Structural modelling and mutagenesis of human cytomegalovirus alkaline nuclease UL98

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Human cytomegalovirus (CMV) is one of eight human pathogens in the family Herpesviridae. It causes disease in immune-compromised patients, including retinitis in human immunodeficiency virus patients, pneumonitis in transplant patients and, when acquired during pregnancy, severe birth defects characterized by deafness and mental retardation (Vancikova & Dvorak, 2001). Current options for treating CMV infections are limited and suboptimal. The approved drugs ganciclovir, its prodrug valganciclovir, foscarnet and cidofovir target the viral DNA polymerase to block viral DNA synthesis. Each is associated with significant toxicity, and studies in laboratory animals suggest that these drugs may be teratogenic (Fouda et al., 1991; Henderson et al., 1993; Klug et al., 1991). Hence, none are approved for treatment of fetal CMV infections during pregnancy. Because prolonged treatment can give rise to viral strains that are resistant to one or more of these drugs (Schleiss, 2005), there is a need for the development of new CMV antivirals, particularly those that target alternative processes and are safe for use during pregnancy.

The CMV alkaline nuclease (AN), UL98, represents one such novel target. ANs are conserved throughout the family Herpesviridae. In vitro, ANs exhibit both endonuclease (endo) and 5'→3' exonuclease (exo) activities that are optimal at alkaline pH, sensitive to high salt concentrations and require a divalent cation, with a preference for Mg2+ (Hoffmann & Cheng, 1978). Their exonuclease activities can hydrolyse either ssDNA or dsDNA to form mono-, di- and trinucleotides (Sheaffer et al., 1997). Although DNA structural and sequence preferences have been noted (Henderson et al., 1998; Kehm et al., 1998), neither activity appears to be sequence specific.

ANs play an important, but poorly understood, role in herpesvirus replication. Herpes simplex virus type 1 (HSV-1) AN null mutants are viable, although they exhibit a growth
deficit of 2–3 logs (Gao et al., 1998; Martinez et al., 1996; Patel et al., 1996; Weller et al., 1990), whereas transposon insertions that disrupt the CMV UL98 ORF are lethal (Yu et al., 2003), thus suggesting that UL98 may be essential for CMV replication.

The fact that UL98 is important for CMV replication and possesses enzymic activities that can be assayed easily makes this protein an attractive target for antiviral development. Here, we used homology-based modelling and mutagenesis to identify the active site residues of UL98 and construction of mutant viruses to determine the importance of UL98 in CMV replication. The results provide a foundation for additional studies to elucidate the role of AN in herpesvirus replication and to explore drug discovery efforts targeting UL98.

RESULTS

Prediction of UL98 structure, DNA interactions and active site residues

Herpesvirus ANs are classified within the λ exo family of DNases, which is within the PD-(D/E)XK superfamily of DNA-modifying enzymes (Kovall & Matthews, 1998). The catalytic sites of these enzymes contain a conserved aspartic acid (D), an aspartic or glutamic acid (D/E) and a lysine (K) (Kovall & Matthews, 1998). PSI-BLAST identified the AN of Kaposi’s sarcoma-associated herpesvirus (KSHV), known as shutoff and exonuclease protein (KSHV-SOX), as being the closest match to UL98. The catalytic residues of KSHV-SOX are D221 and E244, which coordinate Mg2+, and K246, which stabilizes the leaving group (Dahlroth et al., 2009). An adjacent 5′-phosphate-binding pocket formed by R139, S146 and S219 was suggested by the presence of a sulphate ion in the crystal structure (Dahlroth et al., 2009). CLUSTAL X version 2 was used to identify CMV AN amino acids D254, E278 and K280 of UL98 as being the corresponding catalytic residues and R164, T171 and S252 as forming the putative 5′-phosphate-binding pocket (Supplementary Fig. S1a, available in JGV Online). When ANs were compared across the family Herpesviridae, the catalytic residues were 100% conserved, as were R164 and S252 of the phosphate-binding pocket, while the third putative phosphate-binding residue, T171, was conserved among betaherpesviruses but replaced by serine in the alpha- and gammaherpesviruses (Supplementary Fig. S1b, available in JGV Online).

