The human cytomegalovirus UL98 gene encodes the conserved herpesvirus alkaline nuclease

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The human cytomegalovirus (HCMV) UL98 gene is predicted to encode a homologue of the conserved herpesvirus alkaline nuclease. To determine if the HCMV UL98 gene product is functionally homologous to other herpesvirus alkaline nucleases, the HCMV UL98 protein was purified and its activity characterized in vitro. Extracts of HCMV-infected cells were fractionated using Q Sepharose, phosphocellulose and native DNA cellulose chromatography. UL98 immunoreactivity copurified with alkaline pH-dependent nuclease activity. The purified protein migrated at its predicted size of approximately 65 kDa in denaturing polyacrylamide gels, and displayed nuclease activity in an activity gel assay. Enzyme activity was characterized by a high pH optimum, an absolute requirement for divalent cation, salt sensitivity, and 5′ to 3′ exonuclease activity. DNA digestion resulted in 5′ monophosphoryl mono- and oligodeoxyribonucleotides. Kinetic analyses revealed a turnover rate of greater than 200 per min, and similar apparent affinity and rate constants on single- and double-stranded DNA. These results indicate that a functional alkaline nuclease activity is conserved among distant members of the herpesvirus family, and are consistent with a highly conserved role in the virus life cycle.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous, opportunistic herpesvirus responsible for life-threatening illnesses in immunocompromised individuals, including AIDS patients and tissue transplant recipients. In addition, primary infection during the first trimester of gestation often results in serious birth defects (reviewed by Britt & Alford, 1996). For productive infection, all herpesviruses depend on the controlled, sequential expression of a number of conserved viral genes. Upon infection, de novo expressed immediate early proteins regulate subsequent viral early and late gene expression. Many early gene products are responsible for replication of the double-stranded DNA genome. This replication triggers expression of viral late gene products, which include components of the virion as well as proteins which package the newly replicated DNA (Mocarski, 1996).

In addition to conserved enzymes responsible for DNA replication, herpesviruses encode a deoxyribonuclease, termed alkaline nuclease due to its high in vitro pH optimum. The alkaline nuclease was first described (Morrison & Keir, 1968), expressed and mapped (Moss et al., 1979; Preston & Cordingley, 1982) using herpes simplex virus types 1 (HSV-1) and 2 (HSV-2). The exact function of the alkaline nuclease during infection remains unclear, although the HSV-1 alkaline nuclease has been implicated in the processing of complex replicative DNA intermediates prior to their packaging into capsids (Weller, 1995; Martinez et al., 1996a).

On the basis of similar biological properties, the herpesviruses have been grouped into the alpha-, beta- and gamma-subfamilies. Enzymatic activity has not previously been demonstrated for a beta-herpesvirus alkaline nuclease, although the purified enzymes from the alpha-herpesviruses HSV-1 and HSV-2, and the gamma-herpesvirus Epstein–Barr virus (EBV) have been characterized in vitro (Morrison & Keir, 1968; Hoffman & Cheng, 1978; Bayliss et al., 1989; Knopf & Weishart, 1990; Stolzenberg & Ooka, 1990).

The UL98 gene of beta-herpesvirus HCMV shares positional as well as limited amino acid sequence homology with the alkaline nucleases of other known herpesviruses (Chee et al., 1990). UL98 protein is synthesized at early times of infection, but its levels increase significantly after the onset of viral DNA replication (Adam et al., 1995). The protein is translated from a 3.0 kb mRNA, a member of a family of 3′ co-terminal transcripts encoding open reading frames UL93–

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UL99 (Adam et al., 1995; Wing & Huang, 1995). The translated protein migrates close to its predicted size of 65 kDa in SDS–PAGE gels, and has been reported to be phosphorylated and glycosylated in infected cells. Additionally, the UL98 protein is localized in the nucleus late in infection, and has been identified as a component of extracellular viral particles (Lahijani et al., 1992).

To determine whether the HCMV UL98 protein is enzymatically homologous to the alpha- and gamma-herpesvirus alkaline nucleases, we have purified the protein from HCMV-infected fibroblasts and assayed its activity in vitro. We find that UL98 directly directs the expression of a functional alkaline nuclease. Our characterization of the enzyme reveals many similarities with the alkaline nucleases of other herpesviruses. Our results indicate that expression of deoxyribonuclease activity is conserved among alpha-, beta- and gamma-herpesviruses, and that HCMV UL98 encodes the human cytomegalovirus alkaline nuclease.

