Characterization of the Myristylated Polypeptide Encoded by the UL1 Gene That Is Conserved in the Genome of Defective Interfering Particles of Equine Herpesvirus 1†

RONALD N. HARTY,^{1,‡} GRETCHEN B. CAUGHMAN,² V. ROGER HOLDEN,¹
AND DENNIS J. O'CALLAGHAN^{1*}

Department of Microbiology and Immunology, Louisiana State University Medical Center, 1501 Kings Highway, P.O. Box 33932, Shreveport, Louisiana 71130-3932,¹ and Department of Oral Biology/Microbiology, School of Dentistry, Medical College of Georgia, Augusta, Georgia 30912-1126²

Received 25 January 1993/Accepted 19 April 1993

Equine herpesvirus 1 (EHV-1, Kentucky A strain) preparations enriched for defective interfering particles (DIPs) can readily establish persistent infection. The UL1 gene, which is conserved in the genome of DIPs that mediate persistent infection, maps between nucleotides 1418 and 2192 (258 amino acids) from the L (long) terminus. UL1 has no homology with any known gene encoded by herpes simplex virus type 1 but has limited homology to open reading frame 2 of varicella-zoster virus and the "circ" gene of bovine herpesvirus type 1. Previous work showed that the EHV-1 UL1 gene belongs to the early kinetic class and is transcribed as a 1.2-kb polyadenylated mRNA (R. N. Harty, R. R. Yalamanchili, and D. J. O'Callaghan, *Virology* 183:830-833, 1991). In this report, the UL1 protein was identified and characterized as a 33-kDa polypeptide in EHV-1-infected cells by using rabbit polyclonal antiserum raised against a TrpE-UL1 fusion protein (amino acids 7 to 258 of UL1) synthesized in *Escherichia coli*. Results from Western blot (immunoblot), immunoprecipitation, indirect immunofluorescence, and biochemical analyses indicated that the UL1 polypeptide (i) is more abundant in cells infected with DIP-enriched virus than in cells infected with standard EHV-1, (ii) is synthesized as early as 3 h postinfection (p.i.) in infection with standard virus or in infection with DIP-enriched virus preparations and increases in abundance up to 12 h p.i., (iii) appears to be associated with the rough endoplasmic reticulum-Golgi apparatus early in infection (3 to 4 h p.i.), while a diffuse cytoplasmic pattern of fluorescence is observed late in infection (7 to 8 h p.i.), (iv) is modified by myristic acid as it contains a consensus N-terminal myristylation site and is readily labeled with [³H]myristic acid, and (v) is associated with mature EHV-1 virions.

The standard genome of equine herpesvirus (EHV-1, Kentucky A strain) is a linear double-stranded DNA molecule which is approximately 144 kbp in length (1, 2, 22, 31, 39, 48). The genome is composed of unique sequences (UL, unique long; US, unique short) as well as inverted repeat (IR) sequences that bracket the US segment, thus allowing the formation of two isomeric forms of the genome. As observed for other alphaherpesviruses, EHV-1 genes are temporally regulated into immediate-early, early, and late kinetic classes (4, 5, 13, 14).

A standard EHV-1 cytolytic infection is characterized by rapid lysis of infected cells; however, infection of cells with EHV-1 preparations enriched for defective interfering particles (DIPs) results in persistent infection in which standard EHV-1 and DIPs are released continuously from infected cells (8, 17, 32, 38). Since EHV-1 DIP have been implicated in mediating persistence, identification and characterization of viral proteins encoded by EHV-1 genes maintained within the DIP genome may provide a better understanding of the molecular events involved in establishing and maintaining persistent EHV-1 infection.