A structural model of UL98 was constructed based on the crystal structure of KSHV-SOX (Dahlroth et al., 2009) and in silico docking was used to determine the optimal binding of a dsDNA dodecamer in the predicted UL98 binding site crevice (Supplementary Materials, available in JGV Online). KSHV-SOX comprises an amino-terminal domain consisting of ten α-helices and a carboxy-terminal domain formed by five-stranded β-sheets flanked by five α-helices (Dahlroth et al., 2009). The UL98 structure has a very similar pattern and active site crevice (Fig. 1). The model again identifies catalytic site residues D254, E278 and K280, and 5′-phosphate binding residues R164 and S252, in locations precisely analogous to their counterparts in KSHV-SOX. The predicted position of DNA shows the 5′ phosphate held deep into the active-site crevice through hydrogen bonding with R164 and S252 (Fig. 1c). The scissile phosphodiester bond is appropriately positioned proximally to the metal coordination region formed by D254, E278 and K280 (Fig. 1c).

Purification of recombinant wild-type and mutant UL98 proteins

To examine the role of these amino acids in the enzymic activity of UL98, recombinant UL98 or proteins containing single alanine substitutions (R164A, S252A, D254A, E278A or K280A) were expressed with amino-terminal hexahistidine (His6) tags. His6-tagged β-glucuronidase (GUS) was
expressed as a negative control. The seven proteins were expressed and partially purified by immobilized metal affinity chromatography (IMAC) as described in Methods. With the exception of the E278A mutant, each protein was eluted in fractions 30–31 (150–200 mM imidazol), which is typical for His6-tagged proteins. The E278A mutant protein was eluted predominantly in fraction 16, suggesting that the structure of this mutant protein and/or the accessibility of the His6 tag were altered compared with the other proteins. Based on SDS-PAGE and immunoblot analysis, the His6-tagged UL98 and GUS proteins migrated at rates consistent with the calculated masses for these proteins of 68.4 and 75.9 kDa, respectively (Supplementary Fig. S2, available in JGV Online). Although the preparations contained some contaminants, these were similar across all samples. Moreover, the amounts of mutant proteins eluted were similar to the wild-type sample, with the exception of S252A.

**Residues D254, E278, K280, R164 and S252 are important for exo activity**

The exo activities of the IMAC-purified proteins were quantified by release of acid-soluble radioactivity from 14C-labelled DNA. Wild-type UL98 exhibited both time- and concentration-dependent exo activity (Fig. 2a and data not shown). Mutant R164A digested DNA but at a significantly reduced rate (Fig. 2a). In fact, all of the mutant proteins failed to digest substantial amounts of substrate DNA in a 1 h period (Fig. 2b). Exo activity of the R164A mutant was 10.6 % of wild-type and statistically higher than those of the other mutants and GUS (unpaired t-test, \( P \leq 0.05 \)), whereas activities for D254A, E278A, K280A and S252A were <5 % that of wild-type and not statistically different from each other or from GUS \((P>0.1)\).

**Residues D254, E278, K280 and S252, but not R164, are important for endo activity**

Endo activity was determined qualitatively by incubating IMAC-purified proteins with closed-circular pUC19 plasmid DNA and evaluating its conversion to open-circular and linear forms by agarose gel electrophoresis (Goldstein & Weller, 1998). Following 12 h incubation, the wild-type and R164A proteins were not only capable of nicking the supercoiled substrate, they degraded all of the DNA substrate, presumably to products too small to visualize on the gel (Fig. 3a). In contrast, the K280A and S252A mutant proteins nicked essentially all of the supercoiled DNA, although large amounts of nicked open-circular and full-length linear forms remained undigested (Fig. 3a). The smearing below the full-length linear fragment may represent the cumulative effects, over the 12 h incubation period, of endonucleolytic cleavage or low-level exo activity in these preparations. Although D254A and E278A preparations were able to convert some of the supercoiled DNA into open-circular and full-length linear DNA, the majority of the supercoiled DNA substrate remained intact after the 12 h incubation. It is possible that the low-level endo activity exhibited by the latter mutants derives from contaminating nucleases in the IMAC-purified preparations, although the similarly purified control protein, GUS, had no discernible nuclease activity (Fig. 3a).