Methods

- Cells and virus. Cell-free HCMV strain AD169 stocks were grown in human foreskin fibroblast cells (HFFs; Viromed) at an m.o.i. of 0–1–5 p.f.u. per cell, released from cell debris by freeze–thawing, sonication and centrifugation, and stored at −80 °C. Plaque assays used HFF cells in 12-well dishes overlaid with 0.2% pooled human gammaglobulin (Michigan Department of Public Health). Eleven to 14 days after infection, cells were fixed with formaldehyde and stained with crystal violet in ethanol to facilitate plaque counting. For enzyme purification, HFFs, grown to confluence in 65 850 cm² petri dishes, were pelleted, washed once in PBS, and frozen at −80 °C.

- Reagents. Monoclonal antibody (MAb) L2 (Adam et al., 1995) was a gift from J. Nelson (Oregon Health Sciences University, USA). The polyclonal anti-peptide antisera anti-P21 (Lahijani et al., 1991) was a gift from S. St. Jeor (University of Nevada at Reno, USA). Sephadex G25, native DNA cellulose and Q-Sepharose were from Pharmacia Biotech. Phosphocellulose P11 was from Whatman BioSystems and was prepared according to the manufacturer’s instructions. Protease inhibitors were from Boehringer Mannheim.

- Purification. A frozen cell pellet of 5–5 × 10⁹ HCMV-infected cells was thawed and resuspended in 10 ml lysis buffer containing 40 mM Tris pH 7.5, 2 mM DTT, 0.1 mM EDTA, 1 mM Pefabloc, 10 µg/ml leupeptin, 20 µg/ml aprotinin and 10% glycerol. Cells were incubated for 20 min on ice, then lysed in a Dounce homogenizer. An equal volume of extraction buffer (3 M KCl, 50 mM NaCl, 2.5 mM KCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol) was added to the lysate, followed by incubation on ice for 20 min. The lysate was clarified by centrifugation at 100 000 g for 30 min, and desalted over a Sephadex G25 column equilibrated in buffer A (50 mM Tris pH 7.5, 0.1 mM PMSF, 0.1 mM EDTA, 1 mM DTT, 10% glycerol). Absorbance was monitored (A₂₈₀), and fractions containing protein were pooled and applied to a 50 cm² Q-Sepharose column equilibrated in buffer A. Protein was eluted with a linear gradient from 0–1 M NaCl. Western blot-positive (MAb L2) fractions were pooled, dialysed to equilibrium against buffer C (50 mM Tris pH 7.5, 0.1 mM EDTA, 1 mM DTT, 20% glycerol) and loaded onto a 120 cm² column of phosphocellulose equilibrated in buffer C. A linear gradient from 0–1 M NaCl was used to elute the protein. UL98-containing fractions were pooled, dialysed against buffer C to less than 50 mM NaCl and loaded onto a 5 ml column of native DNA cellulose. This column was developed with a linear gradient from 0–0.6 M NaCl and UL98 protein-containing fractions were pooled and dialysed against storage buffer (20 mM Tris pH 7.5, 0.1 mM EDTA, 1 mM DTT, 50% glycerol). The purified enzyme was aliquotted and stored at −80 °C. Protein concentrations were determined using the Bio-Rad protein assay kit.

- SDS–PAGE and Western immunoblot. Proteins, denatured by boiling in SDS and DTT, were separated by electrophoresis in 12% polyacrylamide SDS–PAGE gels using standard techniques. For immunoblotting, gels were electroblotted to nitrocellulose, blocked for 1 h in 5% non-fat milk in TBS (25 mM Tris pH 7.5, 50 mM NaCl, 2.5 mM KCl), incubated at room temperature for 1–2 h with primary antibody diluted in TBS, washed five times in TBS-T, incubated with a 1:1000 dilution of either alkaline-phosphatase-labelled rabbit anti-mouse or goat anti-rabbit immunoglobulins (Dako). After five washes in TBS-T, immunoreactive bands were developed by incubation with BCIP-NBT solution (Kirkegaard and Perry). Coomassie staining used 0.2% Coomassie R-250, 10% glacial acetic acid and 10% methanol. Gels were destained in 10% glacial acetic acid and 10% methanol. For silver staining, gels were incubated for 30 min at room temperature in 10% glutaraldehyde, washed for 2 h with multiple changes of water, incubated for 30 min at room temperature in 15 mM DTT, followed by 30 min in 0.1% (w/v) silver nitrate solution. Gels were washed quickly in water. Bands were developed with 3% sodium carbonate and 0.019% formaldehyde. Development was stopped with 3–38% citric acid and washing in water.