Recently, the UL2 gene of EHV-1 (a homolog of UL55 of herpes simplex virus type 1 [HSV-1]), which is conserved in the DIP genome, was shown to direct the synthesis of a 0.9-kb mRNA and a 23-kDa polypeptide that are overexpressed in DIP-enriched infection (18). In addition to UL2 and sequences that contain the origin of replication and the cleavage-packaging elements (50), other sequences conserved in the DIP genome were shown to generate a hybrid gene composed of sequences that encode the N-terminal portion of the IR4 protein (ICP22 homolog [23]) joined to sequences encoding the C-terminal portion of the UL3 protein (ICP27 homolog [53]). This hybrid IR4-UL3 gene is conserved in all clones of the DIP DNA analyzed to date (51) and has been shown to be expressed in DIP-enriched infection (16).

Restriction endonuclease, Southern blot, and partial DNA sequencing analyses of DIP DNA revealed that the UL1 gene of EHV-1 is also conserved in the genome of DIP (2, 16, 52). Interestingly, the UL1 gene of EHV-1 (Kentucky A strain) exhibits no homology to genes encoded by HSV-1; however, UL1 does exhibit homology to gene 3 of the Ab4 strain of EHV-1 (46), open reading frame 2 (ORF2) of varicella-zoster virus (9), and the "circ" gene of bovine herpesvirus 1 (BHV-1) (11, 41). The gene products encoded by these homologs of UL1 have yet to be identified and characterized. Previously, the UL1 gene was shown to be transcribed as a 1.2-kb polyadenylated mRNA at early stages of infection (20). Northern (RNA) blot and S1 nuclease analyses were utilized to map the UL1 transcript (20),

* Corresponding author.

† D.J.O. dedicates this paper to his mentor and colleague, Charles C. Randall, on the occasion of his 80th birthday and 53rd year of continuous and active research.

‡ Present address: Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029-6574.

and in vitro transcription-translation and hybrid-arrest translation analyses were employed to identify the UL1 polypeptide synthesized from a cloned copy of the UL1 gene (19).

The studies described in this report have extended our characterization of the UL1 protein synthesized in cells infected with standard EHV-1- or DIP-enriched EHV-1. The data revealed that the UL1 polypeptide (i) has a molecular size of approximately 33 kDa and is synthesized as early as 3 h postinfection (p.i.), (ii) is modified by myristic acid (a saturated tetradecanoic fatty acid), (iii) is stable and does not rapidly turn over during infection, (iv) appears to be associated with cellular membranes, (v) is expressed more abundantly in cells infected with DIP-enriched virus than in cells infected with standard EHV-1, and (vi) is a component of mature EHV-1 virions.

To our knowledge, this is the first report to identify a myristylated polypeptide encoded by EHV-1. However, myristylated proteins have been identified and characterized for many RNA- and DNA-containing viruses (for a review, see reference 47), including the gene product encoded by UL1 of HSV-1 (26, 28). These initial studies to characterize the myristylated UL1 polypeptide may provide for a better understanding of its function and potential role in persistent EHV-1 infection.

MATERIALS AND METHODS

Virus and cell culture. The Kentucky A strain of EHV-1 was propagated in murine L-M cells and assayed by plaque titration as described previously (33, 34). The generation of DIPs of EHV-1 and the properties of cell lines persistently infected with EHV-1 have been described previously (2, 8, 21, 22, 32). DIP-enriched EHV-1 preparations employed in these experiments include DIP passage 63 (3.9×10^7 PFU/ml), DIP passage 64 (1.9×10^6 PFU/ml), and DIP passage 69 (9.4×10^7 PFU/ml). Standard EHV-1 and DIP-enriched EHV-1 preparations were propagated in murine L-M cells and hamster embryo (HE) fibroblasts (33).