To quantify endo activity independent of exo activity, a fluorescence-based assay was developed in which fluorescence increases when endonucleolytic cleavage of an ssDNA substrate releases a 3’ quencher from a 5’ fluorophore. One millimole of substrate was incubated with increasing amounts of each protein for 14 h at 37°C (Fig. 3b). No significant difference was observed between the endo activities of R164A and wild-type UL98 \((P>0.05)\) and both wild-type and R164A endo activities were significantly different from those exhibited by GUS and the other mutant proteins (Tukey’s multiple comparison test, \( P<0.001 \)). The activities of S252A, D254A or E278A were not different from each other or from GUS (Tukey’s multiple comparison test, \( P>0.05 \)), although the extremely low activity of K280A was statistically higher \((P<0.01)\) than GUS. Thus, the residual endonuclease activities associated with S252A, D254A or

![Image](https://example.com/image.png)
UL98 is important but not essential for viral replication

UL98 has been classified as an essential CMV gene, based on lethality of mutations that disrupt UL98 by transposon insertion (Yu et al., 2003) or gene deletion (Dunn et al., 2003). However, the mutations in both these studies had the potential to impact upon the expression of neighbouring essential genes. To definitively determine the importance of UL98 for CMV replication, we used Escherichia coli genetics to modify UL98 sequences within bacterial artificial chromosomes (BAC) HB15-t178b, an infectious clone of the CMV strain AD169 genome that also contains an expression cassette for GFP (Saccocio et al., 2011). In BAC clone ΔUL98/galK, the entire UL98 ORF was deleted and replaced by the E. coli galK gene. Consistent with the results of Dunn et al. (2003), this mutation was lethal, as transfection of MRC-5 fibroblasts with ΔUL98/galK BAC DNA resulted in isolated green cells that never expanded to form viral plaques (Fig. 4a). In contrast, both HB15-t178b and ΔUL98/Rev, a derivative of ΔUL98/galK in which galK was replaced by wild-type UL98 sequence from CMV strain Towne, gave rise to GFP+ foci that expanded over time (Fig. 4a).

A second mutant, UL98/Amb, was constructed in which galK in ΔUL98/galK was replaced by UL98 sequences containing an amber stop codon/frameshift mutation at codon 5. Transfection of UL98/Amb DNA resulted in GFP+ foci that expanded very slowly (Fig. 4a). Production of UL98/Amb stocks required 4–6 weeks and achieved peak titres of 10^7 p.f.u. ml^-1. A growth curve starting at a very low m.o.i. (0.0001) confirmed that UL98/Amb is extremely growth impaired – while the parental virus reached 10^8 p.f.u. ml^-1 by day 33 post-infection, UL98/Amb was not detected in the culture supernatants until day 33, when the viral titre was <10 p.f.u. ml^-1 (Fig. 4b). Although we could not detect any UL98 product in UL98/Amb-infected cells by Western blot (not shown), we cannot exclude the possibility of extremely low-level amber codon read-through or infrequent translational initiation beyond the mutation. PCR/restriction analysis detected no evidence that the mutation, which disrupts an Xhol site, had spontaneously reverted to wild-type (restoring the Xhol site) in UL98/Amb viral DNA (not shown). These results indicate that UL98 is not essential for viral replication in tissue culture; however, in the absence of UL98 viral replication is extremely attenuated.

To determine the impact of the R164A mutation on viral replication, galK in ΔUL98/galK was replaced by UL98 sequences containing the R164A mutation to yield BAC UL98/R164A. Virus UL98/R164A was readily reconstituted from this BAC and replicated in MRC-5 fibroblasts with kinetics and efficiency indistinguishable from the parental virus (Fig. 4c). PCR/restriction analysis detected no evidence that the mutation, which disrupts a BstAPI site, had spontaneously reverted to wild-type (restoring the BstAPI site) in UL98/R164A viral DNA (not shown).

