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The Human Cytomegalovirus UL94 Open Reading Frame Encodes a Conserved Herpesvirus Capsid/Tegument-Associated Virion Protein That Is Expressed with True Late Kinetics

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In this report, we provide a detailed characterization of the human cytomegalovirus (HCMV) UL94 gene product. Northern (RNA) blot analysis of infected cell RNA demonstrated that UL94 message was found only at late times of infection and was not synthesized in the presence of the viral DNA replication inhibitor ganciclovir. Expression of the UL94 open reading frame in vitro and in vivo yielded a protein with the predicted molecular mass of 36 kDa. A monoclonal antibody raised to a UL94-specific peptide reacted specifically with a 36-kDa protein in HCMV-infected fibroblasts. This protein was found only at late times of infection and was also present in purified HCMV virions. Fractionation of purified virions and HCMV-infected cells revealed an association of UL94 immunoreactivity with the capsid/tegument and nuclear fractions, respectively. The evolutionary conservation of UL94 protein sequence and an analysis of potential functional regions of the protein are discussed.

Human cytomegalovirus (HCMV) is the most widely studied member of the beta family of herpesviruses and is ubiquitous within the human population. It is associated with myriad clinical manifestations resulting from primary, persistent, or reactivated infection, including retinitis, coronary restenosis, and birth defects (2, 19, 28, 35, 36, 41). Because of its restrictive host range, studies of the HCMV replicative cycle have been limited primarily to tissue culture or in vitro work; however, much insight into the functions of important viral gene products and their roles during infection has been gained within these systems (19, 28, 36).

Analysis of the structure, function, and regulation of HCMV genes has been facilitated by the publication of the entire genomic sequence for laboratory strain AD169 (8). Sequence analysis of the genome has revealed a number of core open reading frames (ORFs) which are conserved with other herpesviruses as well as numerous HCMV-specific genes (7, 8). Some of this latter group have been demonstrated to bear homology to cellular proteins of known function, including G-protein-coupled receptors (10, 38), the T-cell receptor γ subunit (8), and the major histocompatibility class I antigen (6). The functions of many of the estimated 200 viral gene products, however, remain obscure.

In an effort to add to our understanding of the function and regulation of expression of uncharacterized viral gene products, our laboratory has recently provided initial characterizations of a number of novel viral proteins, including the UL84 and US18-20 gene products (15, 16). Analysis of these proteins indicated that UL84 was an HCMV nuclear compartment-associated early protein (16), while the US18-20 proteins were multiply hydrophobic membrane-spanning proteins (15). Subsequently, the UL84 gene product was shown to be essential for viral DNA replication (30) and to physically interact with the viral immediate-early protein IE2-86 kDa (34).

With this in mind, we recently characterized a family of novel overlapping transcripts which code for HCMV ORFs UL93 to UL99 (39). These ORFs are located within a region of the HCMV genome which is conserved in all other human herpesviruses (7, 8, 9, 12, 31, 33). Very little information is currently available concerning the function and/or regulation of expression of these viral gene products; however, at least two of the gene products are thought to play a role in viral DNA replication: the protein product of the UL97 ORF was recently shown to be involved in the phosphorylation of ganciclovir during a productive HCMV infection (22, 37), and the UL98 ORF is thought to encode a DNase, as judged from its homology with a positionally conserved ORF in herpes simplex virus (HSV) (7, 8, 12). In addition, the UL99 gene product (pp28) is an extensively studied virion phosphoprotein which is expressed as a true late gene (20, 21, 24, 25, 29). Kinetic analysis of the transcripts specific for ORFs UL93 to UL98 suggested that ORFs UL95, UL96, and UL97 (ganciclovir kinase) and UL98 (DNase) were likely to be early or early-late gene products, while ORF UL94 (and possibly UL93) was likely to be a late gene (39). This kinetic analysis provides an estimation of the phase during the replicative cycle in which these gene products are needed by the virus and thus provides initial clues as to possible functions of these uncharacterized viral proteins. Follow-up studies for the UL98 transcription unit have subsequently confirmed and extended these predictions for the UL98 protein (1).

In an effort to extend the observations made on the basis of transcription data cited above, we have begun characterization of additional novel gene products associated with the UL93 to UL98 gene region. In this report, we provided an initial characterization of the UL94 protein. We demonstrate that UL94 is regulated as a true late gene and that UL94 protein is found in HCMV virions and in the nuclei of infected cells. Addition-

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ally, we provide an analysis of the UL94 protein sequence with respect to potential functional regions which are conserved in the UL94 homologs of other herpesviruses.

MATERIALS AND METHODS

Cells and virus. Human embryonic lung cells (HELs) and HCMV (Towne strain) were maintained and propagated as previously described (18, 39). Human 293-T cells (a gift of Colin Duckett) were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (13).

DNA cloning and plasmids. UL94 coding sequences were cloned by PCR amplification from 50 ng of *Eco*RI-digested HCMV (Towne) genomic DNA, using 20 pmol of the following primers: 5′-GCATGCATGGATCCATGGCTT GGCGCAGCGGGCTTTGCGAG-3′ and 5′-GCATGCATGAATCCGTGCAC TAGGTTCTTAAGCACCG-3′. A *Bam*HI site was incorporated into the 5′ primer (underlined) to facilitate cloning. Amplification was carried out by using Vent DNA polymerase (New England Biolabs) and a Perkin-Elmer Cetus DNA thermocycler. The ends of the amplified product were flushed with T4 polynucleotide kinase (Boehringer Mannheim) and Klenow DNA polymerase (Promega); the product was digested with *Bam*HI and cloned into *Bam*HI-*Sma*I-digested pBS(+) (Stratagene). The resultant plasmid (pBSUL94) was used in in vitro transcription-translation analysis (described below).

