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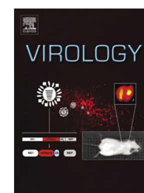

UL84-independent replication of human cytomegalovirus strains conferred by a single codon change in UL122

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ABSTRACT

The UL84 gene of human cytomegalovirus (HCMV) is thought to be involved in the initiation of viral DNA replication, and is essential for replication of strains AD169 and Towne. Hence, discovery that strain TB40-BAC4 is viable in the absence of UL84 presented an enigma requiring an explanation. Data reported here show that strain TR also tolerated loss of UL84, whereas strains FIX, Merlin, Ph, and Toledo did not. UL84-independent growth required the viral replication origin. The genetic locus in TB40 that controls UL84 dependence was mapped to codon 388 of the UL122 gene, which encodes the immediate early 2 (IE2) 86 kD protein. Introduction of this TB40-BAC4 variant (H388D) into FIX and Toledo clones converted these strains to UL84 independence. These results provide genetic evidence in virus-infected cells that supports the hypothesis that UL122 participates in the initiation of viral DNA replication by a mechanism involving transcription-mediated activation of the origin.
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Introduction

Synthesis of human *Herpesvirus* DNA requires a conserved set of six viral replication fork proteins (core) and one or more viral initiation proteins that are not conserved among the alpha, beta, and gamma subfamilies. For the beta-herpesvirus human cytomegalovirus (HCMV), the UL84 gene product was implicated as a candidate for an initiation protein because it is required for replication of a plasmid containing a cloned lytic origin of replication, *ori*Lyt (Anders et al., 1992; Anders and Punturieri, 1991; Hamzeh et al., 1990), in a transient transfection assay (Pari and Anders, 1993; Sarisky and Hayward, 1996). UL84 protein associates with *ori*Lyt in infected cells and virions (Colletti et al., 2007); however, there is no evidence that UL84 protein binds directly to DNA. Rather, it is probably recruited to *ori*Lyt by binding to one or more of the proteins encoded by UL122 (Colletti et al., 2004; Sanders et al., 2008; Spector and Tevethia, 1994; Xu et al., 2004a) and/or the polymerase subunit product of the UL44 gene (Gao et al., 2008; Strang et al., 2009), one of the core replication fork components.

UL84 is essential for growth of bacterial artificial chromosome (BAC) clones of strains AD169 (Xu et al., 2004b; Yu et al., 2003; Gao et al., 2010; Strang et al., 2012) and Towne (Dunn et al., 2003) in tissue culture, and in its absence viral DNA synthesis cannot be detected (Xu et al., 2004b). In contrast, a clone of HCMV strain TB40E [TB40-BAC4 (Sinzger et al., 2008, 1999)] with a deletion of the UL84 reading frame is viable (Spector and Yetming, 2010). This mutant virus replicates with the same kinetics as wild type TB40-BAC4 and produces about one-third as much viral DNA and infectious virus (Spector and Yetming, 2010). These observations prompted investigation both of the replication dependence of other strains on UL84 and of the genetic variation responsible for the different behaviors of the different strains.

Results

Evaluation of additional HCMV strains for UL84 dependence

UL84-deletion mutations were produced in BAC clones of five different HCMV strains by replacing the 587 codon UL84-reading frame (He et al., 1992) with a bacterial galactokinase expression cassette (*galK*). Strains FIX, Merlin, PH, Toledo, and TR (Murphy et al., 2003; Stanton et al., 2010) each retain the consensus genome structure of fresh viral isolates (Prichard et al., 2001). In this respect, they are similar to the genome of TB40-BAC4, but not to those of AD169 and Towne, which were derived after extensive propagation of the viruses in cell cultures, resulting in major genomic alterations (Cha et al., 1996; Dunn et al., 2003; Murphy and Shenk, 2008; Murphy et al., 2003).

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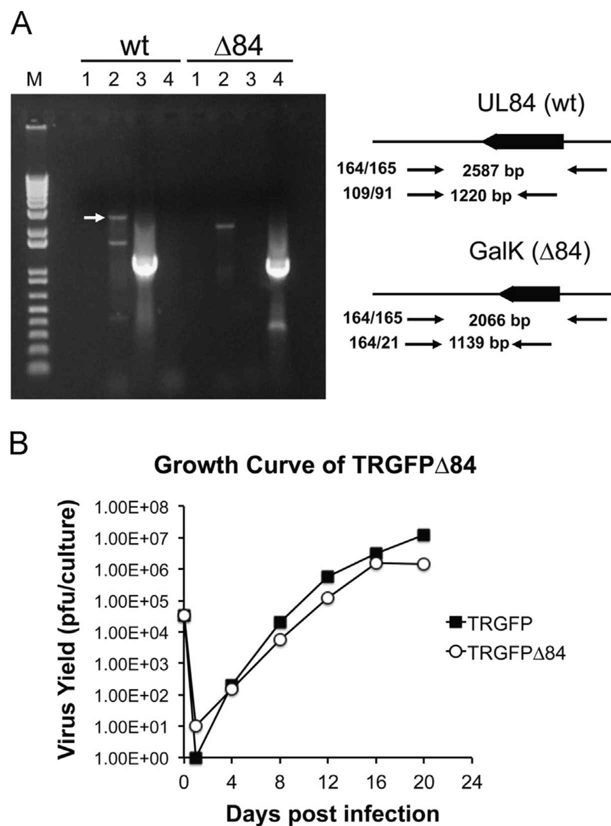

Fig. 1. Structure and replication of TRGFPBACΔ84galK. (A) PCR analysis of viral DNA. DNA was prepared from TRGFP (wt) or TRGFPΔ84galK (Δ84) virus stocks for amplification as described in Materials and methods. The positions of UL84-region primer pairs (numbers correspond to primers listed in Table 2) are indicated along with the sizes of the expected amplification products from each genome. M: 1 kb+ (New England Biolabs, Inc.) DNA length standards. Lanes 1: No DNA; lanes 2: primer pair 164/165; lanes 3: primer pair 109/91; lanes 4: primer pair 164/21. The arrow in lane 2 of the "wt" panel indicates the expected product; the other products in this lane are PCR artefacts. (B) Replication kinetics of TRGPPΔ84galK. MRC5 cells were infected at a MOI of 0.01 and virus titers were determined by plaque assay of samples collected at the times indicated after infection. The zero time represents the input virus amount. The results shown are from one of two duplicate experiments.

