - - N G [illegible] - - - S - - - - - L F N E K - E D S E S D D S M H F 378
HHV7 323 Y [illegible] T K - - - - - N G G [illegible] - - - N - - - - F E N E K - E D S E S D D S A H F 351
HCMV 422 E F V - - P N T K K Q K C G - 433
MCMV 398 F E Y T - - P N T K K Q K C A A M P Y S G P G I D R L T T E S D G G G D D D L S P P 437
HHV6 379 D Y S S N R N F K R Q R C V V - 393
HHV7 352 D Y S - - P N S K R Q R C G M - 364
HCMV 0 433
MCMV 438 L L S P A V N R T N E R T S R R M T W Y V C L S P N R D K F P E R A V F L S V R G G 479
HHV6 0 393
HHV7 0 364
HCMV 0 433
MCMV 480 S E P P S G A G S 488
HHV6 0 393
HHV7 0 364

Fig. 4.6. Structure of RC2284. The top line represents the restriction map of the UL44 region, with the ORFs encoding UL44 and its neighbors shown below. The bottom line represents the same region in RC2284 showing the position of the 1.1 kb insertion containing the *E. coli gpt* gene (white box) under control of the HSV *tk* promoter (hatched box). This insertion disrupts the UL44 protein after amino acid 115. pA, simian virus 40 polyadenylation signal.


X
Xh
Pv
Xh
K
Pv
Bg
E
N
P
B
S
H
E
P
X
UL45
ppUL44
UL43
Towne XbaI-L
1000 bp

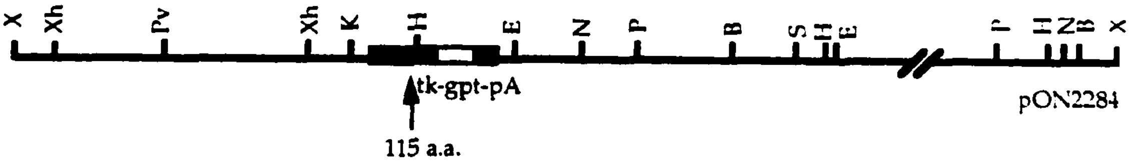
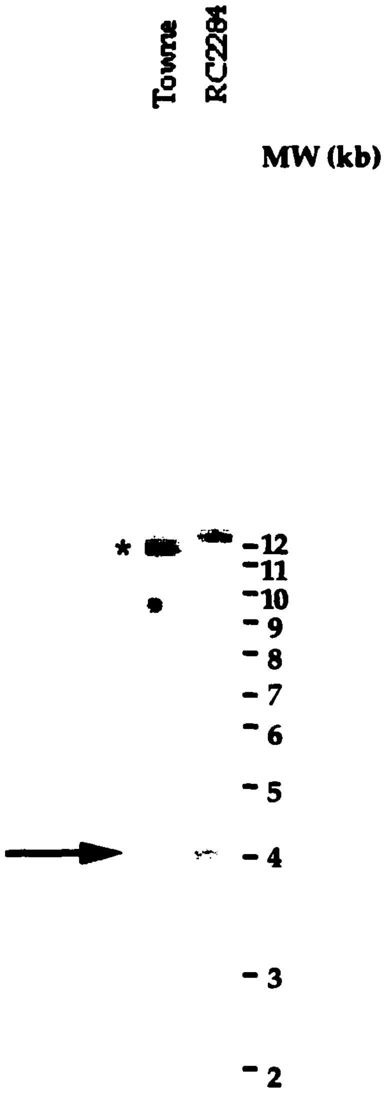

X
Xh
Pv
Xh
K
H
E
N
P
B
S
H
E
X
tk-gpt-pA
115 a.a.
pON2284

Fig. 4.7. DNA blot of viral DNA from parental Towne virus and RC2284. Viral DNA was digested with *Hin*DIII, separated on a 0.6% agarose gel and transfered onto nylon membrane. The membrane was probed with pON1101 containing the *E. coli gpt* gene. A new fragment of the predicted size hybridized to this probe and is indicated by the arrow. Asterisk denotes a cross-hybridizing fragment of unknown origin. The positions of the molecular size standards are shown on the right in kilobases.


Towne
RC2284
MW (kb)
*
12
11
10
9
8
7
6
5
4
3
2

REFERENCES

Achacoso, P. and Nolan, G. manuscript in preparation. .

Chee, M.S., Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerny, R., Horsnell, T., Hutchison, C.A.I., Kouzarides, T., Martignetti, J.A., Preddie, E., Satchwell, S.C., Tomlinson, P., Weston, K.M. and Barrell, B.G. (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol*, **154**, 125-170.

Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol*, **7**, 2745-2752.

Coen, D.M. (1996) Viral DNA polymerases. In DePamphilis, M.L. (ed.) *DNA replication in eukaryotic cells.* Cold Spring Harbor Press, Cold Spring Harbor, pp. 495-523.

de Bruyn Kops, A. and Knipe, D.M. (1988) Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. *Cell*, **55**, 857-868.

Digard, P., Chow, C.S., Pirrit, L. and Coen, D.M. (1993) Functional analysis of the herpes simplex virus UL42 protein. *J Virol*, **67**, 1159-1168.

Ertl, P.F. and Powell, K.L. (1992) Physical and functional interaction of human cytomegalovirus DNA polymerase and its accessory protein (ICP36) expressed in insect cells. *J Virol*, **66**, 4126-4133.

Gao, M., DiTusa, S.F. and Cordingley, M.G. (1993) The C-terminal third of UL42, a HSV-1 DNA replication protein, is dispensable for viral growth. *Virology*, **194**, 647-653.