For expression in vivo, UL94 coding sequences were PCR amplified from pBSUL94 with the following primers: 5′-GCATGCATGGTACCATGGCTTG GCGCAGCGGGCTTTGCGAG-3′ and 5′-GCATGCATGGATCCTTACACC ACCACCACCACCACGTGCACTAGGTTCTTAAGCACCG-3′. *Kpn*I and *Bam*HI sites (underlined) were incorporated into the 5′ and 3′ primers, respectively, to facilitate cloning. A six-histidine tag (also underlined) was incorporated into the 3′ primer prior to the UL94 stop codon to facilitate purification of the expressed protein. The amplified product was digested with *Bam*HI and *Kpn*I and cloned into *Bam*HI-*Kpn*I-digested pCEP4 (Invitrogen). The resultant plasmid (pCEPUL94) was used for in vivo expression of UL94-His protein driven by the HCMV major immediate-early enhancer located within pCEP4.

Expression of UL94 coding sequences. For expression in vitro, plasmid pBSUL94 was linearized with *Eco*RI and in vitro transcribed with T3 RNA polymerase (Promega) by using the Promega in vitro transcription kit. In vitro transcripts were translated by using rabbit reticulocyte lysate (Promega) and labeled with [^{35}S]methionine (1,280 Ci/mmol; Amersham). In vitro-translated proteins (1 μl) were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels; the gels were subsequently incubated in 1 M sodium salicylate (Boehringer Mannheim) for 30 min, vacuum dried for 30 min, and exposed to film overnight at −70°C.

For expression in vivo, one 100-mm-diameter dish of human 293-T cells was transfected with either 5, 10, or 15 μg of pCEPUL94, control plasmid pCEP4 (empty vector), or pCEPgB (HCMV glycoprotein B gene; a gift from Jay Nelson) via calcium phosphate precipitation (3). After an overnight transfection, the precipitate was washed away with DMEM and the cells were incubated with DMEM supplemented with 10% fetal calf serum. After 6 h, the medium was changed to DMEM supplemented with 10% fetal calf serum and 200 μg of hygromycin B (Boehringer Mannheim) per ml. After 3 days, hygromycin-resistant cells were pooled and passaged into 100-mm-diameter dishes, using the same medium. After two passages, cells were expanded to T150 flasks and analyzed for UL94 RNA and protein expression.

RNA isolation and Northern (RNA) blot analysis. Whole cell RNA was isolated from 293-T cell lines and mock- or HCMV (Towne)-infected HELs by the guanidinium isothiocyanate-cesium chloride method (3). Infected cells were harvested at 0, 4, 24, 48, 72, and 144 h postinfection (hpi) as well as at 72 hpi in the presence of 10 μM 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG). Northern blots were prepared and probed with end-labeled UL94-specific oligonucleotide UL94-1 as described previously (39).

Cell and virion fractionation. HCMV-infected HELs and 293-T cell lines were separated into crude nuclear and membrane/cytoplasmic fractions by lysis of cells with 0.1% Nonidet P-40 in hypotonic buffer. The intact nuclei were pelleted at 1,500 rpm and washed with phosphate-buffered saline (PBS), and the membrane/cytoplasmic fraction was clarified by centrifugation at 14,000 rpm. Fractions were probed by Western blotting (immunoblotting) with antibodies to cellular membrane/cytoplasmic and nuclear fraction-specific proteins Ras-GAP and p53 (both from Santa Cruz Biotechnology), respectively, to determine purity of fractions.

HCMV (Towne) virions were separated into envelope and capsid/tegument fractions by addition of 1% Nonidet P-40 to the virions and incubation on ice for 30 min. Samples were subsequently layered onto 30% sucrose cushions and centrifuged at 45,000 rpm for 90 min at 4°C. The envelope fraction was collected from the top of the sample. The capsid/tegument pellet was resuspended in 1× PBS. To assess the purity of the fractions, Western blots of both fractions were probed with antisera to HCMV IE1-72 kDa (a capsid/tegument-associated protein) and HCMV glycoprotein B (an envelope-specific protein).

Western blot analysis. Whole cell protein lysates from 293-T cell lines as well as mock- and HCMV-infected HELs were prepared by lysing cells in equal volumes of 1× PBS and 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (1.52 mg of Tris base per ml, 20% glycerol, 2 mg of SDS per ml, 2% β-mercaptoethanol, 1% bromophenol blue); 2% β-mercaptoethanol in the sample buffer was substituted with 150 mM dithiothreitol (DTT; Boehringer Mannheim) or 150 mM iodoacetimide (Sigma) for some experiments. Purified HCMV virions were prepared as described previously (18). Proteins were separated by electrophoresis on 4% stacking–12% separating SDS-containing polyacrylamide gels, using minigel rigs (Bio-Rad). Gels were subsequently transferred overnight to Immobilon-P membranes (Millipore) and probed with the appropriate primary antisera followed by secondary peroxidase-linked antibodies. Blots were developed by using an enhanced chemiluminescence detection system (Amersham).

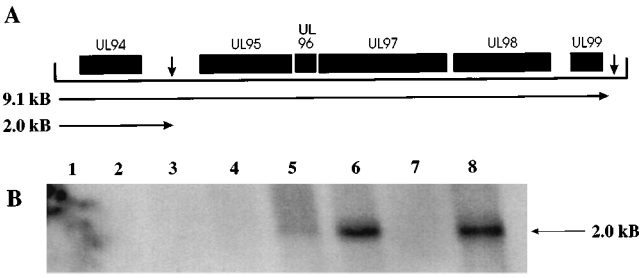

FIG. 1. Expression of UL94 mRNA during HCMV infection of HELs. (A) Schematic of the UL94 to UL99 gene region showing the structures of the UL94 9.1- and 2.0-kb RNAs as determined previously (58). Downward arrows indicate consensus polyadenylation signals. (B) Northern blot analysis of the UL94 2.0-kb mRNA from HCMV-infected HELs probed with UL94-specific oligonucleotide UL94-1. RNA samples: mock infected (lane 1), 0 hpi (lane 2), 4 hpi (lane 3), 24 hpi (lane 4), 48 hpi (lane 5), 72 hpi (lane 6), 72 hpi plus DHPG (lane 7), and 144 hpi (lane 8).