- Enzyme assays. Exonuclease activity was measured using activated calf thymus DNA (Sigma) substrate, unless noted. DNA was labelled with [α-³²P]dATP (New England Nuclear) using Klenow polymerase (New England Biolabs). Unincorporated label was removed using a NAP 10 column (Pharmacia). Unless noted, 50 µl reactions contained 50 mM Tris pH 9.0, 1 mM DTT, 6 mM MgCl₂, 50 µg/ml BSA and 6 µM (in nucleotides) activated calf thymus DNA (average specific activity 28 mCi/µmol). Reactions were incubated at 37 °C for the times indicated, and terminated with 50 µl 20% TCA and incubation on ice for 30 min. Soluble, released nucleotides were separated from undigested, insoluble DNA by filtration through Millipore Multiscreen filtration plates (0.45 µm HVPP membrane). Filtrate was collected into 200 µl Packard Microscint-40 scintillation fluid, and quantified by scintillation counting in a Wallac Microbeta plate reader.

Kinetics of nucleotide release were measured using 0–343 pmol UL98 protein and various concentrations of native or denatured activated calf thymus DNA. Denatured activated calf thymus DNA was prepared by heating for 10 min at 100 °C and rapid cooling on ice. Time-points resulting in hydrolysis of less than 20% of input DNA were used. For activity gel analysis, 5 pmol of purified proteins were boiled in SDS–PAGE gel loading buffer and electrophoresed through 10% polyacrylamide SDS–PAGE gels containing 10³ c.p.m./ml of ³²P end-labelled duplex 50-mer oligonucleotide DNA. 25 pmoles of oligonucleotides were labelled with T4 polynucleotide kinase (New England Biolabs) and 10 µl [³²P]dATP (3000 Ci/ml, 10 mCi/ml) and desalted over a Sephadex G25 spin column. After electrophoresis, the gel was incubated in buffer I (20 mM 2-mercaptoethanol, 1 M NaCl, 50 mM Tris pH 7.4) for 1 h, then overnight, then for 2 h at 4 °C with rocking. Catalysis was allowed to proceed in situ by incubation of the gel in buffer II (10% glycerol, 1 mM DTT, 6 mM MgCl₂, 50 mM Tris pH 9.0). Buffer II was changed after 1 h, 2 h, and overnight at 4°C. Gels were fixed, stained as above, dried and autoradiographed.
The direction and final products of the exonuclease were determined using a duplex oligodeoxyribonucleotide (oligo) template. Oligo #166 (5’-CGCGTCGACCATGATGCTCCGAGCGACTGAGCAGGAT-TCTATGA 3’) and oligo #167 (5’-CGCGTCGACCATGATGCTCCGAGCGACTGAGCAGGAT-TCTATGA 3’) were partially complementary such that annealing leaves a four nucleotide 5’ overhang at each end. Annealed oligos were treated with T4 polynucleotide kinase to phosphorylate the 5’ end, and with Klenow and dNTPs to fill in the 3’ end. For a 5’-labelled oligo, the polynucleotide kinase reaction utilized [α-32P]ATP; for a 3’-labelled oligo, the Klenow reaction included [α-32P]dCTP, [α-32P]dGTP and unlabelled dATP and dTTP (see Fig. 4) or [α-32P]dCTP, unlabelled dGTP, dATP and dTTP (see Fig. 5). Hydrolysis was terminated with stop solution (final concentration 100 µM EDTA, 0.1% SDS, 20 µg/ml proteinase K, 5% glycerol, 0.04% xylene cyanol and 0.04% bromophenol blue), and incubation at 37 °C for an additional 30 min. E. coli exonuclease I, exonuclease III, micrococcal nuclease, phage T7 gp6 and snake venom phosphodiesterase were from US Biochemical and were used as suggested by the manufacturer. Reaction products were assayed degradation of DNA at both pH 7.0 and pH 9.0.