Generation of viral DNA clones. Plasmid pSVL1 was constructed by digesting the pGEML1 construct (described elsewhere [19]) with *AvrII* (nucleotide [nt] 1367) and *EcoRI* (nt 2513) and ligating this 1,146-bp fragment into an *XbaI-EcoRI*-digested pSV12 vector (43). Thus, plasmid pSVL1 contains the entire UL1 ORF cloned downstream of the simian virus 40 (SV40) promoter-enhancer. Plasmid pATH23L1 was constructed by digesting the pGEML1 construct (19) with *XbaI* (nt 1440) and *EcoRI* (nt 2513) and ligating this 1,073-bp fragment into an *XbaI-EcoRI*-digested pATH23 vector (24). Thus, the pATH23L1 construct contains a TrpE-UL1 fusion ORF inserted downstream of the inducible TrpE promoter. The UL1 ORF within plasmid pATH23L1 begins with codon 7 and ends with codon 258 (total number of UL1 codons, 252). All clones were generated by using standard protocols (27) and were screened by restriction endonuclease analyses. Plasmid pSVL1 was maintained in *Escherichia coli* DH5 α , and plasmid pATH23L1 was maintained in *E. coli* TB1. Plasmid DNA was prepared by using the Qiagen (Chatsworth, Calif.) plasmid isolation kit.

Isolation of the TrpE-UL1 fusion protein. The pATH23L1 construct was transformed into TB1 cells, and the TrpE-UL1 fusion protein was purified and isolated by using the protocol of Koerner et al. (24) as described in detail elsewhere (18, 29). The TrpE-UL1 fusion protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with

Coomassie brilliant blue (0.25%), and the appropriate TrpE-UL1 protein band was sliced from the gel.

Preparation of TrpE-UL1 antiserum. The gel slice containing the TrpE-UL1 fusion protein was mixed with an equal volume of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, Mo.), and 2.0 ml of the mixture was used to immunize two male New Zealand White rabbits (0.5 ml per hind leg). Preimmune serum was collected from both rabbits before the administration of the antigen. The administration of booster injections and the isolation of the TrpE-UL1 antiserum were done as described by Harlow and Lane (15).

Transfection of COS-1 cells with pSVL1. COS-1 cells were seeded onto 60-mm-diameter dishes or coverslips and transfected by the lipofectin (Bethesda Research Laboratories, Gaithersburg, Md.) method as described in detail elsewhere (43). Following 48 h of incubation at 37°C, the transfected cells in 60-mm-diameter dishes were lysed with 0.5 ml of radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1.0% SDS, 0.5% deoxycholate, 1.0% Nonidet P-40) containing protease inhibitors (aprotinin, 50 μ g/ml; leupeptin, 50 μ g/ml, and phenylmethylsulfonyl fluoride, 300 μ g/ml; Sigma Chemical Co.). Transfected cells on coverslips were fixed for immunofluorescence assays as described below. Cell extracts were stored at -70°C until use.

Western blot and SDS-PAGE analyses. The protocols utilized for Western blot (immunoblot) and SDS-PAGE analyses have been described in detail elsewhere (18, 19, 43). The specific dilution of the antiserum used in each experiment is indicated in the appropriate figure legends.

Infection of cells and indirect immunofluorescence. Semi-confluent monolayers of HE cells grown on coverslips (25 by 75 mm) were mock infected or infected with standard EHV-1 or DIP-enriched EHV-1 preparations (multiplicity of infection [MOI], 10 PFU per cell) in the presence or absence of 200 μ g of phosphonoacetic acid (PAA) per ml and maintained at 37°C for times ranging from 1 to 7 h p.i. Coverslips were rinsed in phosphate-buffered saline (PBS), fixed in ice-cold acetone (5 min at -20°C), air dried, and stored at -20°C until use. Semiconfluent EHV-1 persistently infected DI-3 cell monolayers (passage 83) were incubated for 24 h with no PAA treatment and fixed in the same manner. Coverslips were rehydrated for 10 min in PBS and incubated in a humid atmosphere for 30 min with normal goat serum (1:10 dilution in PBS) to block nonspecific antibody binding. Coverslips were washed with PBS and incubated for 45 min with preimmune normal rabbit serum or TrpE-UL1 antiserum (1:1,000 dilution in PBS containing 1.0% bovine serum albumin). The coverslips were washed extensively in PBS, incubated for 45 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Grand Island Biological Co.), and washed again in PBS. All incubations were performed at room temperature. The coverslips were mounted on glass slides in 50% glycerol in PBS and examined for fluorescence with a Nikon Optiphot microscope equipped with an episcopic-fluorescence attachment and a B-1A filter block.