Electroporation of *galK*-containing, UL84-deleted versions of FIXGFPBAC, MerlinBAC, PhBAC, and ToledoBAC into MRC5 cells did not yield any virus. However the corresponding mutant derived from TRGFPBAC (TRGFPBACΔ84galK) generated numerous foci of infected cells, and the UL84 region of viral DNA isolated from the infected cultures had the sequence content expected for a *galK* replacement of UL84 with no evidence of the wild type UL84 gene (Fig. 1A). The replication kinetics of TRGFPBACΔ84galK were similar to that of TRGFPBAC (Fig. 1B), although the virus yields were about a half-log lower. TRGFPBACΔ84galK-infected cells did not make any detectable UL84 proteins (Fig. 2A and B). These properties were indistinguishable from those reported for TB40GFPBACgalKΔ84 (Spector and Yetming, 2010). Hence, it was quite clear that TRBAC, like TB40-BAC4, is UL84-independent for growth.

To prove that the UL84 deletions, rather than other genetic changes, were responsible for the non-viability of the other four strains, these mutants were repaired with UL84 derived from TB40-BAC4. A three-step recombineering protocol [(Zhao et al., 2011); see Materials and methods for details] was employed after numerous attempts to revert the mutations directly with a UL84-containing DNA fragment were unsuccessful (data not shown). In the first step, *galK* was removed leaving a UL84 deletion. Then a copy of the UL84 gene of TB40-BAC4 with a *galK* insertion was introduced. Finally, *galK* was removed again in a way that restored the intact TB40-BAC4 UL84 sequence. Each of the repaired BACs was viable (data not shown, but summarized in Table 1). That they were derived from a repair event was verified by examining the sequence of the UL84 genes; all were TB40-BAC4 (data not shown).

Identification of the genetic locus controlling UL84 independence

Two possible mechanisms for UL84 independence were explored. The first was that independent strains contain an undiscovered origin of replication that does not require UL84 for its function. If so, then replication of TB40-BAC4-derived viruses would also be independent of *ori*Lyt. To test this hypothesis, a 1.5 kb segment of *ori*Lyt in TB40mChBAC or TB40mChBACΔ84 (Spector and Yetming, 2010) was replaced with *galK* to produce TB40mChBACΔorigalK and TB40mChBACΔ84ΔorigalK, respectively. This region of *ori*Lyt is required for AD169 origin function (Borst and Messerle, 2005). Neither strain with an *ori*Lyt deletion and *galK* insertion was viable, and replication was rescued by restoring the wild type *ori*Lyt sequence (data not shown). Thus, it appeared that *ori*Lyt is required for replication of TB40-BAC4-derived viruses.

The second possible mechanism of UL84 independence was variation in a viral gene product that enables it to promote viral DNA replication in place of, or at least in the absence of, UL84. Initially, the known nucleotide sequences of the eight strains listed in Table 1 were inspected, but too many differences were found to identify ones that co-segregate with the phenotypes of the ΔUL84 strains. Survey of ninety-one different orfs, including all of those with demonstrated or suspected nuclear localization (Salsman et al., 2008), was similarly unproductive. Accordingly, different strategies were explored for producing recombinants of UL84-dependent and -independent strains that would map the critical locus, if there were only a single one.

A marker rescue approach was successful. For this experiment, the BAC containing UL84-dependent strain MerlinΔ84 was electroporated into MRC5 cells along with TRGFPBAC DNA fragments generated by digestion with *Kpn*I (see Materials and methods). Some viable recombinants produced fast-spreading CPE, and these were predicted to arise by direct repair of the UL84 deletion in the Merlin strain with the wild-type gene from TR. However, other viable recombinants were obtained that exhibited slower-spreading CPE, consistent with the predicted phenotype of a MerlinBACΔ84 genome that had acquired the TR region that confers UL84-independence. Twenty virus clones were isolated by limiting dilution, and viral DNA was prepared from each. The UL84 status of each genome was determined by PCR analysis. Seventeen of the clones had UL84 sequences restored whereas three, designated MerlinΔ84recTR-1, 2 and 3, had the same UL84 deletion as MerlinBACΔ84.

The three recombinants were sequenced, and the genomic regions derived from Merlin or TR DNA were analyzed by comparing the sequence of the recombinants with the published sequences of MerlinBAC and TRBAC (Dolan et al., 2004; Murphy et al., 2003; Stanton et al., 2010). The genome sequences were from Merlin except for a region of TR sequence from the largest *Kpn*I DNA fragment. The TR DNA regions in clones 2 and 3 were larger and indistinguishable from each other so they were likely to have been sister clones (data not shown). The TR DNA in clone 1 was a 4.5-kbp sub-region of the TR DNA in the other two clones, and it extended from within UL117 to within UL122 (Fig. 3A). This TR DNA segment in the MerlinΔ84recTR-2 genome seemed likely to be the source of the rescued viability of the MerlinΔ84 genomes; however, this interpretation was uncertain because the virus stock that was amplified and used to generate DNA for

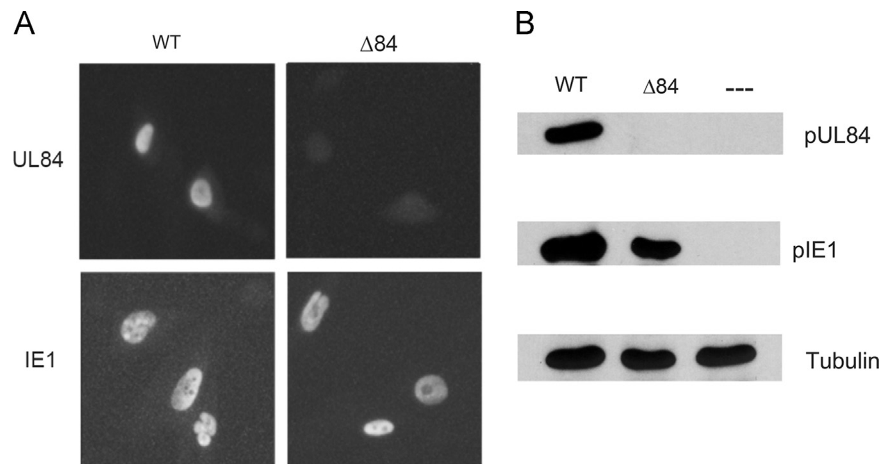

Fig. 2. UL84 protein synthesis in TRGFPΔ84galK-infected cells. MRC5 cells were infected with TRGFP (wt) or TRGFPΔ84galK (Δ84) virus at a MOI of 0.5, or mock-infected (–), for 48 h. (A) Infected cells on cover slips were processed for immunofluorescence with antibody 9G3 (UL84) or 3A9 (IE1). Mock-infected cells produced background levels of fluorescence with either antibody (not shown). Background fluorescence in these images may have been produced, at least in part, by spillover of GFP into the red channel used to detect the cy3 fluor. (B) Infected cells in dishes were harvested and protein extracts were prepared. Immunoblotting was performed with 9G3, 3A9, or anti-tubulin antibodies.