Monoclonal antibody production. UL94 monoclonal antibodies were generated by using standard techniques (3). Primary immunization was carried out with a branched peptide corresponding to UL94 amino acid residues 26 to 40 (KLVGKSRKHREYRAV) (purchased from Research Genetics). Hybridoma supernatants were screened for UL94 peptide reactivity by enzyme-linked immunosorbent assay (ELISA); ELISA-positive samples were tested for UL94 reactivity by Western blotting with purified UL94-His as well as HCMV-infected cell extracts. For antibody competition experiments, monoclonal antibodies were preincubated with 0 to 100 mg of either UL94 or nonspecific protein kinase C (PKC) peptide per ml prior to use in Western blot analysis.

Affinity purification of histidine-tagged UL94. UL94-His expressed in permanently transfected 293-T cells was purified by incubating three T150 flasks of cells lysed in IMAC-5 (20 mM Tris-HCl [pH 7.9], 0.5 M NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mM imidazole)–1% Nonidet P-40 with 20 to 50 μl of nickel-agarose beads (Novagen) in a total volume of 10 ml. Following incubation, the beads were washed five times with 1 ml of IMAC-5, and proteins were eluted stepwise by incubating beads with IMAC-5 supplemented with 24.5 to 200 mM imidazole. Elutions were subjected to SDS-PAGE followed by either Coomassie blue staining or Western blot analysis.

RESULTS

Kinetics of UL94 RNA expression. We previously reported that coding sequences for HCMV ORFs UL93 to UL99 were present on a series of overlapping transcripts found in productively infected cells (39). UL94-associated transcripts were demonstrated to initiate 335 nucleotides (nt) upstream of the UL94 initiation codon and to utilize two distinct polyadenylation sites. Use of the first site, located 512 nt downstream of the UL94 termination codon, results in a 2.0-kb UL94-specific message; use of the second polyadenylation site, located 176 nt downstream of the UL99 termination codon, results in a 9.1-kb message which also hybridizes to probes specific for ORFs UL95 to UL99 (Fig. 1a). This larger message was shown to be expressed only at late times of infection and to be sensitive to inhibitors of viral DNA replication. The kinetics of the smaller (2.0-kb) message, however, were not shown in the previous study (39). To determine the pattern of expression of the 2.0-kb message, we prepared RNA from mock- and HCMV (Towne)-infected HELs and performed Northern blot analysis using an end-labeled UL94-specific oligonucleotide probe. The results of this experiment indicate that the 2.0-kb UL94-specific transcripts are detected only at late times of infection (Fig.

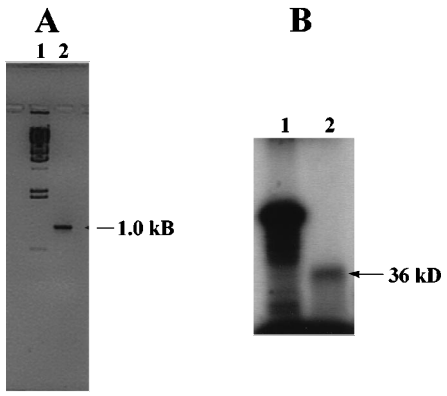

FIG. 2. Expression of UL94 coding sequences in vitro. (A) PCR amplification of the UL94 ORF from *Eco*RI-cut HCMV (Towne) genomic DNA (lane 2) and *Hin*dIII-digested λ markers (lane 1). (B) In vitro translation of UL94 PCR product cloned into vector pBS(+) showing the 36-kDa UL94 protein (lane 2) and the control luciferase translation (lane 1).

1B, lanes 5, 6, and 8). In addition, no 2.0-kb transcripts are detected at 72 hpi in the presence of ganciclovir (lane 7). These results suggest that the 2.0-kb UL94-specific transcripts are expressed with true late kinetics.

Exogenous expression of UL94 coding sequences. To more closely examine the viral gene product(s) associated with ORF UL94, the predicted coding sequences for UL94 were subcloned from HCMV (Towne) genomic DNA by PCR amplification using primers spanning sequences between the predicted N-terminal and C-terminal ends of the UL94 reading frame; the putative stop codon for UL94 was also included in the amplified product (see Materials and Methods for primer sequences). Using these primers, we were able to specifically amplify a double-stranded DNA product approximately 1 kb in size from total HCMV genomic DNA (Fig. 2A). The size of this product was consistent with the predicted size of 1,038 bp for the UL94 reading frame (7, 8). To determine the coding capacity of the amplified product, we subcloned the PCR fragment and expressed the cloned HCMV sequences by in vitro transcription-translation. Analysis of the proteins produced in this reaction revealed that a 36-kDa protein was specifically produced in the UL94 in vitro translation reaction (Fig. 2B). This size was in agreement with the predicted molecular mass of 36 kDa for the 346-amino-acid UL94 reading frame (7, 8). DNA sequence analysis of the termini of the DNA insert used in the in vitro transcription-translation reaction confirmed that the expected UL94 coding sequences had been successfully subcloned (40).