Immunoprecipitation analyses of infected cell extracts. Mock- or EHV-1-infected L-M cells (2×10^6 cells per flask) were harvested in radioimmunoprecipitation assay buffer with protease inhibitors as stated above. Cells were radiolabeled with either [35 S]methionine (50 μ Ci/ml; New England Nuclear, Boston, Mass.) or [3 H]myristic acid (20 μ Ci/ml; New England Nuclear). Cell extracts were stored at -70°C until use. The protocol used for immunoprecipitation has been described in detail elsewhere (18). 14 C-labeled molec-

FIG. 1. Diagram of the EHV-1 genome (top) with nt 1 to 3000 from the L terminus expanded below. The arrows represent the UL1 and UL2 ORFs of 258 and 200 amino acids (aa), respectively. The UL1 ORF begins at nt 1418 and ends at nt 2192, while the UL2 ORF begins at nt 2825 and ends at nt 2225. The viral insert within plasmid pSVL1 maps from the *AvrII* site (nt 1367) to the *BglIII* site (nt 2513). Note that the *BglIII* site in the original clone (p1-111) from which pSVL1 and pATH23L1 were derived was converted to an *EcoRI* site upon cloning. The viral insert within plasmid pATH23L1 maps from the *XbaI* site (nt 1440) to the *EcoRI* site (nt 2513). The first six N-terminal codons of the UL1 ORF are deleted in the pATH23L1 construct. Abbreviations: UL, unique long; IR, inverted repeat; US, unique short; TR, terminal repeat.

ular weight standards (Bethesda Research Laboratories) were utilized to determine molecular size.

Pulse-chase analysis. Pulse-chase analysis was performed as described previously (18). Briefly, L-M cell monolayers (2×10^6 cells) were infected with DIP passage 64 at an MOI of 2.0 PFU per cell. The cells were pulse labeled with [35 S]methionine (50 μ Ci/ml; New England Nuclear) for 30 min at 5 h p.i. and chased with a 100,000-fold excess of cold methionine (30 mg/liter; Sigma Chemical Co.) for the times indicated. Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.), and equal amounts of protein were immunoprecipitated and fractionated by SDS-PAGE.

Purification of EHV-1 virions. EHV-1 virions were purified by polyethylene glycol precipitation and rate-velocity centrifugation in dextran-10 gradients as described previously (34). To remove the virion envelope, the appropriate amount of purified virions (collected by centrifugation at 50,000 rpm for 30 min with a TLA 100.3 rotor in an Optima TL ultracentrifuge) was suspended in sterile PBS containing 1.0% Nonidet P-40 and 1.0% deoxycholate and incubated for 1 h at 37°C. The disrupted virions were centrifuged at 27,000 rpm for 30 min with the TLA 100.3 rotor, and the supernatant represented the envelope fraction, while the pellet represented the nucleocapsid-tegment fraction. Proteins present in each fraction were fractionated by SDS-PAGE and analyzed by Western blot hybridization.

RESULTS

Generation of TrpE-UL1 polyclonal antiserum. To characterize the UL1 polypeptide synthesized during EHV-1 infection, polyclonal antiserum was raised against a TrpE-UL1 fusion protein. The UL1 ORF (Fig. 1) was cloned previously into the transcription expression vector pGEM-3Z to generate plasmid pGEML1 (19). A portion of the UL1 ORF (codons 7 through 258) was excised from pGEML1 as an

XbaI-EcoRI fragment and ligated into an *XbaI-EcoRI*-digested pATH23 vector containing the bacterial TrpE gene and its inducible promoter (Fig. 1). The resultant construct, pATH23L1 (Fig. 2A), contains the TrpE-UL1 fusion ORF under the control of the inducible TrpE promoter.