DISCUSSION

Nucleases classified within the λ exo family are encoded by a diverse range of large dsDNA viruses, including bacteriophages, insect baculoviruses and herpesviruses. While their functions remain poorly understood, the high level of conservation of these enzymes across broad groups suggests that they are important for a common replication process. The best-studied member of this family, λ exo, is thought to play a role in recombination by creating ssDNA ends that are subsequently utilized by the λ ssDNA-binding protein for strand invasion. Although in vitro the HSV-1 AN, UL12, can participate in similar strand-transfer reactions mediated by the HSV-1 ssDNA-binding protein in association with host recombination repair Mre11–Rad50–Nbs1 complex components (Balasubramanian et al., 2010; Reuven et al., 2003, 2004), the replication defect of herpesvirus AN null mutants has not as yet been linked to a defect in DNA recombination.
To date, the phenotypes of null mutants suggest a role for AN in resolving branched or abnormally structured DNA. This appears to have downstream effects on DNA packaging, capsid stability, nuclear egress and the infectivity of virions that reach the extracellular compartment (Martinez et al., 2002; Porter & Stow, 2004a, b; Shao et al., 1993; Weller et al., 1990). Although the mechanistic details remain unclear, genetic studies clearly show that herpesvirus ANs are important for viral replication. Thus, AN inhibitors are predicted to have a significant impact on viral replication.

The λexo family belongs to a superfamily of DNA-modifying enzymes that include restriction enzymes, Holliday-junction resolvases, DNA-nicking enzymes, Vsr and the mismatch repair enzyme MutH, which all share a conserved active-site structure characterized by a PD-(D/E)XK motif (Bujnicki & Rychlewski, 2001; Venclovas et al., 1994). In λexo four additional residues, an arginine, two serines and a glutamic acid, form a pocket that is believed to stabilize the 5’ end of the DNA substrate and position the scissile phosphodiester bond proximal to the catalytic residues (Kovall & Matthews, 1998). In the KSHV-SOX crystal structure, an analogous pocket is formed by R139, S146 and S219 and contains a sulphate ion that presumably occupies the pocket in the absence of DNA (Dahlroth et al., 2009). The UL98 model predicts that this pocket is formed by R164, T171 and S252. In a panel of seven ANs from viruses representing the three herpesvirus subfamilies, the predicted catalytic residues of UL98 (D254, E278 and K280) were fully conserved, as were two of the predicted phosphate-binding pocket residues (R164 and S252); the third (T171) was conserved as a threonine in the β subfamily and an serine in the α and γ subfamilies.

Fig. 5 shows predicted active-site structures for wild-type UL98 and each of the five mutants. The DNA is positioned in the active-site crevice through hydrogen-bond interactions with R164, T171 and S252. Carboxylate bearing side chains of D254 and E278 coordinate the catalytically important magnesium ion, and a conserved water molecule acts as the base for hydrolysis. K280 is proximal to the scissile phosphodiester bond and serves to stabilize the leaving group. Loss of hydrogen-bonding interactions resulting from substitutions R164A or S252A may result in improper alignment of the DNA in the active site, resulting in improper positioning of the scissile phosphodiester bond relative to the catalytic K280 residue (Fig. 5b and 5c). This
was further supported by significant changes in the predicted DNA-binding energies for R164A, S252A and K280 mutants (Supplementary Table S1, available in JGV Online). Similarly, loss of charged carboxylate residues in D254A or E278A mutants would result in failure to coordinate the magnesium ion and water molecule (Fig. 5d and 5e). Changes in predicted DNA-binding energies for mutants D254A and E278A are minimal (Supplementary Table S1), consistent with these residues interacting with Mg$^{2+}$ rather than by directly interacting with the DNA. The K280A mutation would be unable to carry out the hydrolysis of the phosphodiester bond and stabilize the leaving group (Fig. 5f). This mechanistic model therefore predicts that D, E or K$\rightarrow$A substitution mutants should lack both exo and endo activity and that R or S$\rightarrow$A mutants may have impaired exo activity. As no 5$'$ phosphate is present on the substrate in endonucleolytic cleavage, endo activity might be unaffected in R or S$\rightarrow$A mutants.