Table 1
Growth phenotypes of cloned HCMV strains.

Strain	ΔUL84	UL84 repair	References
AD169BAC	Non-viable	Viable	(Xu et al., 2004b; Yu, Silva, and Shenk, 2003)
FIXGFPBAC	Non-viable	Viable	This work
MerlinBAC	Non-viable	Viable	This work
PhBAC	Non-viable	Viable	This work
TB40-BAC4	*Viable*	NA	(Spector and Yetming, 2010)
ToledoBAC	Non-viable	Viable	This work
TowneBAC	Non-viable	ND	(Dunn et al., 2003)
TRGFPBAC	*Viable*	NA	This work

NA: not applicable.
ND: not evaluated.

sequencing had not been plaque purified, and there was evidence that it was contaminated with recombinant Merlin virus containing a TR UL84 gene (see Materials and methods).

To seek definitive evidence that the TR DNA segment from the UL117-UL122 region could restore viability to dependent strains that lack UL84 and to map the contributing genetic locus more precisely, additional genetic analyses were performed. UL117 could be ruled out because its predicted amino acid sequences in TB40-BAC4, an independent strain, and MerlinBAC, a dependent strain, are identical. Moreover, the UL118–121 region was unlikely to provide the source of UL84-independence, because individually or in some combinations, UL118 (also designated UL119 exon 2), UL119 (exon 1), UL120, and UL121, are non-essential for growth of HCMV AD169 or Towne (Atalay et al., 2002; Dunn et al., 2003; Yu et al., 2003). However, a complete deletion of the UL118–121 region had not been tested. Accordingly, this region was replaced with *galK* in a UL84-dependent strain (Merlin BAC) and in both UL84-independent strains (TB40mChBACΔ84 and TRGFPBACΔ84, Fig. 3B). All of the clones with *galK* replacements of UL118–121 produced virus (data not shown). Therefore, the locus controlling UL84 dependence did not map in this region, and further attention was directed to UL122.

UL122 alleles that confer UL84 independence

UL122 gene sequences are expressed as a complex series of protein products from a variety of spliced mRNAs (Fig. 4A). Inspection of all the UL122-coding sequences of the eight strains listed in Table 1 did not reveal any single locus where variation in the amino acid sequence co-segregated with the UL84 dependence phenotypes. However, alignments based on the IE2 86 kDa protein (Fig. 4B), which contains 579 aa in strain AD169 (Chee et al., 1990; Hermiston et al., 1987; Malone, Vesole, and Stinski, 1990; Stenberg et al., 1989; Stenberg and Stinski, 1985; Stinski et al., 1983), revealed that the TB40-BAC4 genome encodes an aspartic acid (D) at position 388, whereas all of the other strains encode a histidine (H) (Chee et al., 1990; Murphy et al., 2003; Stanton et al., 2010). Similarly, TRBAC encodes a D at position 545 whereas all of the other strains specify an alanine (A). These changes became candidates for genetic variations that render the two strains UL84-independent.

Two different approaches were used to test whether the H388D variation would convert UL84-dependent strains to UL84 independence. The first was a marker rescue strategy in MRC5 cells. Cells were co-electroporated with DNA from a dependent strain that lacks UL84 and an oligonucleotide derived from the TB40-BAC4 UL122 sequence, which contains the single base-pair change that creates the D388 codon. A focus of viral growth was observed in a culture that received ToledoBACΔ84 DNA, and the virus was designated ToledoΔ84H388D. Viral DNA was prepared and the UL84 and UL122 regions of the genome were assessed by PCR and restriction enzyme digestion. ToledoΔ84H388D still had the UL84 deletion of the ToledoBACΔ84 clone from which it was derived (Fig. 5A). To look for the predicted change in codon 388, a 726 bp PCR product from this region of UL122 was digested with *Ava*II because the base change that converted codon 388 from H-coding

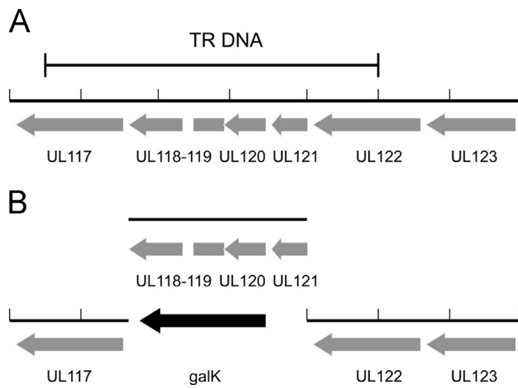

Fig. 3. Recombinant region of MerlinΔ84recTR-1. (A) The segment shown extends from Merlin nucleotide positions 166,796 to 173,796 with tick marks every 1 kbp. The delimited line indicates the segment of Merlin DNA that was replaced by TR DNA in the recombinant. The boundaries are approximate based on the nearest single nucleotide polymorphisms. (B) Structure of Δ118–121galK BAC clones. The *galK* cassette (dark arrow) replaced the region of the BAC delimited by the start codon of UL121 and the stop codon of UL118.

to D-coding also introduced a site for this enzyme. Thus, a genome with the D-coding sequence should have an additional *Ava*II site replacing the 316-bp product of the parental Toledo strain (H388) with 206- and 110-bp products. Moreover, *Bst*N1digestion of Toledo-derived DNA adjacent to the 200 bp fragment used for the marker rescue should produce 420- and 198-bp products, rather than a 618-bp product that would be observed if somehow more extensive TB40-BAC4-derived DNA had been incorporated into the recombinant genome. The observed restriction patterns (Fig. 5B) were precisely those predicted for ToledoΔ84H388D.

In a second approach for testing the importance of H388D, this codon change was introduced into the UL84-dependent FIXGFP-BACΔ84 strain to ascertain whether it would become independent. Three different BAC clones that retained the UL84 deletion were tested for their viability. Numerous foci of CPE were obtained for each clone; their rate of expansion was consistent with that observed previously for UL84-independent strains, with many foci containing fifty to a hundred GFP-positive cells by 17d after electroporation (data not shown). DNA isolated from the resulting virus stocks generated PCR products (Fig. 6A, lanes 3–5) of the sizes expected for genomes that maintained the UL84 deletion (Fig. 6A, lanes 2), as opposed to those with *wt* UL84 (Fig. 6A, lanes 1). Moreover, the UL122 genes of the three clones (Fig. 6B, lanes 3–5) contained the new *Ava*II site introduced by the H388D codon change but maintained the *Fsp*I and *Rsa*I sites expected of the FIX UL122 gene (Fig. 6B, lanes 1) rather than that of TB40-BAC4 (Fig. 6B, lanes 2). The isolation of these viable Toledo and FIX Δ84 variants shows that the H388D conversion in UL122 was sufficient to confer UL84 independence to UL84-dependent HCMV strains.