We also examined the expression of UL94 coding sequences in vivo by generating a stably transfected human kidney-derived 293-T cell line expressing UL94 protein from the HCMV major immediate-early enhancer/promoter. To facilitate analysis and affinity purification of eukaryotically expressed UL94 protein, a six-histidine-codon tag was incorporated onto the 3′ end of the subcloned UL94 product (prior to the stop codon) as described in Materials and Methods. To examine UL94-specific RNA expression, whole cell RNA was isolated from the UL94 cell lines, as well as control cell lines containing either no expression vector, empty expression vector, or HCMV glycoprotein B expression vector, and analyzed by Northern blot hybridization to a UL94 oligonucleotide probe. The results of this experiment indicated that the UL94 cell lines expressed a 1.3-kb transcript which hybridized with the UL94 probe (Fig. 3A, lanes 4 to 7); none of the control cell lines contained UL94-cross-reactive RNA (lanes 1 to 3). To examine UL94 protein expression, UL94-His protein was affinity purified on nickel-agarose beads. Results of this experiment indicated that the UL94 cell lines specifically contained an approximately 36-kDa protein which was purified on the nickel-agarose beads (Fig. 3B, lanes 3 and 5); this protein was not present in cell lysates from control cell lines (lanes 2 and 4). The molecular weight of the nickel-agarose affinity-purified protein corresponded with the size of in vitro-expressed UL94 as well as the expected molecular weight of UL94 protein, indicating that it was likely that the UL94 cell lines expressed histidine-tagged UL94.

To facilitate further analysis of the UL94 gene product, we sought to generate an antiserum specific for UL94 protein. Mice were immunized with a peptide corresponding to amino acids 26 to 40 of the UL94 reading frame (see Materials and Methods for peptide sequences). The presence of codons corresponding to this peptide sequence in our subcloned UL94 DNA fragment was confirmed by DNA sequence analysis of the corresponding region of the pBSUL94 insert (40). One monoclonal antibody (designated UL94-9) that was generated reacted strongly to the UL94 peptide by ELISA (40). To examine the specificity of monoclonal antibody UL94-9, we initially tested its ability to recognize UL94 protein in Western blots of imidazole elutions from nickel-agarose affinity-purified UL94-His cell lines. The results indicated that UL94-9 detected a 36-kDa protein in the imidazole elutions containing

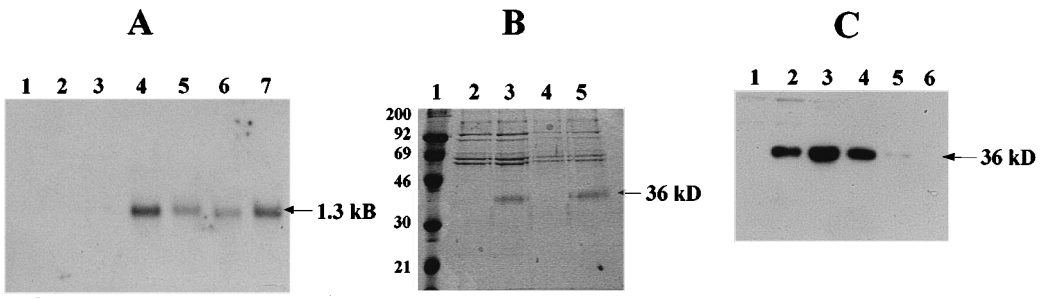

FIG. 3. Expression and purification of UL94 as a histidine-tagged protein from stably transfected human 293-T cells. (A) Northern blot of whole cell RNA isolated from 293-T cells (lane 1) and 293-T cells stably transfected with pCEP4 (lane 2), pCEBgB (lane 3), and four different cell lines stably transfected with pCEPUL94: 94.5 (lane 4), 94.10 (lane 5), 94.15A (lane 6), and 94.15B (lane 7). The 1.3-kb size of the UL94 RNA is indicated. (B) Coomassie-stained SDS-polyacrylamide gel of UL94-His purified by nickel-agarose affinity chromatography from cell lines 94.5 (lane 3) and 94.15B (lane 5) as well as control purifications from untransfected 293-T cells (lane 2) and pCEP4-transfected cells (lane 4). Sizes (in kilodaltons) of protein molecular weight markers (lane 1) are indicated at the left. (C) Western blot of imidazole elutions of nickel-agarose affinity-purified UL94-His from cell line 94.15B. The imidazole concentrations are 24.5 mM (lane 1), 30.5 mM (lane 2), 102.5 mM (lane 3), 141.5 mM (lane 4), 180.5 mM (lane 5), and 200 mM (lane 6).

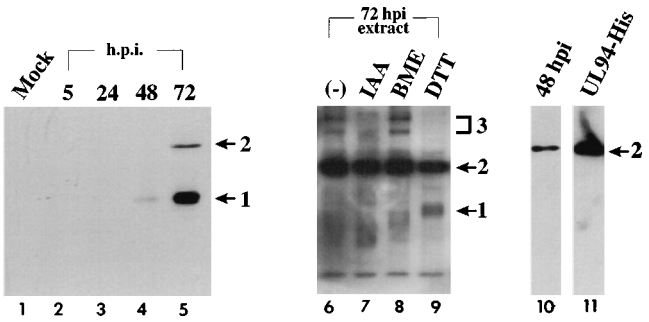

FIG. 4. Western blot analysis of UL94 protein from infected cells. Lanes: 1 to 5, infection time course Western blot analysis using protein extracts prepared with 150 mM DTT from mock-infected HELs (lane 1) as well as infected cells at 5 (lane 2), 24 (lane 3), 48 (lane 4), and 72 hpi (lane 5) probed with monoclonal antibody UL94-9; 6 to 9, 72-hpi cell lysates prepared with no reducing agent (lane 6), 150 mM iodoacetimide (IAA; lane 7), 2% β-mercaptoethanol (BME; lane 8), or 150 mM DTT (lane 9) and analyzed by Western blotting with antibody UL94-9; 10 and 11, Western blot analysis of 48-hpi cell extract (lane 10) and affinity-purified UL94-His (lane 11) prepared with 2% β-mercaptoethanol and probed with antibody UL94-9. The 36-kDa UL94 protein is indicated by arrow 1; the 72-kDa form and larger forms are indicated by arrow 2 and bracket 3, respectively.

