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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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November 1999
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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.
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Chapter 1: Introduction
The herpesvirus family infects a wide variety 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 persistent 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 beta herpesvirus 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 beta herpesvirus 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 (foscarinet) 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 foscarinet 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, iε1 and iε2, 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 G1 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 Ul 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 oriL (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 dispensable 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 from 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).
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).
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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 maintenance 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/Sall 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' TAATACGACTCACTATAGGGATGATGGATCTGTCC or UDG F2 5' CATGGACCTAATCAAGC and T7 UDG R2 5' TAATACGACTCATAGGGGCTCTCCATCCAGGTGG. 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-U4R 5' TAATACGACTCATAGGGGGCTCGCCCGTCGCG 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' GTGGTACCACTGGCGCTTTAAGGTGCG and T7 UL44 R 5' TAATACGACTCATATAGGGCAGGTACATGAAATTACC. 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 x 10⁶ 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 x SSPE, 2 x 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 x SSPE, 3 x 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 x 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

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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

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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 G1 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

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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 10⁵ cells infected with AD169 or RC2620 isolated at the indicated times post-infection. The membrane was probed with ³²P-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.
C  

AD169

RC2620

D

Relative DNA Synthesis

Time (hpi)

0 24 48 72 96 120 144 168 192 216

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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).
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.
Fig. 2.4. Map of the UL114-119 region of the CMV genome. The top line represents a *HindIII* 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).
Previously Mapped transcripts:

<table>
<thead>
<tr>
<th>Size (kb)</th>
<th>AATTAAA</th>
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<tbody>
<tr>
<td>3.1</td>
<td></td>
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<tr>
<td>3.1</td>
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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.
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 transferred 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.
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.
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Chapter 3: Uracil incorporation into HCMV DNA and its consequences.
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 occurring. Consistent with this possibility, 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 credence 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 $3 \times 10^6$ 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 $1,000 \times g$ 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 $\rightarrow$ 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-link). Membranes were prehybridized in $6 \times$ SSPE, $2 \times$ Denhardt's, 0.5\% SDS and 300 $\mu$g/ml salmon sperm DNA for one hour. Blots were hybridized with $^{32}$P-radiolabelled DNA probes overnight at 65°C in $4 \times$ SSPE, $3 \times$ Denhardt's, 0.5\% SDS, 15\% formamide, 10\% dextran sulfate and 400 $\mu$g/ml salmon sperm DNA.
DNA. Filters were washed at 65°C in 0.1 x 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/m²/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 32P-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 nuclease or helicase 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 transferred 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.
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/m² (lane 10); AD169 UV-irradiated at 40 kJ/m² and incubated with TEV (lane 11) and AD169 UV-irradiated at 80 kJ/m² and incubated with TEV. Reaction products were subjected to alkaline denaturing agarose gel electrophoresis, transferred 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.
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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 32P-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.
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9.4-
6.6-
4.4-

2.3-
2.0-

+ UDG

hpi: 0 m 4 8 12 24 48 72 96

85

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B

![Bar chart showing relative intensity (x 10^6) over time (hpi) for -UDG and +UDG conditions.](chart.png)
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 transferred 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.
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 \textit{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.
REFERENCES


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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 beta herpesvirus 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 alpha-herpesvirus homologs is divergent, the C-terminus of HCMV UL44 and other beta-herpesvirus 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 dispensable 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 x 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 titrated 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 titrated 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 XbaI fragment from the Towne cosmid TN23 into the SpeI site of pGEM-3Zf+. Plasmid pON2278 was constructed by ligating a 2.95 kb KpnI fragment from pON2275, representing nucleotides 53754-56701 of the published AD169 strain sequence (Chee et al., 1990), into the KpnI site of pGEM-3Zf+. The plasmid pON2280 was
constructed by ligating a 2.5 kb BamHI fragment from pON2278 into the BglII 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 BamHI/BglII digestion.

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

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 transferred 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 transferred 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, IHFiel.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 iel protein product (IHFiel.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 determined 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 BglII 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 HinDIII 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 \textit{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 \textit{et al.}, 1993; Weiland \textit{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 \textit{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 BstHI (B), BglII (Bg), EcoRI (E), HindIII (H), NcoI (N), SalI (S), XbaI (X) and XhoI (Xh) maps and partial KpnI (K), PstI (P) and PvuII (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 KpnI 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).
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 transferred 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.
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.
Virus yield (p.f.u./ml)

Time (days post-infection)

10^8

10^7

10^6

10^5

10^4

10^3

10^2

0 1 2 3 4 5 6 7 8

HFF

IHF2280

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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.
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.
Fig. 4.7. DNA blot of viral DNA from parental Towne virus and RC2284. Viral DNA was digested with *Hin*III, separated on a 0.6% agarose gel and transferred 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.
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