E. coli TB1 cells were transformed with the pATH23L1 construct, and the TrpE-UL1 fusion protein was identified by fractionating the proteins on an SDS-polyacrylamide gel followed by staining with Coomassie blue (Fig. 2B). Cell extracts from TB1 cells (Fig. 2B, lane 2), TB1 cells transformed with the pATH23 vector and grown under inducing conditions (lane 3), and TB1 cells transformed with pATH23L1 and grown under noninducing (lane 4) or inducing (lane 5) conditions were fractionated by SDS-PAGE. The 37-kDa TrpE protein synthesized from the pATH23 vector is clearly visible in extracts of TB1 cells transformed with pATH23 (lane 3), demonstrating that the TrpE promoter is functional and inducible. A protein with the approximate size expected for the TrpE-UL1 fusion protein was observed in extracts of TB1 cells transformed with pATH23L1 and grown under inducing conditions (lane 5) but was not observed in extracts of either TB1 cells (lane 2) or TB1 cells transformed with the pATH23 vector alone (lane 3). The abundance and level of expression of the TrpE-UL1 protein were relatively low in extracts of pATH23L1-transformed cells grown under inducing conditions (lane 5). However, this level of expression was not surprising, since proteins encoded by large ORFs inserted into pATH23, such as the large UL1 insert of 252 codons, are not expressed from the pATH vectors as efficiently as are proteins encoded by smaller ORFs (24).

Specificity of the TrpE-UL1 antiserum. To demonstrate that the TrpE-UL1 antiserum was specific for the UL1 polypeptide, a UL1 expression vector was employed in indirect immunofluorescence and Western blot analyses. The entire UL1 ORF was inserted downstream of the SV40

FIG. 2. Expression of a TrpE-UL1 fusion protein from the pATH23L1 construct. (A) Diagram of the pATH23L1 plasmid (4.8 kb) containing the TrpE-UL1 fusion gene. The UL1 ORF in this construct begins with codon 7 and ends with codon 258 (252 codons). Expression of the TrpE-UL1 fusion protein is driven by the inducible TrpE promoter. Abbreviations: ORI, origin of replication; Ap, ampicillin resistance gene. (B) SDS-PAGE analysis of Coomassie blue-stained proteins from extracts of TB1 cells (lane 2), TB1 cells transformed with pATH23L1 grown under inducing (*) conditions (lane 3), TB1 cells transformed with pATH23L1 grown under noninducing conditions (lane 4), and TB1 cells transformed with pATH23L1 grown under inducing (*) conditions (lane 5). The positions of the TrpE-UL1 fusion protein (approximately 58 kDa; lane 5) and the TrpE protein (approximately 37 kDa; lane 3) are indicated. Molecular weight standards (M, lanes 1 and 6), in thousands, are indicated on the left.

promoter-enhancer region present in vector pSV12 to generate plasmid pSVL1 (Fig. 1 and 3A). Plasmid pSVL1 was transfected into COS-1 cells by using the lipofectin method (43). Indirect immunofluorescence analyses demonstrated that the TrpE-UL1 antiserum did not react with COS-1 cells transfected with the pSV12 vector alone (Fig. 3B, top) but did react strongly with cells transfected with pSVL1 (Fig. 3B, bottom). As further evidence of the specificity of the TrpE-UL1 antiserum, Western blot analysis was performed with extracts of COS-1 cells transfected with pSV12 (Fig. 3C, lane 1) or with 5 (lane 2), 10 (lane 3), and 15 μ g (lane 4) of pSVL1. The 33-kDa UL1 polypeptide was detected in extracts of pSVL1-transfected COS-1 cells (lane 2 to 4) but not in pSV12-transfected COS-1 cells (lane 1). To confirm that the 33-kDa protein was synthesized from UL1-coding sequences, pSVL1 was first linearized by digestion with either *Kpn*I (which cleaves within the UL1 coding sequences) or *Eco*RI (which cleaves within the vector sequences) and then transfected into COS-1 cells. As expected, the 33-kDa UL1 protein was not observed in COS-1 cell extracts receiving the *Kpn*I-digested pSVL1 (Fig. 3C, lane 5) but was observed in extracts receiving the *Eco*RI-digested pSVL1 (lane 6). These results and the finding (data not shown) that the UL1 antiserum also reacted with the product of an in vitro-transcribed and -translated pGEM construct containing UL1 sequences (pGEMUL1 [19]) indicated that the TrpE-UL1 antiserum could recognize the EHVS-1 UL1 polypeptide.