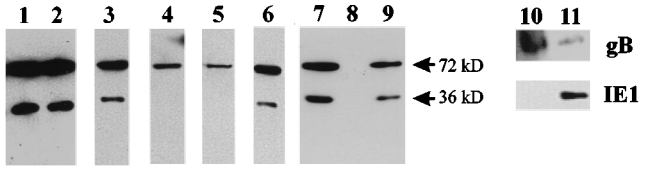

FIG. 5. Western blot analysis of UL94 protein from purified HCMV virions. Lanes: 1 and 2, Western blot of Towne (lane 1) and AD169 (lane 2) virions; 3 to 6, peptide competitions of the UL94-9 antiserum preincubated with 0 (lane 3), 10 (lane 4), and 100 (lane 5) mg of UL94 peptide per ml or 10 mg of PKC peptide per ml (lane 6) and used to probe Western blots of HCMV (Towne) virions; 7 to 9, Western blot of HCMV (Towne) whole virions (lane 7), virion envelope (lane 8), and virion capsid/tegument (lane 9) fractions (both the 36- and 72-kDa forms of UL94 are indicated); 10 and 11, Western blot controls of virion envelope (lane 10) and capsid/tegument (lane 11) fractions probed with antisera specific for HCMV glycoprotein B (gB) and IE1-72 kDa proteins.

UL94-His protein (Fig. 3C, lanes 2 to 4). Additional assays for UL94-9 antibody specificity are detailed below.

Western blot analysis of UL94 protein in HCMV-infected fibroblasts. To examine expression of UL94 protein during a productive infection by HCMV, protein extracts were prepared from HCMV-infected HELs at 5, 24, 48, and 72 hpi, as well as from mock-infected HELs, and examined by Western blotting with antibody UL94-9. The results of this experiment indicated that the UL94-9 antiserum reacted specifically with a 36-kDa protein (band 1) found exclusively in infected cells (Fig. 4, lanes 2 to 5); no UL94 reactivity was seen in mock-infected cells (lane 1). The 36-kDa protein could be detected only at late times of infection and was maximal at 72 hpi (lane 5). This result is consistent with the Northern blot data (Fig. 1), which demonstrated that UL94-specific transcripts were detected only at late times of infection. In addition to the 36-kDa protein, a 72-kDa species (band 2) was also detected at 72 hpi (lane 5). The presence of this band in Western blots appeared to be primarily dependent on the reducing conditions used to prepare extracts for SDS-PAGE (lanes 6 to 9). When extracts from 72-hpi-infected cells were prepared with no reducing agent in the SDS-PAGE sample buffer (lane 6), or when samples were prepared with 150 mM iodoacetimide (lane 7) or 2% β-mercaptoethanol (lane 8), the UL94-reactive species migrated predominantly at a molecular mass of 72 kDa; however, in the presence of 150 mM DTT (lane 9), a noticeable decrease in the 72-kb species (band 2) was accompanied by an increase in the 36-kDa species (band 1). Variable amounts of the 72-kb band persisted in cell extracts following treatment with DTT (lanes 5 and 9). In addition, several larger-molecular-weight species were observed when no reducing agent was added to the sample buffer or in the presence of 2% β-mercaptoethanol (lanes 6 and 8, bracket 3). We could also detect the 72-kDa species exclusively when UL94 from the UL94 cell lines (lane 10) or in infected cell extracts at 48 hpi (lane 11) was analyzed by Western blot under less than ideal reducing conditions, indicating that no novel viral gene product is required to detect the 72-kDa species. Taken together, these results suggest that the appearance of the 72-kDa species is dependent on the reducing conditions of sample preparation and that it may, in fact, represent a disulfide-linked dimer of 36-kDa UL94 monomers. This conclusion is supported by the significant number of cysteine residues present in the primary amino acid sequence of UL94 which are conserved in the UL94 homologs of other herpesviruses (see Fig. 7). The possible significance of the 72-kDa species is addressed in Discussion.

Virion association of UL94 protein. Predominantly late proteins from herpesviruses are often virion structural components; we therefore looked for UL94 protein in HCMV particles. Purified, enveloped HCMV virions were probed in Western blots with the UL94-9 antiserum as shown in Fig. 5. The results of this experiment indicate that UL94 protein is associated with HCMV particles (Fig. 5, lanes 1 and 2). Proteins of identical size were detected in virions from both Towne (lane 1) and AD169 (lane 2) strains, indicating that the peptide sequence used to generate antibody UL94-9 is conserved in the two strains. We also examined the specificity of the antibody for the 36- and 72-kDa UL94 bands in virions by preincubating the antiserum with increasing amounts of UL94 peptide, or an unrelated peptide, and probing Western blots of HCMV (Towne) virion proteins (lanes 3 to 6). The results of this experiment indicated that the UL94 peptide specifically competed for both the 36- and 72-kDa bands (lanes 4 and 5), while a peptide to protein kinase C had no effect on UL94 immunoreactivity (lane 6). Thus, both protein species are specific for UL94 protein, and the antibody specifically recognizes a UL94-specific peptide sequence.

HCMV virion envelope proteins are often found in disulfide-linked complexes (14). Since UL94 protein had an apparent propensity to form disulfide-linked dimers in vitro, we suspected that UL94 protein would be associated with the HCMV virion envelope. To test this hypothesis, whole HCMV virions were separated into envelope and capsid/tegument fractions and analyzed by Western blot analysis (Fig. 5, lanes 7 to 9). Analyses with control antisera indicated that the capsid/tegument fraction contained only minor levels of envelope proteins and that the envelope fraction contained exclusively envelope proteins (lanes 10 and 11). When these fractions were probed with a UL94 antiserum, we detected UL94 immunoreactivity exclusively in the capsid/tegument fraction (lane 9), indicating that, surprisingly, UL94 protein was not associated with the viral envelope. This result suggests that UL94 protein is a structural component of the capsid or tegument. Since the major structural proteins of the viral capsid have been documented, UL94 is more likely to be associated with the tegument.