The experimental data are generally consistent with these predictions. The D, E or K$\rightarrow$A mutants lacked measureable exo activity. Endo activity was greatly reduced, but low-level activity was still apparent with the supercoiled dsDNA substrate. Similar findings have been reported for substitution mutants of ANs from KSHV (SOX), Epstein–Barr virus (BGLF5) and HSV-1 (UL12). In SOX mutants D221S and E244S, substitutions of the catalytic aspartic acid and glutamic acid residues inactivated exo activity (Bagneris et al., 2011). In BGLF5 substitutions D203(E/S) or E225Q inactivated exo and endo activities, while E225D changed the divalent cation preference from Mg$^{2+}$ to Mn$^{2+}$ (Buisson et al., 2009; Liu et al., 2003). In UL12, substitution D340E inactivated exo activity but not endo activity (Goldstein & Weller, 1998). This was surprising given that both activities were inactivated in the BGLF5 D203E mutant (Liu et al., 2003). Of the two UL98 mutations targeting putative phosphate-binding residues, R164A behaved as predicted. Exo activity was reduced by 90 %, but there was no impact on endo activities detected with supercoiled dsDNA or end-blocked ssDNA substrates. In contrast, the S252A mutation eliminated exo activity, and reduced both ssDNA and supercoiled dsDNA endo activities to levels similar to those of the K280A mutant. While single amino acid changes in the putative phosphate-binding residues of other ANs have not been evaluated, a double substitution mutant of HSV-1 UL12 (G336A/S338A, where S338UL12 is equivalent to S252UL98) was significantly impaired for both exo and endo activity (Goldstein & Weller, 1998). In both UL98 and UL12, the proximity of the serine-to-alanine mutation, one residue from the catalytic aspartic acid, may have perturbed the local geometry of the active site enough to impair catalytic function, and hence to have impacted not only exo but also endo activity.

The crystal structure of KSHV-SOX solved by Dahlroth et al. (2009) suggests that its active-site canyon probably accommodates four nucleotides. They successfully modelled a duplex DNA 11-mer into the pocket with the 5$'$ end in the active site. Consistent with these findings, our model suggests that the active-site canyon of UL98 is able to accommodate duplex DNA, and that the active-site residues show significant interactions with the scissile phosphodiester group. Bagneris et al. (2011) have suggested that exonucleolytically processed substrates are bound as duplexes, and that the only rearrangements required for cleavage are those necessary for recognition of the 5$'$-phosphate group in a serine-rich pocket. Our model shows a similar potential 5$'$-phosphate-binding pocket.
and docking of dsDNA exhibited a close approximation of the 5’ phosphate within the pocket. However, upon minimization, the dsDNA was minimally altered to conform to the hydrophobic cleft, suggesting that some conformational change of the DNA owing to steric influences may be necessary to access the 5’-phosphate-binding pocket. That active-site–dsDNA interactions are predicted mainly with phosphate groups of the 5’–3’ strand further suggests that ssDNA could bind in a manner similar to dsDNA. Consistent with this, Sheaffer et al. (1997) observed that UL98 hydrolyses ssDNA and dsDNA with similar turnover rates, affinities and rate constants.

There have been suggestions that the ‘bridge’ architecture plays an important role in exonucleolytic cleavage by inducing strand separation (Bagnéris et al., 2011; Buisson et al., 2009). The bridge region is also proposed to promote endonucleolytic processing by developing local regions of ssDNA within the substrate, which are the likely targets for endo cleavage. However, the high degree of disorder in the structure of the bridge region in both KSHV-SOX and the KSHV-SOX–DNA complex structures made it difficult to model the corresponding region in UL98. Thus, our modelling focused on the active-site region and cannot be extrapolated to the role of ‘the bridge’ in the functions of UL98.

Two previous studies classified UL98 as being essential for CMV replication (Dunn et al., 2003; Yu et al., 2003). This was surprising given that the HSV-1 UL12 mutants are viable, although significantly attenuated for growth in cell culture (Gao et al., 1998; Martinez et al., 1996; Patel et al., 1996; Weller et al., 1990). However, UL98 is contained within a UL93–UL99 gene cluster that exhibits complex 5’-nested transcripts that are 3’ coterminal owing to the shared use of a polyadenylation signal downstream of UL99 (Wing & Huang, 1995). Consequently, the transposon insertions in UL98 described by Yu et al. (2003) could have had polar effects on transcripts for other genes in this cluster, most notably UL99, an essential gene that encodes the pp28 tegument protein (Silva et al., 2003). Similarly, because the deletion described by Dunn et al. (2003) removed the entire UL98 ORF, and UL98 overlaps the 5’ end of UL99 (Adam et al., 1995), this mutation must have eliminated expression of both UL98 and pp28. We therefore constructed mutant UL98/Amb to selectively inactivate UL98 expression with a minimal risk of perturbing expression of nearby genes. That UL98/Amb was able to replicate, albeit minimally, indicates that UL98, while extremely important for replication, is not essential in cell culture. However, the slow rate of replication of this mutant suggests that it might easily be cleared in vivo. Thus, UL98 remains a viable antiviral target as drugs inactivating its functions should dramatically reduce the ability of CMV to replicate.