Results

Purification of UL98 from HCMV-infected cells

UL98 was purified from HCMV strain AD169-infected HFF cells as described under Methods. A high-salt lysate of infected cells was prepared as described (Powell & Purifoy, 1977). The lysate was desalted over a Sephadex G25 column, then subjected to Q Sepharose, phosphocellulose and native DNA cellulose chromatography. Elution of UL98 from the Q Sepharose and phosphocellulose columns was monitored by Western blotting. Since alpha- and gamma-herpesvirus alkaline nuclease activity is dependent on a high-reaction pH, we separated the alkaline pH-dependent exonuclease activity were resolved upon elution from the phosphocellulose column. Western blots showed that the majority of UL98 coincides with the first peak (peak 1, centred around fraction 71), eluting at 200 mM NaCl. A comparatively smaller amount of UL98 was eluted at 250 mM NaCl (peak 2, centred around fraction 81). No Western blot-detectable UL98 was present in the third peak (400 mM NaCl, centred around fraction 103).

At all steps during the purification, elution of immunoreactive UL98 corresponded with elution of an alkaline pH-dependent exonuclease activity. Fractionation on Q Sepharose resulted in the partial resolution of UL98 from a somewhat less alkaline pH-dependent exonuclease activity which peaked in fraction number 105 (Fig. 1A). UL98, identified by Western blotting, eluted later in the gradient and peaked at fraction number 119, at 330 mM NaCl. UL98-containing fractions were pooled, dialysed and applied to a phosphocellulose column. A slight peak of exonuclease activity was observed in the flow-through (Fig. 1B, fractions 1–43); however, UL98 protein was not detected in these fractions by Western blotting. Three peaks of alkaline pH-dependent exonuclease activity were resolved upon elution from the phosphocellulose column. Western blots showed that the majority of UL98 coincides with the first peak (peak 1, centred around fraction 71), eluting at 200 mM NaCl. A comparatively smaller amount of UL98 was eluted with stop solution (final concentration 100 µM EDTA, 0.1% SDS, 20 µg/ml proteinase K, 5% glycerol, 0.04% xylene cyanol and 0.04% bromophenol blue), and incubation at 37 °C for an additional 30 min. E. coli exonuclease I, exonuclease III, micrococcal nuclease, phage T7 gp6 and snake venom phosphodiesterase were from US Biochemical and were used as suggested by the manufacturer. Reaction products were assayed degradation of DNA at both pH 7.0 and pH 9.0.

Results of these assays are presented in Fig. 1.

Fig. 1. Copurification of UL98 with alkaline pH-dependent nuclease activity. Exonuclease activity of fractions collected from Q Sepharose (A), phosphocellulose (B) and native DNA cellulose columns (C) was measured on [35S]dAMP-labelled activated calf thymus DNA at pH 9.0 (●) and pH 7.0 (○). Activity is expressed in c.p.m. of [35S]dAMP released from labelled DNA. 5000 c.p.m. was equivalent to 3.6 fmol [35S]dAMP. Solid black line, salt gradient used to elute proteins from each column (mM NaCl). Open bars, fractions containing UL98 as determined by Western immunoblotting with MAb I2.
Fig. 2. Electrophoretic analysis of UL98 purification. (A) Silver-stained 12% SDS–PAGE analysis of pooled peak fractions from each purification step. Lane 1, soluble fraction of high-salt extract from infected cells; lane 2, Sephadex G25 desalted extract; lane 3, Q Sepharose peak; lane 4, phosphocellulose peak 1; lane 5, native DNA cellulose peak (purified HCMV alkaline nuclease). The migration of molecular mass standards (in kDa) are noted. (B) Western immunoblot of purified UL98 using MAb I2. (C) Coomassie blue-stained 12% SDS–PAGE of phosphocellulose peak 1 (lane 1) and peak 2 (lane 2). (D) Activity gel analysis of purified UL98 (lane 1) and similarly purified HSV-1 alkaline nuclease (lane 2).