Subcellular localization of UL94 protein. To examine the localization of UL94 protein in infected cells, 72-hpi-infected cells were fractionated into nuclear and cytoplasmic/membrane fractions by Nonidet P-40 lysis and probed for UL94

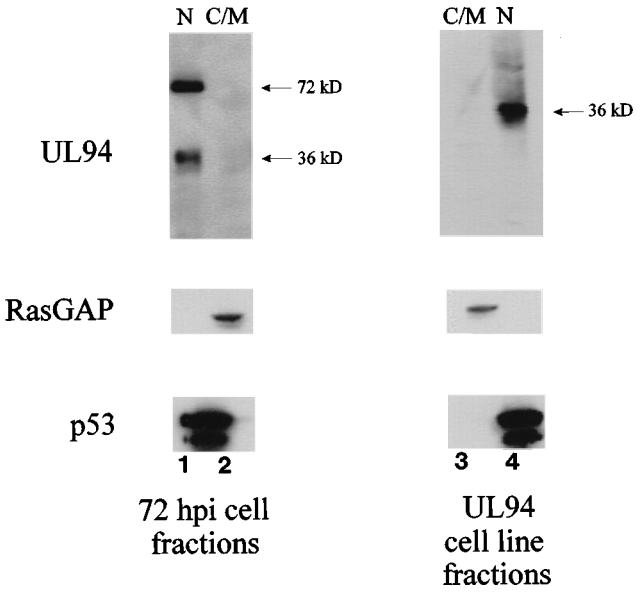

FIG. 6. Western blot analysis of UL94 protein from 72-hpi-infected HEL and 95.15B cell nuclear and membrane/cytoplasmic fractions. The top panel shows Western blot analysis of UL94 from 72-hpi-infected cell nuclear (N; lane 1) and cytoplasmic/membrane (C/M; lane 2) fractions, as well as 94.15B cell cytoplasmic/membrane (lane 3) and nuclear (lane 4) fractions. The bottom panels show the same extracts probed with antisera to cellular proteins Ras-specific GTPase-activating protein (RasGAP) and p53.

reactivity in Western blots. The results (Fig. 6, lanes 1 and 2) demonstrate that UL94 protein is localized to the nuclear fraction of the infected cell, since UL94 immunoreactivity was seen only in the nuclear fraction. Control experiments using either Ras-specific GTPase-activating protein (a membrane associated protein) or p53 (a nuclear protein in HCMV-infected cells and some transformed cell lines) indicated that the fractions were highly enriched (Fig. 6, bottom two panels). Since no UL94 reactivity was seen in the membrane/cytoplasmic fraction, no additional fractionation of infected cells was carried out. We also fractionated UL94-expressing cell lines to determine whether a similar localization would be observed. The results of this experiment (Fig. 6, lanes 3 and 4) indicate that UL94 protein is localized to the nucleus of the UL94 cell lines, as well. Analysis of the UL94 amino acid sequence indicates a potential nuclear localization signal at residues 80 to 84 which could be involved in transport of the protein to the nucleus (Fig. 7).

DISCUSSION

In this report, we identify a novel HCMV virion protein which is conserved in all other human herpesviruses for which sequence information in this region is available. The UL94 ORF was shown to encode a 36-kDa protein which was found in productively infected cells only at late times of infection. In the presence of viral DNA replication inhibitors, UL94-specific message is not transcribed, indicating that UL94 is a true late gene product. This establishes UL94 as one of the few true late HCMV gene products documented to date. Since very little is known about the regulation of true late genes during a productive HCMV infection, an examination of the regulatory mechanisms controlling UL94 expression should provide valuable information concerning the nature of HCMV late gene expression. Such studies are under way in our laboratory.

Using Western blot analysis, we have demonstrated that

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    44-Vzv .........M ELQRIFPLY. .TATGAARKL TPEAVQRLCD A......LTL
Ul16-Hsveb ......MMAA ASDSCLSLWE GSASSPNRQL TPEAVNCLTE A......LTE
  Ul16-Hsv MAQLGPRRPL APPGPPGTLP RPDSRAGARG TRDRVDDLGT DVDSIARIVN
  Bgl2-Ebv .......... ....MASAAN SSREQLRKFL NKECLWVLSD ASTPQMKVYT
  Ul16-Sam .......... ........MD DFRNKLRNFL NNECLWVKNV ACTSFTKVYC
Ul16_Hhv6u .......... .MA.ISTFSI GDLGYLRNFL QNECNWFRIC KKTFY.REYR
  Ul94-Cmv .......... .MAWRSGLCE TDSRTLKQFL QEECMWKLVG KSRKH.REYR

          51                                                 100
    44-Vzv DMGLWKSILT DPRVKIMRST AFITLRIAPF IPLQTDTTNI A.VVVATIYI
Ul16-Hsveb DVAVLRLIRS DPRVKIFMAV SVLTPRLARF APPPPKLTHT AKCAVIMIYL
  Ul16-Hsv SVFVWRVVRA DERLKIFRCL TVLTEPLCQV ALPNPDPGR. ALFCEIFLYL
  Bgl2-Ebv ATTAVSAVY. .VPQIAGPPK TYMNVTLIVL KPKKKPTYVT VYINGTLATV
  Ul16-Sam ATTAVSPFFK PISPQGVPDK HYINVTLIIL KPKKSHPYIT VYINDLAVDC
Ul16_Hhv6u SVATSSPTF. ..SLNNKPKK FCMHCEIVIF K.RSEEFMFS LAVNGIHFGQ
  Ul94-Cmv AVACRSTIF. ..SPEDDSS. .CILCQLLLL Y.RDGEWIIC FCCNGRYQGH