Discussion

Genetic and molecular evidence have implicated the UL84 gene of HCMV as being essential for the initiation of viral DNA replication. Hence, the discovery that strain TB40-BAC4 replicates without the need for UL84 (Spector and Yetming, 2010) raised questions about the actual role of this protein and provided a potential opportunity to genetically probe the mechanism of initiation in infected cells. Data presented here shows that the UL84-independent growth phenotype is shared by at least one other strain, TRGFPBAC, and that this property is conferred by genetic variation in UL122. Introduction of the UL122 388D variant from TB40-BAC4 into UL84-dependent strains Toledo and FIX converted

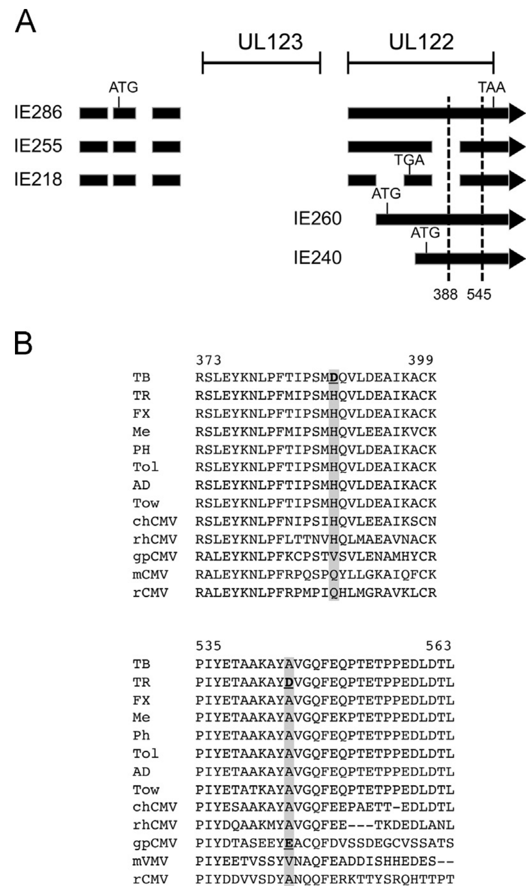

Fig. 4. UL122 region of HCMV. (A) UL122 mRNAs and proteins. The exon structure of UL122-containing mRNAs (boxes and arrows) is indicated along with the designations of the translation products and the initiation and termination codons that produce them. The dashed lines indicate the position of codons 388 and 545. (B) Encoded amino acid sequences in cytomegalovirus UL122 genes for conserved regions adjacent to histidine 388 and alanine 545 [AD169 IE2 86 amino acid designations (Chee et al., 1990)]. Positions 388 and 545 are shaded and the replacement of conserved residues by acidic residues is indicated by underline and bold. The single amino acid code is used. TB: TB40-BAC4 (Sinzger et al., 2008); TR: TRBAC (Murphy et al., 2003); FIX: FIXBAC (Murphy et al., 2003); Me: MerlinBAC (Dolan et al., 2004; Stanton et al., 2010); Ph: PhBAC (Murphy et al., 2003); Tol: ToledoBAC (Murphy et al., 2003); AD: AD169BAC (Chee et al., 1990; Murphy et al., 2003); Tow: TowneBAC (Murphy et al., 2003); chCMV: chimpanzee cytomegalovirus (Davison et al., 2003); rhCMV: rhesus cytomegalovirus (Hansen et al., 2003); gpCMV: guinea pig cytomegalovirus (Schleiss et al., 2008); mCMV: mouse cytomegalovirus (Rawlinson et al., 1996); rCMV: rat cytomegalovirus (Vink et al., 2000).

both to UL84 independence. There is a good circumstantial case that the TR 545D variant also has the same capacity. First, the genetic analysis showed that the portion of the TR genome that is required for replication in the absence of UL84 maps to UL122 or the UL121–122 intergenic region (Fig. 3), and A545D is the only difference in that region that co-segregates with independence. Second, the amino acids flanking residue 545 are conserved among primate and rodent cytomegaloviruses (Fig. 4B); and in the UL122 homolog of guinea pig cytomegalovirus, which is also UL84-independent for growth (McGregor et al., 2011), an acidic residue (E rather than D) replaces the highly conserved alanine (Schleiss et al., 2008).

The mechanism underlying UL84-independence is yet to be determined. UL122 is an essential gene (Heider et al., 2002; Marchini et al., 2001; White et al., 2004), which is implicated in a wide variety of regulatory functions including viral and cell gene activation and repression (Bresnahan et al., 1998; Caswell et al., 1993; Cherrington et al., 1991; Hagemeier et al., 1992; Hermiston et al., 1990; Hermiston et al., 1987; Jupp et al., 1993; Liu et al., 1991;