Table 1. Summary of UL98 purification

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (units)*</th>
<th>Protein (mg)</th>
<th>Specific activity†</th>
<th>% Yield‡</th>
<th>Fold purification§</th>
</tr>
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<tr>
<td>Desalted lysate</td>
<td>18565</td>
<td>138</td>
<td>135</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Q Sepharose</td>
<td>21665</td>
<td>35</td>
<td>619</td>
<td>117</td>
<td>4 ± 5</td>
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<tr>
<td>Phosphocellulose</td>
<td>6071</td>
<td>0.3</td>
<td>20236</td>
<td>32.7</td>
<td>153</td>
</tr>
<tr>
<td>Native DNA cellulose</td>
<td>3423</td>
<td>0.09</td>
<td>38033</td>
<td>16.4</td>
<td>282</td>
</tr>
</tbody>
</table>

* 1 unit of activity represents 1 nmol DNA (in nucleotides) hydrolysed in 30 min at 37 °C.
† Specific activity in units mg protein.
‡ Percentage yield = the total units at each purification step divided by the total units in the desalted lysate.
§ Fold purification = specific activity at each purification step divided by the specific activity of the desalted lysate.

column. UL98 eluted from the column at 280–440 mM NaCl in one peak of exonuclease activity (Fig. 1C). Coomassie-stained SDS–PAGE and Western blotting indicated that the peak of exonuclease activity correlated with purified UL98 (data not shown); these fractions were pooled, dialysed against storage buffer, and frozen in aliquots at −80 °C.

A summary of the extent of UL98 purification is presented in Table 1. Phosphocellulose chromatography resulted in the most significant purification of UL98, as judged by the large decrease in total protein and increase in specific activity. Application of this protein to the DNA cellulose column resulted in only a modest increase in purification (150- to 282-fold), but yielded UL98 free of detectable contamination by other proteins, as judged by silver-stained SDS–PAGE (Fig. 2A, lane 5). The peak of exonuclease activity obtained from DNA cellulose contained one predominant band migrating at about 65 kDa, as well as several very light bands both larger and smaller in apparent molecular mass. Western blots using MAb I2 confirmed that the 65 kDa band is indeed UL98. The minor bands larger and smaller than the major product also reacted with I2 by Western blot (Fig. 2B), although the photograph of the blot did not reproduce these bands well. These light bands were probably the result of slight degradation of UL98 or insufficient denaturation prior to electrophoresis. The identity of the 65 kDa band as UL98 was further confirmed using a rabbit polyclonal antibody directed against a peptide near the C terminus of UL98 (anti P2-1; Lahijani et al., 1991) (data not shown). The darkly stained 65 kDa band seen in Fig. 2A, lane 1 (soluble infected-cell extract) was not UL98, but rather the HCMV pp65 protein, as determined by immunoblot analysis (data not shown). This protein also migrated slightly faster than the UL98 protein (compare lanes 1 and 5).

Exonuclease activity was correlated with the 65 kDa UL98 protein species by using the activity gel assay described under Methods. After electrophoresis in a gel containing radiolabelled DNA, purified protein was renatured in the gel and exonuclease activity was measured as a clearing of the radioactivity. A single band was obtained in this assay, which corresponded exactly with the 65 kDa band of UL98 protein seen by both Western blotting and silver staining (Fig. 2D, lane 1). In comparison, lane 2 of Fig. 2D shows the slower migration and similar activity of purified HSV-1 alkaline nuclease.
The pooled peak from DNA cellulose is considered to represent a pure source of UL98, and was used for subsequent experiments.

**Optimal reaction conditions**

Reaction conditions were varied to determine those under which UL98 exhibited optimal activity (Fig. 3). The pH was varied from 7.0 to 10.0. Activity was optimal at pH 9.0, with less than 10% of activity remaining at pH 7.0 (Fig. 3A). Titration of NaCl at concentrations greater than 10 mM progressively inhibited enzyme activity (Fig. 3B). In the absence of a divalent cation, nuclease activity was undetectable (data not shown). MgCl₂ exhibited a broad optimum from 1–4 mM (Fig. 3 C); acetate was similarly effective as a counter-ion (data not shown). The effect of other divalent cations was also measured. MnCl₂ was optimal at about 125 µM, while cobalt, zinc and copper failed to support enzyme activity in this assay; CaCl₂ not only failed to support the exonuclease reaction, but concentrations greater than 1 mM were inhibitory when added to magnesium-containing reactions (data not shown). ATP failed to stimulate the exonuclease reaction and was in fact 50% inhibitory when included in reactions at a final concentration of 1 mM (in reactions containing 6 mM MgCl₂) (data not shown).