          101                                                150
    44-Vzv TRPRQMNLPP KTFHVIVNFN YEVSYAMTAT LRIYPVENID HVFGATFKNP
Ul16-Hsveb TRPKALALQP KQFHMLVTFN KASVYSLVVR VKTKPFPVGT QRFRAVFQDP
  Ul16-Hsv TRPKALRLPP NTFFALFFFN RERRYCAIVH LRSVTHPLTP LLCTLTFARI
  Bgl2-Ebv ARPEVLFTKA VQGPHSLTLM Y...FGVFSD AVGEAVPVEI RGNPVVTC..
  Ul16-Sam CSTEILQVKP VPCSH.FSLI Y...FGPL.. .IAPPHNVQI PANLSIKASK
Ul16_Hhv6u FLTGKMKFNK KAVPEGLYYY I...LELGS. ITPIDLGFIP RYNSDCVTNM
  Ul94-Cmv YGVNHVHRRR RRICHLPTLY Q...LSFGGP LGPASIDFLP SF.SQVTSSM

          151                                                200
    44-Vzv IAYPLPTSIP DPRADPTPAD LTPTPNLSNY LQPPRLPKNP YACKVISPGV
Ul16-Hsveb EFIGLPSDIP DPAAENIPTE INDRLDVSNF ATPAQPPKDK YDCCVLAPGV
  Ul16-Hsv RAATPPEETP DPTTEQLAEE PVVGELDGAY LVPAKTPPEP GACCALGPGA
  Bgl2-Ebv .TDLTTAHVF TTSTAVKTVE ELQ..... ...DITPSEI IPLGRGGAWY
  Ul16-Sam KSHLTKNQVI FTSKVIHPER LPD..... ...GYKSASL IG...ACAWY
Ul16_Hhv6u RC..VTPEVI YENCSIVCPE EANRLTVKGS GDNKLT.... .PLGGGAWC
  Ul94-Cmv TCDGITPDVI YEVCMLVPQD EAKRILVKGH GAMDLTCQKA VTLGGAGAWL

          201                                                250
    44-Vzv WWSDERRRLY VLAMEPNLIG LCPAGWHARI LGSVLNRLLS HADG....CD
Ul16-Hsveb WWSNANKAIY FLQMDVALLA LCPAGWKARG LGIILGRLLN HQEG....CA
  Ul16-Hsv WWHLPSGQIY CWAMDSDLGS LCPPGSRARH LGWLLARITN HPGG....CE
  Bgl2-Ebv ....AEGALY MFFVNMDMLM CCPNMPTFPS LTHFINLLTR CDNGECVTCY
  Ul16-Sam ....SEGAIF QHFLSTDYMS LCPAFKEFPS LSRILSLLTR CDDLSCVPCY
Ul16_Hhv6u LKNGGDLYIY TFALAYDLFL TCYDKSTFPS LAKIIFDMIA CESEDCVFCK
  Ul94-Cmv LPRPEGYTLF FYILCYDLFT SCGNRCDIPS MTRLMAAATA CGQAGCSFCT

          251                                                300
    44-Vzv ECNHRVHVGA LYALPHVTNH AEGCVCWAEC MWRKAGQREL KVEVDIGATQ
Ul16-Hsveb TCRFTEHSDP LNATADSVAT PESCLCWAEC LWRKAHQREL TVEGDRYLFR
  Ul16-Hsv SCAPPPHIDS ANALWLSSVV TESCPCVAEC LWAKMAQCTL AVQGDASLCP
  Bgl2-Ebv GAGAHVNILR GWTEDDSPGT SGTCPCLLPC ..TALNNDYV PITGHRALLG
  Ul16-Sam GEKIHVNCQS GYTDSDCDGK SNSCPCITSC ..TALKKDIV PITGHRNLLS
Ul16_Hhv6u DHNKHVSQAG QIV..GCVSN QETCPCYTSC ..KKKMAN.I ...NNPELIS
  Ul94-Cmv DHEGHVDPTG NYV..GCTPD MGRCLCYVHC ..GPMTQSLI ...HNEEPAT

          301                                                350
    44-Vzv VLFVDVTTC. IRITS.TKNP RITANLGDVI AGTNASGLS. .VPVNSSGWQ
Ul16-Hsveb VLFMDAVER. VRLTGLRRSP KITANLADLV VGIGPHGQQ. .IPVNNAGWK
  Ul16-Hsv LLFGHPVDT. VTLLQAPRRP CITDRLQEVV GG.RCGADN. .IPPTSAGWR
  Bgl2-Ebv LMFKPEDAPF VVGL.RFNPP KMHPDMSRVL QGVLANGKE. .VPCTAQPWT
  Ul16-Sam LLFDATIQHN ITSI.KFFSP QTPTTVNNVF CGVLDTGET. .VECTCEAWN
Ul16_Hhv6u LLCDQEINK. IDIMYPKIKA SLSLDINSYA HGYFGDDP.. .YALKCVNWI
  Ul94-Cmv FFCESDDAKY LCAVGSKTAA QVTLGDGLDY HIGVKDSEGR WLPVKTDVWD

          351                                          390
    44-Vzv LYMFGETLSR AIINGCGLLQ RICFP.ETQR LSGEPEPTTT
Ul16-Hsveb LVALDADISR LIVCGCYALR YICPPTNSKH QPSSPDEYA.
  Ul16-Hsv LCVFSSYISR LFATSCPTVA RAVARAS... .SSDPE....
  Bgl2-Ebv LLRFSDLYSR AMLYNCQVLK RQVLHSY... ..........
  Ul16-Sam LLMFSDFISR QMIYNCQIMK RFCLRSC... ..........
Ul16_Hhv6u PVRISAALSR LIVLSCPVCK RVVMD..... ..........
  Ul94-Cmv LVKVEEFVSR MIVCSCPVLK NLVH...... ..........
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FIG. 7. PILEUP analysis of UL94 protein sequence and its homologs from varicella-zoster virus (44-Vzv), equine herpesvirus (Ul16-Hsveb), HSV (Ul16-Hsv), Epstein-Barr virus (Bgl2-Ebv), herpesvirus saimiri (Ul16-Sam), human herpesvirus 6 (Ul16_Hhvbu), and human cytomegalovirus (Ul94-Cmv). Conserved features as well as other notable sequences are boxed and are discussed fully in the text.