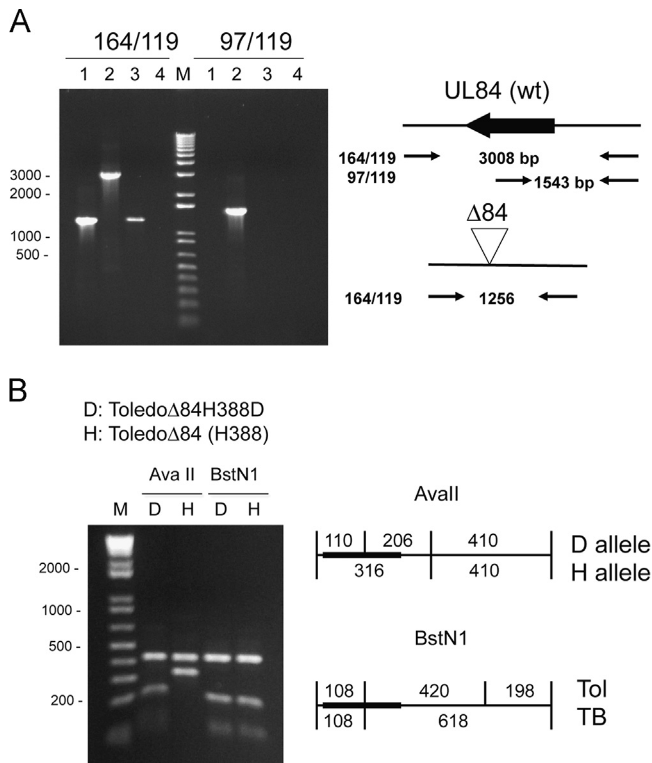

Fig. 5. Restriction endonuclease site analysis of UL84 and UL122 alleles in ToledoΔ84 virus strains. Viral DNA was extracted from ToledoΔ84H388D, Toledo, or TRGFPΔ84 virus preparations as described in Materials and Methods. (A) Each sample was amplified with the UL84 primer pairs indicated (see Table 2). The products expected for the wt UL84 or Δ84 alleles are shown on the right. Lanes 1: ToledoΔ84H388D; lanes 2: Toledo; lanes 3: TRGFPΔ84; lanes 4: no template. M: 1 kb+ DNA length standards. (B) A 726-bp region of UL122 was amplified by PCR using the primer pair 196/189 (see Table 2). The PCR product was digested with the restriction endonuclease indicated. The left panel shows the banding pattern from each sample. M: 1 kb+ DNA length standards. The right panel shows the products expected from each UL122 allele. Tol: Toledo allele; TB: TB40-BAC4 allele. The thick part of the line indicates the position of the 198-bp PCR product derived from TB40-BAC4 and used to produce ToledoΔ84H388D.

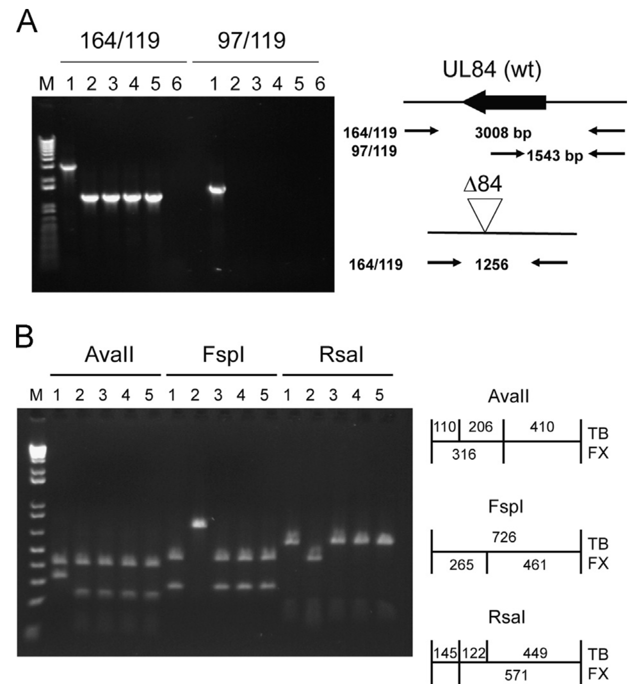

Fig. 6. Analysis of genomes from single amino acid change variants of FIXGFPΔ84 that conferred UL84 independence. (A) Viral DNA samples were amplified with the UL84 region primer pairs indicated. Lanes 1: TB40mCh; lanes 2: TB40mChΔ84; lanes 3: FIXGFPΔ84H388Dcl1; lanes 4: FIXGFPΔ84H388Dcl2; lanes 5: FIXGFPΔ84H388Dcl3; lanes 6: no DNA. M: 1 kb+ DNA length standards. (B) Viral DNA samples were amplified with the UL122 primer pair 196/189 (Table 2) and digested with the restriction endonucleases indicated. Lanes 1: FIXGFP; lanes 2: TB40mCh; lanes 3: FIXGFPΔ84H388Dcl1; lanes 4: FIXGFPΔ84H388Dcl2; lanes 5: FIXGFPΔ84H388Dcl3; M: 1 kb+ DNA length standards

Malone et al., 1990; Pizzorno and Hayward, 1990; Pizzorno et al., 1988; Song and Stinski, 2002; Tevethia et al., 1987), cell cycle progression (Bresnahan et al., 1996; Murphy et al., 2000; Sinclair et al., 2000; Sommer et al., 1994; Weibusch and Hagemeier, 1999), apoptosis (Zhu et al., 1995), disruption of ND10 nuclear structures (Ahn and Hayward, 1997; Ishov et al., 1997), and inhibition of cellular DNA synthesis (Bresnahan et al., 1996; Song and Stinski, 2005). Among the overlapping proteins expressed from UL122 (Fig. 4A), most studies have focused on the 86 kD immediate early (IE) protein, designated IE86 or IE2 86, which contains two amino-terminal segments encoded by exons shared with the major product of the UL123 gene (Hermiston et al., 1987; Pizzorno et al., 1991; Stenberg et al., 1989; Stinski et al., 1983). At least two other proteins, 60 kD (IE2 60) and 40 kD (IE2 40), are encoded exclusively by UL122 and accumulate later in infection (Plachter et al., 1993; Puchtler and Stamminger, 1991). Selective genetic ablation of their synthesis results in a severe growth defect (White et al., 2007), and they, too, have gene regulatory activities (Jenkins et al., 1994; Sanders et al., 2008; Sanders and Spector, 2010; White et al., 2007). Like the 86 kD product (Spector and Tevethia, 1994; Gebert et al., 1997), the 60 and 40 kD products can interact individually with the UL84 protein (Sanders et al., 2008), and IE2 40 in particular enhances the expression of UL84 (Sanders et al., 2008; Sanders and Spector, 2010; White et al., 2007). All three of these UL122 products contain the H/D388 and A/D545 residues. Little is known about the functions of two additional UL122-encoded proteins, IE2 18 and IE2 55 (Kerry et al., 1995; Stenberg et al., 1989). These proteins contain the segment including amino acid 545 but not amino acid 388. Viruses expressing subsets of UL122 products should help identify the species whose variants are capable of conferring UL84 independence.

Regardless of which UL122 product(s) is responsible, it is probable that the gain-of-function phenotype of the variants (i.e., the ability to grow without UL84) arises from a quantitative change in one or more activities of the protein, rather than acquisition of a new function. As suggested by previous studies in transfected cells (Pari and Anders, 1993; Reid et al., 2003; Sarisky and Hayward, 1996; Xu et al., 2004a), it seems most likely that the substitutions enhance the ability of UL122 to participate in the initiation of viral DNA replication. How might this work? Over 20 years ago, it was shown that elimination of various acidic residues in the 550–573 region reduces the ability of IE2 to transactivate early gene promoters (Yeung et al., 1993); hence, it is reasonable to predict that the acquisition of an additional acidic residue at position 388, or at position 545, increases transactivation of a promoter by IE2. This is a particularly attractive hypothesis because the mechanism of unwinding during the initiation of DNA synthesis for HCMV has been proposed to be a transcriptionally driven process [see (Pari, 2008)], similar to that proposed for the initiation of mitochondrial DNA replication (Lee and Clayton, 1996; Xu and Clayton, 1995; Xu and Clayton, 1996). The promoter region for the nearby UL57 gene activates *ori*Lyt (Kiehl et al., 2003), and a region of *ori*Lyt itself (*ori*Lyt$_{PM}$) that contains a functional IE2 binding site also has promoter activity (Xu et al., 2004a). Moreover, when the SV40 early promoter replaces *ori*Lyt$_{PM}$, the resulting replicon still functions in a transient replication assay, and replication is rendered independent of IE2 (Xu et al., 2004a). Also, several RNA species (vRNA) are made from *ori*Lyt and captured in RNA-DNA hybrid structures at the origin

(Prichard et al., 1998). UL84 and UL122 synergistically activate *ori*Lyt$_{PM}$ (Xu et al., 2004a). Thus, it will be of interest to ascertain whether the variants reported here are capable of increasing activation of *ori*Lyt$_{PM}$ by UL122 alone to an extent sufficient to relieve the requirement for UL84.