**UL98 exhibits 5’ to 3’ exonuclease activity**

The effects of UL98 protein on 5’ or 3’ end-labelled DNA substrates were examined in order to ascertain whether the protein was predominantly an exonuclease, as well as its direction of DNA hydrolysis. Fig. 4 shows that within 5 s the UL98 protein released labelled nucleotide from the 5’ but not the 3’ end of labelled DNA. At 30 s, a significant proportion of the 5’ end label was released, while the 3’ end label remained associated with larger DNA products. These results indicate that hydrolysis by UL98 protein proceeds in the 5’ to 3’ direction along DNA. In contrast, *E. coli* exonuclease III released labelled nucleotides first from the 3’ end of DNA, as previously reported (Kornberg & Baker, 1992). Digestion with the HCMV DNase appears to cause progressive shortening of the substrate DNA, consistent with a predominant exonuclease rather than endonuclease activity under these conditions.

Several previous reports have demonstrated a low but measurable endonuclease activity using purified herpesvirus alkaline nucleases (Hoffman & Cheng, 1979; Hoffman, 1981; Strobel-Fidler & Franke, 1980; Banks *et al.*, 1983; Clough, 1980; Weber & Bronstein, 1996). We also observed endo-
nuclease activity on supercoiled DNA templates using our enzyme preparations, although at much lower levels than the corresponding exonuclease activity on linear templates. For example, approximately 30-fold more enzyme was required to relax a 3 kb supercoiled plasmid (one hydrolytic event) than is required to completely digest the linear plasmid (3000 hydrolytic events) (data not shown). The optimal reaction conditions for the endonuclease activity were similar to those for the exonuclease activity, with the exception that low concentrations of MnCl2 were optimal (data not shown), similar to the HSV-1 and HSV-2 alkaline nucleases (Hoffman, 1981). Under conditions where the HSV-1 alkaline nuclease may act as either an exo- or endonuclease, the enzyme preferentially uses DNA termini as a substrate (Hoffman, 1981). We find that excess cold, linear plasmid DNA more effectively competes for HCMV UL98 exonuclease activity than does supercoiled plasmid DNA using our standard reaction conditions (approximately fivefold, data not shown), suggesting that UL98 protein is primarily an exonuclease in vitro. However, a quantitative kinetic comparison of the endo-and exonuclease activities under conditions optimized for each has not been performed.

**Products of the exonuclease reaction**

We next examined the products generated by complete digestion of DNA with the UL98 protein (Fig. 5). The products of digestion were identified by co-migration with nucleotide products generated using well-characterized nucleases (Kornberg & Baker, 1992). The gel conditions used resolved 5′ α-monophosphorylated mononucleotides and oligonucleotides (compare lanes 5 and 6), as well as 5′ α-phosphoryl dCTP, 5′ α-phosphoryl dCMP and 5′ α-phosphoryl dGMP (lanes 7–9). UL98 digestion of oligonucleotide DNA, labelled at the α-phosphate of the 5′ dCMP residue, mainly resulted in products which co-migrated with 5′ α-phosphoryl dCMP (compare lanes 4 and 8). These products also co-migrated with 5′ α-phosphoryl dCMP generated by the digestion of 3′ end-labelled DNA with E. coli exonuclease III (lane 5), and clearly differed from the migration of 5′ α-phosphoryl dCMP (lane 6), generated using micrococcal nuclease. Digestion with UL98 protein also generated a small amount of 5′ α-phosphoryl dGMP (compare lanes 4 and 9). It is possible that the 5′ α-phosphoryl dGMP results from labelling of a partially degraded oligonucleotide, since the 5′ penultimate nucleotide present in the oligonucleotide is deoxyguanosine.

When the UL98 protein was incubated with 3′ end-labelled DNA, some 5′ α-phosphoryl dCMP was generated, but the majority of the reaction products migrated more slowly through the gel (lane 3). The major band co-migrates with a 5′ α-monophosphoryl dinucleotide product generated by digestion of 3′ end-labelled DNA with E. coli exonuclease I (lane 1). The identity of the products was further confirmed by comparison to a ladder generated by the digestion of the same DNA substrate with T7 gp6 (lane 2). The products of UL98 digestion co-migrate with mono-, di-, tri- and tetra-deoxyribonucleotides. In most experiments, UL98 protein did not completely digest 3′ end-labelled DNA to single 5′ dNMP products. The majority of UL98 protein reaction products were di- and tri-deoxyribonucleotides, present along with lesser amounts of mono- and tetra-deoxyribonucleotide products. Since T7 gp6 generates co-migrating 5′ monophosphoryl deoxyribonucleotides, we conclude that these small oligonucleotides are also 5′ phosphorylated. In any event, a dinucleotide appears to be the smallest possible substrate for the nuclease, as there was no detectable phosphatase activity when the enzyme was incubated with γ-32P-labelled triphosphates with snake venom phosphodiesterase.