Surprisingly, virus UL98/R164A exhibited no growth impairment in cultured fibroblasts. This suggests that CMV can tolerate a 90% reduction in exonuclease activity without losing replication efficiency. Alternatively, given the wild-type kinetics of UL98/R164 replication, it may be the endo activity of UL98 that is important for replication. Even so, wild-type levels of exo activity may be important in vivo, as CMV infects a variety of other cell types and the importance of exo activity may be cell-type dependent.

In the absence of a crystallographic structure for UL98, these results indicate that in silico modelling can provide structural models that are sufficient to predict which residues are important for nucleolytic functions. Such models should prove valuable for continued structure/function dissection of ANs and for informing structure-based drug discovery. The ability to mutagenically target the 5’-phosphate-binding pocket to inhibit exo but not endo activity may provide a useful tool for exploring the biological roles of each activity in herpesvirus replication.

**METHODS**

**Plasmid construction.** Oligonucleotide sequences are listed in Supplementary Table S2 (available in JGV Online). The UL98 ORF was PCR-amplified from CMV strain AD169 DNA by using primers GW8-F and GW8-R and a High Fidelity EasyA PCR mixture (Bioline), cloned into vector pCR-8/GW/TOPO (Invitrogen) and moved, by using a clonase reaction, to Gateway destination vector pDest17 (Invitrogen), thus generating E. coli expression vector pD17-98(wt). Mutations were constructed by overlap extension PCR. Template DNA from pD17-98(wt) was amplified by using primer GW8-F paired with primers DR, ER, KR, RR or SR, or primer GW8-R paired with primers DF, EF, RF, SF or SF to generate overlapping products containing the desired nucleotide changes. For each mutant the two PCR products were purified, mixed and amplified again with primers GW8-F and GW8-R. The products were cloned into pCR-8/GW/TOPO and transferred as above to generate plasmids pD17-98(D), pD17-98(E), pD17-98(K), pD17-98(R) and pD17-98(S), which contain the mutations specified in Supplementary Table S2. Plasmids pGW8-98(Towne)L, pGW8-98(S5Amber)L and pGW8-98(R164A)L were constructed by similar methods, except that primers UL98-Forward and UL98-Reverse replaced primers GW8-F and GW8-R, respectively, in order to generate mutations in UL98 with flanking 500 bp homologies for use in recombinant BAC construction. For pGW8-98(Towne)L, DNA from CMV strain Towne was used as the PCR template. The entire UL98 ORF in each vector was sequenced to confirm the predicted changes and to exclude the possibility of other mutations. A control pDest17 vector expressing GUS was constructed as directed by the manufacturer’s (Invitrogen) protocol.

**Expression of recombinant proteins in E. coli.** Optimal expression of soluble UL98 was obtained by using BL-21(DEQ) pLysS (Bioline) cells. Overnight culture was diluted 1:30 with Luria broth containing 50 μg carbenicillin ml⁻¹ and grown to mid-exponential phase (A₅₉₅ of 0.6) with shaking at 37 °C. Cultures were induced with 1 mM IPTG and incubated with shaking for an additional 3 h at 22 °C. Bacteria were harvested by centrifugation at 4000 g for 20 min at 4 °C and cell pellets were stored at −80 °C.

**IMAC purification of recombinant proteins.** Bacterial cell pellets were thawed on ice and lysed by incubation for 30 min on ice in buffer A (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 15% glycerol, 1% NP-40 and 0.1 mM PMSF) containing 10 mM imidazole. Lysates were homogenized by intermittent ultrasonic disruption on ice over a 20 min period then clarified by centrifugation at 20,000 g for 20 min at 4 °C. Supernatants were applied to a 5 ml Ni-NTA agarose column.
equilibrated with buffer A containing 10 mM imidazole and washed extensively with the same buffer. Proteins were eluted with an 80 ml linear gradient of imidazole (10–300 mM) in buffer A. Fast protein liquid chromatography fractionation was conducted at room temperature and all fractions were collected at 0 °C. UL98-containing fractions were identified by immunoblotting with UL98-specific antibody 12 [a gift from Jay Nelson, Oregon Health and Science University, Portland, OR, USA (Adam et al., 1995)], pooled and dialysed against B-2 buffer (50 mM KH₂PO₄, pH 8.0, 50 mM NaCl, 15 % glycerol, 0.1 mM EDTA). Protein concentrations were determined by Bio-Rad assay as described by the manufacturer.