UL94 protein is associated with HCMV particles and, specifically, with the capsid/tegument fraction of the virion. We had originally anticipated an envelope association for UL94 protein, given its tendency to form what appear to be disulfide-linked dimers in cell extracts from infected cells and when

purified as a histidine-tagged protein from UL94-expressing cell lines. Viral envelope proteins have been previously shown to form disulfide-linked complexes in cell extracts (14). The localization of UL94 protein to the capsid/tegument fraction of the virions as well as its localization to the nucleus of infected and transfected cells, however, make the significance of these disulfide-linked dimers unclear. While it is possible that virion capsid or tegument proteins would be able to exist as disulfide-linked dimers, it is highly unlikely that such an association would exist in the nucleus of the infected cell since the reducing environment of the cell generally precludes formation of such complexes. It is possible that the dimer form of UL94 seen in cell extracts simply represents a nonspecific aggregation of UL94 protein, since the protein is fairly abundant in both infected and transfected cell extracts. Alternatively, it is possible that the native conformation of UL94 protein produces an affinity of UL94 protein for itself and that these associations are normally maintained by another mechanism. In particular, the arrangement of the conserved cysteine residues in the C-terminal half of UL94 is somewhat reminiscent of that seen in zinc-binding proteins. Other intracellular viral proteins have been demonstrated to exist in an active form as zinc-bridged dimers (26), and it should be possible to test experimentally whether UL94 protein has an affinity for zinc binding. In addition, immunohistology of infected cells as well as electron microscopy of virus particles should provide useful information with respect to UL94 protein localization once reagents for such studies become available.

The exact function of the UL94 protein remains to be elucidated. Reports from other laboratories have indicated that the Epstein-Barr virus homolog of UL94 (BGLF2) is produced during a lytic infection, but no virion-association was demonstrated (11). Additionally, the HSV type 1 homolog of UL94 (UL16) has been shown to be dispensable for replication in tissue culture, although as much as a log-fold drop in virus titer can be seen in a cell-type-specific manner in the absence of UL16 protein (4, 5). Assuming a conservation of function among these proteins, this observation would seem to suggest that UL94 is not an essential structural protein, despite its presence in the mature virions. Rather, it may contribute to the maintenance of a cellular environment conducive to viral replication, maturation, or egress that is nonessential in completely permissive cell types. Since UL94 is present in purified virions, it is also likely to be introduced into the infected cell at the earliest stages of viral infection. Within this context, it could potentially play a role in regulation of viral and/or cellular gene expression, as has been demonstrated for HCMV tegument protein pp71 (23, 32), although what that role might be is unclear. In this regard, it might prove interesting to determine whether virion-associated UL94 protein localizes to the nucleus following capsid uncoating.

Conserved features of UL94 and its homologs in other herpesviruses. It was previously reported that the UL94 coding sequences were conserved in all other human herpesviruses (7, 8). To determine similarities in amino acid sequence, the predicted UL94 amino acid sequence was compared with those of its homologs by using the PILEUP program (17). The results indicated that the amino acid similarity is confined primarily to the C-terminal halves of the proteins (Fig. 7). The UL94 protein was most similar to the UL16 protein of betaherpesvirus human herpesvirus 6, while it was least similar to ORF44 of varicella-zoster virus and UL16 of equine herpesvirus. Consistent with the overall similarity among the herpesviruses, Epstein-Barr virus, herpesvirus saimiri, and HSV occupied middle positions with respect to percent homology of the UL94-like proteins. A highly conserved three-residue sequence encompassing UL94 amino acids 175 to 177 (GAW) demarcates the N-terminal boundary of the highly conserved region (boxed in Fig. 7). Within this region there are also seven conserved cysteine residues as well as two conserved histidine residues (boxed in Fig. 7). The position of these residues is reminiscent of that seen in zinc finger or zinc ring proteins, suggesting a possible zinc and/or nucleic acid binding function for UL94. In addition, the UL16 proteins of equine herpesvirus and HSV type 1 as well as ORF44 of varicella-zoster virus contain a 5- to 10-residue C-terminal extension which is serine-threonine rich, suggesting a possible region of phosphorylation in these proteins. This region is not present in the other UL94 homologs; however, a putative phosphorylation site of sequence aliphatic-S-R is found at UL94 residues 311 to 313 and is 100% conserved. The putative UL94 nuclear localization signal at amino acids 80 to 84 (RRRRR) is also highlighted in Fig. 7. The exact significance of this sequence is unclear since it is not well conserved in the other UL94 homologs. It was previously reported (11) that the Epstein-Barr virus BGLF2 protein contained a putative nuclear localization signal at residues 65 to 69 (KPKKK). As with the putative UL94 nuclear localization signal, however, this sequence is not well conserved. Since both of these sequences are within the less conserved region of these proteins, it is possible that different mechanisms have evolved for signaling nuclear localization of these proteins. Additional characterization of UL94 and its homologs, however, is needed to support this conclusion.

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