**Fig. 5.** HCMV alkaline nuclease hydrolysis of DNA yields 5′ monophosphorylated mononucleotides and oligonucleotides. Oligonucleotide DNAs contained [α-32P]dCMP labels at the 5′ (lanes 1, 4 and 11) or 3′ (lanes 2, 3, 5, 6 and 10) ends of the DNA. Reactions were performed in the absence of enzyme (lanes 10 and 11) or in the presence of E. coli exonuclease I (lane 1); phage T7 gp6 exonuclease (lane 2); HCMV alkaline nuclease (lanes 3 and 4); E. coli exonuclease III (lane 5); or micrococcal nuclease (lane 6). Lane 7 contains [α-32P]dCTP. Lanes 8 and 9 contain [α-32P]dCMP and [α-32P]dGMP, respectively, generated by digestion of the corresponding α-32P-labelled triphosphates with snake venom phosphodiesterase.

**Kinetics of exonuclease activity**

The rate of DNA digestion was measured using both native and denatured activated calf thymus DNA as substrates. As
shown in Table 2, the purified enzyme hydrolysed native and denatured DNA at similar rates. Similar apparent affinity constants were also obtained for both substrates. These results indicate that the HCMV UL98 protein displays an equivalent capacity to hydrolyse both single-stranded and double-stranded DNA templates under the assay conditions used. The turnover number for our purified enzyme was greater than 200 per min, which is within the range of representative values reported in the literature for other exonucleases: 27 per min for the proofreading activity of *E. coli* Pol (Kornberg & Baker, 1992), and 120 and 750 per min for the recombination exonucleases *Schizosaccharomyces pombe* exonuclease II (Szankasi & Smith, 1992b) and *S. pombe* exonuclease I (Szankasi & Smith, 1992a).

**Discussion**

All herpesviruses sequenced to date contain a gene encoding a protein with homology to the HSV-1 alkaline nuclease (see for example Nicholas, 1996). The protein predicted to be encoded by HCMV UL98 is 26% identical and 47% similar to the HSV-1 homologue UL12. In this work, we have purified the protein encoded by HCMV UL98 and show it to be the functional herpesvirus alkaline nuclease homologue. The nuclease activity of purified UL98 protein was characteristic of the alkaline nucleases previously purified from other herpesvirus species, confirming that the UL98 protein is indeed the HCMV alkaline nuclease. These results suggest that the alkaline nuclease and its role in infected cells is conserved over all three subgroups of herpesviruses.

The reported activities of the HSV-1, HSV-2 and EBV purified alkaline nucleases are similar to our findings with the HCMV alkaline nuclease. All four purified enzymes exhibit a high pH optimum, an absolute requirement for divalent cation, and maximal activity under conditions of low ionic strength. The HSV-1 and EBV alkaline nucleases degrade DNA in the 5’ to 3’ direction, generating single 5’ monophosphonucleotide products (Knopf & Weishart, 1990; Lin et al., 1995). In agreement with these results, we find the HCMV alkaline nuclease degrades DNA 5’ to 3’. HCMV alkaline nuclease degradation products include 5’ monophosphoryl nucleotides and some small oligonucleotides near the 3’ end of DNA. Small oligonucleotide products may be a result of a loss of affinity of the enzyme for small DNA substrates, and may be reflective of the size of the enzyme’s DNA binding site. In support of this hypothesis, the HCMV alkaline nuclease was unable to hydrolyse the single nucleotide substrate dCTP.

The HCMV UL98 alkaline nuclease behaved similarly to those characterized from other herpesviruses in its binding to anion exchange media, phosphocellulose and DNA cellulose. During purification, UL98 was found to bind to native DNA cellulose more tightly than to denatured DNA cellulose. Kinetic analyses with purified alkaline nuclease, however, showed that native and denatured DNA molecules were nearly equally hydrolysed by the HCMV DNase. This result is similar to results obtained by Hoffman & Cheng (1978) who found that the HSV-1 and HSV-2 enzymes hydrolysed native DNA only slightly better than denatured DNA.