Geballe, A.P., Leach, F.S. and Mocarski, E.S. (1986) Regulation of cytomegalovirus late gene expression: γ genes are controlled by posttranscriptional events. *J Virol*, **57**, 864-874.

Greaves, R.F., Brown, J.M., Vieira, J. and Mocarski, E.S. (1995) Selectable insertion and deletion mutagenesis of the human cytomegalovirus genome using the Escherichia coli guanosine phosphoribosyl transferase (gpt) gene. *J Gen Virol*, **76**, 2151-2160.

Greaves, R.F. and Mocarski, E.S. (1998) Defective growth correlates with reduced accumulation of a viral DNA replication protein after low multiplicity infection by a human cytomegalovirus *ie1* mutant. *J. Virol.*, **72**, 366-379.

Halbert, C.L., Demers, G.W. and Galloway, D.A. (1991) The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J Virol*, **65**, 473-478.

Johnson, P.A., Best, M.G., Friedmann, T. and Parris, D.S. (1991) Isolation of a herpes simplex virus type 1 mutant deleted for the essential UL42 gene and characterization of its null phenotype. *J Virol*, **65**, 700-710.

Kemble, G.W., McCormick, A.L., Pereira, L. and Mocarski, E.S. (1987) A cytomegalovirus protein with properties of herpes simplex virus ICP8: partial purification of the polypeptide and map position of the gene. *J Virol*, **61**, 3143-3151.

Kouzarides, T., Bankier, A.T., Satchwell, S.C., Preddy, E. and Barrell, B.G. (1988) An immediate early gene of human cytomegalovirus encodes a potential membrane glycoprotein. *Virology*, **165**, 151-164.

Leach, F.S. (1990) . Stanford.

Leach, F.S. and Mocarski, E.S. (1989) Regulation of cytomegalovirus late-gene expression: differential use of three start sites in the transcriptional activation of ICP36 gene expression. *J Virol*, **63**, 1783-1791.

Mocarski, E.S. (1995) Cytomegaloviruses and their replication. In Fields, B.N., Knipe, D.M. and Howley, P.M. (eds.), *Fields Virology*. Lippincott-Raven Publishers, New York, pp. 2447-2492.

Mocarski, E.S., Bonyhadi, M., Salimi, S., McCune, J.M. and Kaneshima, H. (1993) Human cytomegalovirus in a SCID-hu mouse: thymic epithelial cells are prominent targets of viral replication. *Proc Natl Acad Sci U S A*, **90**, 104-108.

Mocarski, E.S., Kemble, G.W., Lyle, J.M. and Greaves, R.F. (1996) A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation. *Proc Natl Acad Sci U S A*, **93**, 11321-11326.

Morgenstern, J.P. and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res*, **18**, 3587-3596.

Pari, G.S. and Anders, D.G. (1993) Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. *J Virol*, **67**, 6979-6988.

Pari, G.S., Kacica, M.A. and Anders, D.G. (1993) Open reading frames UL44, IRS1/TRS1, and UL36-38 are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA synthesis. *J Virol*, **67**, 2575-2582.

Penfold, M.E. and Mocarski, E.S. (1997) Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis. *Virology*, **239**, 46-61.

Plachter, B., Nordin, M., Wirgart, B.Z., Mach, M., Stein, H., Grillner, L. and Jahn, G. (1992) The DNA-binding protein P52 of human cytomegalovirus reacts with monoclonal antibody CCH2 and associates with the nuclear membrane at late times after infection. *Virus Res*, **24**, 265-276.

Prichard, M.N., Duke, G.M. and Mocarski, E.S. (1996) Human cytomegalovirus uracil DNA glycosylase is required for the normal temporal regulation of both DNA synthesis and viral replication. *J Virol*, **70**, 3018-3025.

Quinlan, M.P., Chen, L.B. and Knipe, D.M. (1984) The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell*, **36**, 857-868.

Reddig, P.J., Grinstead, L.A., Monahan, S.J., Johnson, P.A. and Parris, D.S. (1994) The essential in vivo function of the herpes simplex virus UL42 protein correlates with its ability to stimulate the viral DNA polymerase in vitro. *Virology*, **200**, 447-456.

Ripalti, A., Boccuni, M.C., Campanini, F. and Landini, M.P. (1995) Cytomegalovirus-mediated induction of antisense mRNA expression to UL44 inhibits virus replication in an astrocytoma cell line: identification of an essential gene. *J Virol*, **69**, 2047-2057.

Rixon, F.J., Atkinson, M.A. and Hay, J. (1983) Intranuclear distribution of herpes simplex virus type 2 DNA synthesis: examination by light and electron microscopy. *J Gen Virol*, **64**, 2087-2092.

Sarisky, R.T. and Hayward, G.S. (1996) Evidence that the UL84 gene product of human cytomegalovirus is essential for promoting oriLyt-dependent DNA replication and formation of replication compartments in cotransfection assays. *J Virol*, **70**, 7398-7413.

Spaete, R.R. and Mocarski, E.S. (1985) Regulation of cytomegalovirus gene expression: α and β promoters are trans activated by viral functions in permissive human fibroblasts. *J Virol*, **56**, 135-143.

Weiland, K.L., Oien, N.L., Homa, F. and Wathen, M.W. (1994) Functional analysis of human cytomegalovirus polymerase accessory protein. *Virus Res*, **34**, 191-206.

Wilcock, D. and Lane, D.P. (1991) Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. *Nature*, **349**, 429-431.

Wilkinson, G.W., Akrigg, A. and Greenaway, P.J. (1984) Transcription of the immediate early genes of human cytomegalovirus strain AD169. *Virus Res*, **1**, 101-106.