The TrpE-UL1 antiserum reacts with the UL1 protein synthesized in EHVS-1-infected cells. To detect the UL1 polypeptide synthesized in vivo, murine L-M cells were mock infected or infected with standard EHVS-1 or DIP-

passage-69-enriched EHVS-1. Western blot analysis of these infected cell extracts (harvested at 8 h p.i.) revealed that the 33-kDa UL1 polypeptide was present in extracts of cells infected with standard EHVS-1 (Fig. 4A, lane 2) and with DIP-enriched virus (lane 1) but not in extracts of mock-infected cells (lane 3). The intensity of the signal in the cell extract infected with DIP-passage-69-enriched EHVS-1 69 (lane 1) was slightly higher than that observed in cells infected with standard EHVS-1 (lane 2).

Immunoprecipitation analysis was also utilized to detect the UL1 protein in EHVS-1-infected cells. L-M cells were infected with standard EHVS-1 in the presence of [35 S]methionine, and the cell extracts were harvested at 8 h p.i. Equal amounts of the extracts were incubated with either preimmune serum (Fig. 4B, lane 1) or the TrpE-UL1 antiserum (lane 2), and the immunoprecipitated proteins were fractionated by SDS-PAGE. The 33-kDa UL1 protein was immunoprecipitated readily with the TrpE-UL1 antiserum (lane 2), but not with preimmune serum (lane 1).

The UL1 protein is myristylated. Computer analysis of the UL1 amino acid sequence revealed a consensus myristylation site (47) at the N terminus of the protein (Fig. 5A). The myristic acid moiety is linked to proteins containing the consensus site via an amide bond to a glycine residue immediately following the initial methionine. The third amino acid position may be occupied by any of the amino acids indicated, while positions 4 and 5 may be filled by any amino acid. Amino acid position 6 can be occupied by one of the six amino acids shown, however a serine residue is preferred. Lastly, amino acid position 7 cannot be occupied by a proline residue. The first seven amino acids are illustrated for the UL1 protein (homolog of ORF3 of EHVS-1

FIG. 3. Expression of the UL1 polypeptide from an SV40 promoter. (A) Diagram of the pSVL1 construct (3.7 kb) depicting the complete UL1 ORF downstream of the SV40 promoter-enhancer element. The *AvrII-EcoRI* fragment (see Fig. 1) containing the UL1 ORF was cloned into the pSV12 vector digested with *XbaI-EcoRI*. Ap, ampicillin resistance gene. (B) Indirect immunofluorescence analyses of COS-1 cells transfected with either the pSV12 vector (top) or pSVL1 (bottom) and reacted with TrpE-UL1 antiserum (1:1,000). (C) Western blot analysis of extracts of COS-1 cells transfected with pSV12 (lane 1), 5.0 µg of pSVL1 (lane 2), 10.0 µg of pSVL1 (lane 3), 15.0 µg of pSVL1 (lane 4), 10.0 µg of pSVL1 linearized with *KpnI* (lane 5), and 10.0 µg of pSVL1 linearized with *EcoRI* (lane 6). The blot was reacted with TrpE-UL1 antiserum at a 1:7,500 dilution.