The function of the herpesvirus alkaline nuclease in the virus life cycle remains unclear. Because the nuclease is expressed at early times in infection, it has been suggested to function during DNA replication either in the degradation of host DNA to produce dNMPs for use in viral DNA synthesis, or in the removal of RNA primers generated during lagging-strand DNA synthesis (Keir, 1968). Our results show that the HCMV alkaline nuclease produces 5’ monophosphoryl nucleotide products which are suitable for phosphorylation and incorporation into nascent DNA by a polymerase. However, the viral alkaline nuclease is not required in vivo for transient virus origin-dependent DNA synthesis in the HSV-1, HCMV or EBV systems (Wu et al., 1988; Pari & Anders, 1993; Fixman et al., 1992). Additionally, the generation of a null mutant in the HSV-1 alkaline nuclease gene UL12 has allowed detailed analysis of the block in virus replication (Weller et al., 1990; Shao et al., 1993). The null mutant synthesizes viral DNA at levels approaching those in wild-type infection, but egress of DNA-filled capsids from the nucleus is blocked. The lack of a significant reduction in the levels of viral DNA also argues against an integral role for the enzyme in viral DNA synthesis.

Further studies of the HSV-1 alkaline nuclease deletion mutant have revealed that although newly replicated viral DNA is properly cleaved to unit length, the DNA contains an increased proportion of complex structures, perhaps branches, which may prevent efficient packaging of DNA into virions.

**Table 2. Kinetic parameters of the UL98 alkaline nuclease**

<table>
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<tr>
<th>DNA template</th>
<th>$K_v$ (µM)*</th>
<th>$V_{max}$ (nmol per min)†</th>
<th>$k_{cat}$ (per min)‡</th>
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<tr>
<td>Native</td>
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<td>222</td>
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<tr>
<td>Denatured</td>
<td>5.9</td>
<td>87</td>
<td>253</td>
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* $K_v$ is expressed in µM concentration of DNA in nucleotides.
† $V_{max}$ is expressed as nmol nucleotide released per min.
‡ $k_{cat}$ is expressed in nucleotides released per min.
(Martinez et al., 1996a). Based on this characterization of the null mutant virus, the alkaline nuclease has been hypothesized to prevent or resolve complex DNA structures which may prevent the virus from efficiently packaging its DNA into virions (Martinez et al., 1996a). The \textit{in vitro} activity of the HCMV alkaline nuclease is consistent with a role for the enzyme in this process. It is possible that the alkaline nuclease prevents branch generation by degrading 5’ ssDNA tails (generated by incomplete lagging-strand DNA replication) which could otherwise invade duplex DNA. While invading 3’ ssDNA ends can be productively utilized as primers for DNA synthesis, invading 5’ ssDNA ends cannot be extended by the polymerase. An invading 5’ ssDNA end may create unresolvable junctions between single-stranded and double-stranded DNA. A 5’ to 3’ directed exonuclease such as the alkaline nuclease could preferentially degrade these 5’ ended ssDNA tails and prevent their non-productive interaction with duplex DNA.

Alternatively, the alkaline nuclease may act as a resolvase to cleave at Holliday junctions formed by DNA strand-exchange recombination (as discussed by Weller, 1995). Such an activity would require a controlled endonucleolytic cleavage at Holliday junctions. Although we and others have observed low level endonuclease activity by the herpesvirus alkaline nucleases, it is hard to envisage how this cleavage could occur without subsequent degradation of the DNA by the efficient exonuclease activity.

It is conceivable that the activity of the alkaline nuclease is modified in some way or controlled \textit{in vivo} by another protein. Indeed, several reports have identified interactions of the HSV-1 and EBV alkaline nucleases with the viral single-stranded DNA binding proteins (Lin et al., 1995; Vaughan et al., 1984; Thomas et al., 1988, 1992), or the polymerase accessory protein (Daibata & Sairenji, 1993). However, we found no direct evidence for co-purification of a tightly interacting protein using our purification protocol. The second peak of exonuclease activity which eluted from phosphocellulose (see Fig. 1) may represent an interaction between the alkaline nuclease and another protein, such as the viral DNA polymerase. We look forward to investigating this possibility.

The presence of an alkaline nuclease homologue in every herpesvirus sequenced to date, together with the conservation of enzymatic properties among alpha-, beta- and gamma-herpesvirus nucleases, emphasizes that the enzyme’s role in infection must be extremely important. The predicted enzymatic properties of herpesvirus alkaline nucleases, together with the conservation of conserved motifs required for additional shared functions of the herpesvirus alkaline nucleases.

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References


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