The findings of this study have implications for other UL84 activities that have been attributed to origin function. For example, it has been suggested that UL84 protein binds to one or more vRNA species or RNA-DNA hybrids in *ori*Lyt (Colletti et al., 2007), and that UL84 modifies the nucleic acid structure, perhaps through a helicase activity (Colletti et al., 2006, 2007). Clearly, these activities must not be essential for the initiation of DNA replication because single amino acid substitutions in UL122 can eliminate the need for UL84.

In summary, the results presented here provide genetic evidence in infected cells for a direct role of UL122 in oriLyt function. It remains to be seen whether the two novel alleles that have been identified are widespread in nature or whether other changes also can enable UL84 independence. Whether these or other substitutions in UL122 also provide a selective advantage to UL84-containing strains remains to be determined.

Materials and methods

Cells and viruses

MRC5 cells were maintained as described previously (Spector and Yetming, 2010) in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 U of streptomycin per ml, 0.15% sodium bicarbonate and 10% fetal bovine serum (Hyclone Laboratories, Inc.).

BAC clones of the HCMV genomes AD169, and TB40mCherry were described previously (Spector and Yetming, 2010). FIXGFP, Ph, Toledo, Towne and TRGFP genomes (Murphy et al., 2003) were provided by Eain Murphy and Tom Shenk as BAC clones in *E. coli* SW105 (Warming et al., 2005). Expression of the color markers is mediated by an SV40 origin/promoter in a cassette inserted between the US34 and TRS1 genes (E. Murphy, personal communication). Richard Stanton provided pAL1111, a clone of the Merlin genome (Stanton et al., 2010) referred to here as MerlinBAC.

Virus stocks were prepared from MRC5 cells electroporated with BAC DNA from an infectious clone as described previously (Spector and Yetming, 2010).

BAC mutagenesis

BAC sequences were manipulated by recombineering using *galK* as the selectable marker in strains SW102 or SW105 as described previously (Spector and Yetming, 2010). Table 2 lists the primers and oligonucleotides that were used in these experiments. In some cases the homologous recombination activity of BAC-containing strains was lost. When that occurred, BAC DNA was isolated and transferred to a new SW102 or SW105 host for further mutagenesis.

The construction of TB40GFPBACgalKΔ84, a clone of TB40-BAC4 with a GFP expression unit and a *galK* replacement of the smaller UL84 reading frame, was described previously (Spector and Yetming, 2010). An identical strategy using primers 87 and 88 and selection for *galK* was used to produce *galK* replacements of the corresponding UL84 gene sequences in FIXGFPBAC, PhBAC, ToledoBAC, TRGFPBAC, and MerlinBAC.

The *galK* cassette was excised by counter-selection to produce Δ84 BACs essentially as described by Warming et al. (2005). Electro-competent cells were prepared as for the *galK* insertion and electroporated with a double-stranded DNA generated by incubating 10 μg each of oligonucleotides 158 and 159 (Table 2) in 0.1 ml of 1X Standard Taq Buffer (New England BioLabs, Inc.) for 5 min at 100 °C and keeping at room temperature for 30 min. The double-stranded DNA was precipitated with sodium acetate and ethanol, collected by centrifugation and suspended in water at a final concentration of 200 μg/ml. 0.5 μg was used for each electroporation. After electroporation in conditions described previously (Spector and Yetming, 2010) 0.05 ml of transformed cells were diluted to 10 ml with YENB (7.5 g/L yeast extract; 8.0 g/L nutrient broth) and kept at 32° for 4.5 h in a shaking incubator. One ml of cells was collected and washed three times with 1 ml of M9 medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4CL, 0.5 g/L NaCl), suspended in 1 ml of M9, and 0.1 ml was plated on M63 medium (2 g/l $(NH_4)_2SO_4$, 13.6 g/L KH_2PO_4, 0.5 mg/L $FeSO_4 \cdot 7H_2O$, pH 7.0) containing 0.2% 2-deoxy-galactose, 0.2% glycerol, 0.01% leucine, 1 μg/ml biotin, 0.5 mM $MgSO_4 \cdot 7H_2O$ and 12.5 μg/ml chloramphenicol. Colonies were picked and plated on YENB containing 12.5 μg/ml chloramphenicol, or M63 plates with the additions indicated above except that the carbon source was 0.2% galactose, and the glycerol and 2-deoxy-galactose were omitted. Colonies that grew only on the YENB plates were grown in liquid culture and the BAC DNA was isolated as described above. The loss of *galK* and maintained absence of the UL84 reading frame were confirmed by PCR and DNA sequencing of the PCR products.

To restore wild type UL84 sequences in Δ84 BACs a region of the TB40GFPBACUL84galKm7 genome, which has a *galK* replacement of a short segment of UL84, was introduced into the Δ84 BACs by *galK* selection. TB40GFPBACUL84galKm7 was constructed from TB40GFPBAC using primers 142 and 143. The UL84 gene region of TB40GFPBACUL84galKm7, including about 400 bp of genomic sequences at either end of the reading frame, was amplified with primers 164 and 165. Standard electroporation reactions containing 0.75 μg of this DNA fragment and a Δ84 BAC were plated, and *galK*-containing clones were isolated. After identifying UL84galKm7 BACs by PCR, the wild type TB40-BAC4 UL84 sequence was restored by counter-selection after recombineering with 1 μg of a double-stranded DNA fragment produced by the annealing of oligonucleotides 178 and 179. Verification that the wild type TB40-BAC4 UL84 sequence repaired the original FIX, Ph, Merlin, or Toledo Δ84 genomes was performed by using primer 132 to prime sequencing of a PCR product generated with primers 109 and 91.

Replacement of 1.5 kbp of *ori*Lyt in TB40mChBAC with *galK* was performed by recombineering using primers 151 and 152 to produce TB40mChBACgalKΔoriLyt. The *galK* cassette was replaced and the wild type *ori*Lyt restored by counter-selection using a PCR product generated with primers 147 and 180, which amplified the deleted 1.5 kbp region with extensions of about 160 bp on either side.