**Quantitative exo assay.** The exo activities were quantified by release of trichloroacetic acid-soluble radioactivity from uniformly ¹⁴C-labelled E. coli DNA as previously described (Henderson et al., 1998). Reactions (200 μl final volume) were performed at 37 °C in nuclease buffer (50 mM Tris/HCl pH 9.0, 6 mM MgCl₂, 1 mM DTT) and contained 3 μg ¹⁴C-labelled DNA. Reactions were initiated by the addition of protein and terminated by the addition of 25 μg 50 % trichloroacetic acid and 25 μl of salmon sperm DNA (2 mg ml⁻¹). Undigested DNA was precipitated by incubation on ice for 5 min and centrifugation for 10 min at 3000 r.p.m. in a Beckman TJ-6 table-top centrifuge. The supernatant (200 μl) was neutralized with 75 μl of 1.2 M KOH, mixed with 7.5 ml ScintiSafe (Fisher) and the radioactivity measured by liquid scintillation spectrometry.

**Qualitative endo assay.** IMAC-purified proteins (2.5 μg) were mixed with 250 ng supercoiled pUC19 plasmid DNA and incubated in nuclease buffer for 12 h at 37 °C. Reaction products were separated by electrophoresis on 1 % agarose gels, stained with ethidium bromide and visualized by UV light.

**Fluorescence-based endonuclease assay.** A 35 nt ssDNA oligonucleotide substrate containing a 5′-6-carboxyfluorescein (6-FAM) fluorophore and 3′ Iowa Black FQ (IABFQ) quencher (6-FAM-A*GCTAGGACGAAACCTCTATGTGATCATAAATC-IABFQ) was synthesized by Integrated DNA Technologies. A thioate bond at the 5′ end (*) was added to inhibit 5′ exo activity. One millimole of DNA substrate was incubated with increasing amounts of recombinant protein in nuclease buffer for 14 h at 37 °C. Fluorescence was measured at 535 nm with a Beckman Victor² Multilabel plate reader using an excitation wavelength of 485 nm.

**Recombinant virus construction.** Construction of BAC HB15-t178b has been described (Saccoccio et al., 2011). ‘Recombineering’ was used to replace the UL98 ORF in HB15-t178b with galK as described (Warming et al., 2005). Briefly, a linear recombinant fragment containing galK flanked by 50 bp homologies to the 5′ and 3′ ends of the UL98 ORF was generated by PCR using plasmid pGalK as the template for amplification with primers UL98-GalKF and 42 coli SW102 cells containing BAC AUL98/galK following induction at 42 °C for 15 min. GalK-negative colonies were selected for by growth on minimal-media plates containing 0.2 % deoxygalactose and 15 μg chloramphenicol ml⁻¹. Candidate clones were screened for by detecting restoration of the 6.6 kb HindIII fragment and were confirmed by targeted sequencing.

**Viruses reconstitution, propagation and growth analysis.** BAC DNAs were purified and transfected into MRC-5 fibroblasts (ATCC CCL-171) as previously described (Sauer et al., 2010). GFP⁺ foci were observed by using an Olympus LX70 Inverted UV microscope 10–20 days post-transfection. At 15–30 days post-transfection culture supernatants and cells were transferred to 75 cm² flasks of confluent MRC-5 cells and incubated until extensive cytopathic effect was observed. Viral stocks were prepared from cell-culture media that were clarified by centrifugation, adjusted to 0.2 M sucrose, aliquoted, stored at –80 °C and titrated on MRC-5 cells by limiting-dilution in 96-well plates as described (Cui et al., 2008). Growth curves were generated by infecting 75 cm² flasks of confluent MRC-5 cells with carefully matched viral inocula. Cultures were washed 3 h post-infection and samples of culture media were removed periodically for titration as described above.

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