Replacement of UL118 to UL121 by *galK* was performed by recombineering using a DNA product amplified with primers 185 and 186.

Nucleotide 172,262 in the UL22 gene of FIXGFPBACΔ84 was mutated from G to C (205,110 in the TB40-BAC4 sequence), to change codon 388 from specifying histidine to specifying aspartic acid (H388D). In the first step, nt 172,262 was replaced by *galK* using a PCR product generated with primers 194 and 195. In the second step, a synthetic double-stranded TB40-BAC4-derived DNA containing C at a position corresponding to 205,110 (GB1, IDT corp) was used in a counter-selection recombineering to replace *galK* of the FIX clone with the changed base pair. The FIX and TB40-BAC4 sequences in this region of UL122 are identical except for the single base pair difference. Recombinants were designated FIXGFPΔ84H388D.

Recombinant viruses

To produce recombinant viruses between the non-viable BAC clone of MerlinΔ84 and TRBAC DNA, the latter was digested with

Table 2
List of Synthetic DNAs.

DNA source	No.	Sequence Nos.	Sequence
PCR primers			
galK[a]	21	453-482	CGCACAAATCGCGCTTAACGGTCAGGAAGC
TB40-BAC4			
UL84 region	91	157648-157668c	GAATCCTCGTTAGAGACCACG
	109	156448-156469	AGTGTCCGTTTTCCATCACCAG
	132	156667-156690	GAGTGTAGCGTGGATTTGACTTGG
	164	156037-156056	CCAGGATCAGCGAGGGCTGG
	165	158605-158624c	CAGGTGCTGGTCGATGACCG
UL122	189	205704-205723c	TACCATCCAGTACCGCAACA
	196	204998-205018	ACCGCATCCACCTCACTCTTC
	197	205175-205195c	CGGCTCACCTCGTCAATCTTG
oriLyt	147	129085-129103c	CCTTATCCTGGGCGTTGGC
	180	127270-127290	GGGAGTGTCTACAGGGCTACG
FIXBAC (UL84)	97	124628-124648	TCAACAGCGGCGTGATGATAC
Primers for amplification of *galK* cassette with flanking HCMV DNA sequences[b]			
TB40-BAC4			
UL84 region	87	158232-158281 *galK* forward	GCTTGGTGTCTGCGGGCGCGAGAGGGCCGGCTCAGCCTTTAAATATGCAG*CCTGTTGACAATTAATCATCGGCA*
	142	157167-157216c *galK* forward	GTCCGGCGGTGATTCGTTCGGGTCTAGCTCCAATCGTCCAAAGACGGAGG*CCTGTTGACAATTAATCATCGGCA*
	143	157115-157164 *galK* reverse	CGTTGAAACGTAACATGCCGTCTTGGTATAGCGTGAGTGACGACAGCGTC*TCAGCACTGTCCTGCTCCTT*
ori*Lyt*	151	127385-127434 *galK* forward	ACGTCACACTCGCGTGACCACACCCACTCCGGATATACGTCATCCCGTGG*CCTGTTGACAATTAATCATCGGCA*
	152	128936-128985 *galK* reverse	GGAGCCGCCGCGAGACCCGGAAGCCGTCGCGGTCGACCCCGCTCCCGAAG*TCAGCACTGTCCTGCTCCTT*
UL122	194	205060-205109 *galK* forward	GTTCACCTGCATGGTTTTGCAAGCTTTGATGGCCTCATCTAACACCTGGT*CCTGTTGACAATTAATCATCGGCA*
	195	205111-205160c *galK* reverse	CCAATCGCTCTCTTGAGTACAAGAACCTGCCCTTCACGATTCCCAGTATG*TCAGCACTGTCCTGCTCCTT*
FIXBAC			
UL84 region	88	123544-123593 *galK* reverse	CGTATGGCGCGGACGCCTAGTGTCCGTTTCCCATTATCAGGGTCCTCTGT*TCAGCACTGTCCTGCTCCTT*
Merlin BAC			
UL118-121	185	168024-168073 *galK* forward	CGTGTGGAGCCGTAGACGATCTGGACGTGGTCCTGGGAGAACATGACCAT*CCTGTTGACAATTAATCATCGGCA*
	186	170565-171614c *galK* reverse	GCTGTTTAATAAAAGTAGCTTTTTTATACATCTCCGTCTCTGGTCTCCTG*TCAGCACTGTCCTGCTCCTT*
Oligonucleoties			
TB40-BAC4			
UL84	158	156430-156479, 158232-158281	CGTATTGCGCGGACGCCTAGTGTCCGTTTTCCATCACCAGGGTCCTCTGTCTGCATATTTAAAGGCTGAGCCGGC-CCTCTCGCGCCCGCAGACACCAAGC
	159	158232-158281c, 156430-156479c	CGTATTGCGCGGACGCCTAGTGTCCGTTTTCCATCACCAGGGTCCTCTGTACAGAGGACCCTGGTGATGGAAAA-CGGACACTAGGCGTCCGCGCAATACG
	178	157115-157216c	CCTCCGTCTTTGGACGATTGGAGCTAGACCCGAACGAATCACCGCCGGACCTGACGCTGTCGTCACTCACGCTAT-ACCAAGACGGCATGTTACGTTTCAACG
	179	157115-157216	CGTTGAAACGTAACATGCCGTCTTGGTATAGCGTGAGTGACGACAGCGTCAGGTCCGGCGGTGATTCGTTCGGG-TCTAGCTCCAATCGTCCAAAGACGGAGG
UL122	GB1[c]	205011-205210	CACTCTTCACCTCATGATTGCGGGTGTAGATGATCTGGATGCCCTTGTTGTTCACCTGCATGGTTTTGCAAGCTTT-GATGGCCTCATCTAACACCTGGTCCATACTGGGAATCGTGAAGGGCAGGTTCTTGTACTCAAGAGAGCGATTGG-TGTTGCGGAACATGCGGCTCACCTCGTCAATCTTGACGCGACCCCGCCGA

[a] *galK* orf of p*galK* (Warming et al., 2005).
[b] sequences from *galK* cassette in pgalK are in italics.
[c] double-stranded, only one strand is shown.

*Kpn*I and the DNA was purified with an *Illustra* GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The DNA was concentrated to about 1 μg/μl and 10 μl of DNA were added to a standard electroporation reaction containing MerlinΔ84BAC DNA and 1 μg of pp71-expressing plasmid (Murphy et al., 2008; Spector and Yetming, 2010). After about two weeks, two different sizes of foci with virus-infected cells were observed. After one more week, the cells and virus were harvested, dispersed by sonification, serially diluted, and dilutions from 1:10,000 to 1:1,000,000 were distributed to MRC5 cells growing in separate wells of 12-well plates. Again both fast-expanding and slow-expanding foci were observed. From two to three weeks after these infections, contents from twenty wells with only a single visible focus of virus growth (but see below) were transferred to MRC5 cells growing in T25 flasks. Five to eight days after infection of the T25 flasks, the cells and medium from each were collected separately and stored at −80°.

DNAs was isolated from the "cells" sample of each clone using the QIAamp Mini DNA Kit (Qiagen) and evaluated by PCR for the status of the UL84 gene. Three of the twenty clones (1, 14, and 17) had the same UL84 gene deletion as the parent MerlinΔ84BAC and were designated MerlinΔ84recTR-1, 2 and 3. The virus from these recombinants was amplified by transferring a portion of the medium from the original T25 flask to MRC5 cells growing in a T175 flask. One-tenth of the virus yield from the T175 flask was then added to 6 × 15-cm dishes of MRC5 cells. After 6 to 7 days, the cells and medium were pooled and the cells were collected by centrifugation. The pellet was combined with 5 ml of medium and disrupted by sonification in a cup sonicator (Misonix sonicator 3000). Debris was removed by centrifugation and the supernatant was combined with the remaining medium. The suspension was layered over 5 ml of 20% sorbitol in 0.05 M Tris–HCl, pH 7.4, and particulate material including virus was collected by

centrifugation at 68,000 x g for 90 min at room temperature in an SW32 rotor. The supernatant was removed and the pellet was suspended in 0.05 M Tris–HCl, pH 8.1, 0.01 M EDTA, 0.15 M NaCl (TSE), and kept at 4 °C until DNA extraction. A control sample was prepared similarly from cells infected with HCMV Merlin.

To produce a recombinant virus with a G to C mutation in UL122 that changed codon 388 from histidine- to aspartic acid-coding, a 198 bp region of UL122 was amplified with primers 196 and 197. The DNA was electroporated along with FIXGFPBACΔ84, PhBACΔ84, or ToledoBACΔ84 DNA, and pp71 plasmid directly into MRC5 cells. A single focus of virus spreading was observed in the flask that received Toledo DNA. A lysate stock was prepared from the flask 22 days after the transformation and designated ToledoΔ84H388D.

DNA isolation

Partially purified BAC DNA was isolated from 10 ml of bacterial cultures grown for 16–18 h by a modified cosmid isolation procedure described previously (Spector and Yetming, 2010). Half of the sample was used in standard electroporation reactions for virus production. Highly purified BAC DNA was isolated from 100 ml of bacterial cell cultures using the Genopure Plasmid Maxi Kit (Roche Life Sciences) according to the instructions for low copy number plasmid isolations provided by the manufacturer.

Viral DNA was prepared from 0.2 ml of infected cell lysate using the QIAamp DNA Mini Kit (Qiagen) according to instructions provided by the manufacturer. To prepare viral DNA from sorbitol cushion-isolated virus, the suspension was adjusted to 5 mg/ml N-lauroyl-sarcosine and 100 μg/ml proteinase K and mixed gently by inversion at 37 °C for 2 h. The mixture was extracted two times with TSE-saturated phenol and once with chloroform-isoamyl alcohol (24:1). Nucleic acid was precipitated by adding sodium acetate and ethanol and collected by centrifugation. The nucleic acid was dissolved in 0.01 M Tris–HCl, pH 7.4, 0.1 M EDTA (10.1 TE) and treated with 100 mg/ml of RNAse A and 8 u/ml of RNAse T1 for 30 min at 37°. Nucleic acid was extracted with organic solvents and collected by precipitation as described above, and suspended in 10.1 TE for DNA sequencing.

HCMV genomic resequencing

DNA was quantified in a Qubit 2.0 Fluorometer (Life Technologies), and 0.01–0.09 μg of DNA in 0.05 μl of 10.1 TE was sheared to an average fragment size of 200 bp in a Covaris adaptive focused acoustics ultrasonicator (Covaris; 175 W peak incident power; 10%, duty factor; 200 cycles per burst; 120 s total treatment time). After confirming the appropriate DNA fragment size distribution in a 2100 Bioanalyzer (Agilent Technologies), a DNA library was generated for each sample (total of four) using Illumina TruSeq multiplex adapters (Illumina) with the Kapa Library Prep kit (Kapa Biosystems). Quality-checked sequencing libraries were pooled and sequenced by MiSeq Nano Kit (Illumina) to generate a total of one million of 2×150 sequencing reads (250,000 reads per sample). 872 K/mm^2 of clusters were detected and a total of 1.11 million passed filter reads among 1.22 million total reads were obtained. The percentage of high quality reads (Q score greater than 30) was 96.7 (data not shown).

The sample with the lowest read number distribution had 163,000 reads (14.66% of 1.11 million total reads), corresponding to 32.6X coverage of the 240 Kb viral genome after accounting for the proportion of human genome contamination. Initial de novo assembly was performed with the DNASTAR Lasergene Genomics Suite (DNASTAR). Assembly was finished using the Pustell DNA matrix feature (Pustell and Kafatos, 1984) of MacVector v12.5 (MacVector, Inc.) to align assembled sequences with MerlinBAC and TRGFPBAC reference sequences.

The MerlinΔ84recTR-1, 2 and 3 viral DNA preparations contained varying amounts of TR UL84 DNA (data not shown) indicating that the samples also contained Merlin genomes whose viability had been rescued by recombination with TR UL84 region sequences. The repaired UL84 recombinant must have contaminated the Δ84recTR virus, most likely as an unrecognized component of the contents of wells in the 12-well plates in the limiting dilution experiment (see above). Preferential amplification of the UL84-containing variant during subsequent passage of the virus stock, which was not plaque-purified, would be expected because of its presumed growth advantage over the Δ84TRrec virus [this work, (Spector and Yetming, 2010)]. The amplification of UL84 DNA was confirmed by PCR analysis of the DNA from the original and passaged virus stocks (data not shown). The uncertainty in interpretation introduced by the contaminating UL84-containing virus was eliminated by the additional genetic analysis described in the Results section.

Protein analysis

Protocols for immunofluorescence and immunoblotting, including the sources of antibodies, and for imaging of green fluorescent protein or its mCherry variant in live MRC5 cells growing and infected on cover slips were described previously (Spector and Yetming, 2010).

Growth curves

Growth curves were performed on infected cells growing in multi-well tissue culture plates as described previously (Spector and Yetming, 2010). Virus concentrations in harvested samples were determined by plaque titration.

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