Ab4), the circ gene product of BHV-1, and the UL11 gene product of HSV-1. The UL11 gene product is not a homolog of UL1 of EHV-1 but does represent a known myristylated herpesvirus protein (26). The circ gene product of BHV-1 is a homolog of UL1 of EHV-1 (11, 41), and although it possesses a consensus myristylation site, the circ gene product has not yet been shown to be myristylated. To demonstrate whether the UL1 protein is modified by myristic acid, L-M cells were mock infected or infected with standard or DIP-enriched EHV-1 in the presence of 20 µCi of [³H]myristic acid per ml. The cell extracts were harvested at 8 h p.i., and equal amounts of the extracts were incubated with the TrpE-UL1 antiserum. Following fractionation of

FIG. 4. Expression of the UL1 polypeptide in standard EHV-1 and EHV-1 DIP infections. (A) Western blot analysis of extracts harvested at 8 h p.i. from cells infected with either DIP-passage-69-enriched (lane 1) or standard (lane 2) EHV-1- or mock-infected cells (lane 3). The blot was reacted with TrpE-UL1 antiserum at a dilution of 1:10,000. (B) Immunoprecipitation of [³⁵S]methionine-labeled extracts (4×10^5 cell equivalents) from L-M cells infected with standard EHV-1 (8 h p.i.). Proteins were immunoprecipitated with either preimmune serum (15 µl, lane 1) or TrpE-UL1 antiserum (15 µl, lane 2). The position of the 33-kDa band is indicated in each panel.

the immunoprecipitated proteins by SDS-PAGE, a radiolabeled 33-kDa protein was detected in extracts of cells infected with standard EHV-1 (Fig. 5B, lane 3) and DIP-passage-64 (lane 4)- and -69 (lane 5)-enriched EHV-1 following an overnight exposure of the gel. The myristylated 33-kDa protein was not detected in mock-infected cell extracts (lane 2). This posttranslational modification may explain why the UL1 protein synthesized *in vivo* migrates at approximately 33 kDa, although its computer-predicted molecular size is 28,343 Da.

Intracellular localization of the UL1 protein in standard and DIP-enriched EHV-1 infections. The UL1 gene product was first detected at 3 h p.i. in HE cells infected with standard EHV-1 as a polarized, perinuclear region of reactivity (Fig. 6A). The fluorescence appeared to delineate a multilayered structure, consistent with the idea that the UL1 protein was localized to the Golgi apparatus (Fig. 6A). Subsequently, the majority of reactivity was associated with structures which appeared to be intracytoplasmic vesicles (Fig. 6B and C). At 7 h p.i., the UL1 reactivity was intense and evenly distributed throughout the cell such that little intracellular detail was visible. This pattern of reactivity is consistent with the UL1 protein being localized in or near the cytoplasmic membrane. In DIP-enriched EHV-1-infected HE cells, the fluorescence was more intense, and the time course of the intracellular distribution of the UL1 protein was more rapid than that in standard EHV-1-infected HE cells (Fig. 7C and D). By 4 h p.i., extensive, intense cytoplasmic reactivity was observed in DIP-enriched EHV-1-infected cells. This generalized cytoplasmic pattern of reactivity was similar to that observed in standard EHV-1-infected cells at 7 h p.i. and to

FIG. 5. Myristylation of the UL1 polypeptide. (A) The consensus amino acid sequence (single-letter code) necessary for myristylation of polypeptides is shown. The underlined G (glycine) represents the amino acid to which the myristic acid is bonded via an amide linkage. Amino acids within parentheses represent those that are allowed at that particular position, while only proline (in braces) is not allowed at position 7. The S (serine) residue is underlined to indicate that it is the preferred amino acid at this position. The letter X represents any amino acid. The seven N-terminal amino acids of the UL1 protein (EHV-1 Kentucky A strain)-ORF3 (EHV-1 strain Ab4), the circ protein of BHV-1, and the UL11 gene product of HSV-1 are also shown. (B) Immunoprecipitation of [³H]myristic acid-labeled cell extracts. Cell extracts (4×10^5 cell equivalents) from cells that were mock infected (m, lane 2) or infected with standard (STD, lane 3) EHV-1 or DIP-passed-64 (DI 64, lane 4) DIP-passed-69 (DI 69, lane 5)-enriched EHV-1 were incubated with the TrpE-UL1 antiserum (15 μ l), and the immunoprecipitated proteins were fractionated by SDS-PAGE. Molecular weight standards (MW, lane 1) (in thousands) are indicated on the left; the position of the 33-kDa band is indicated on the right.

that observed in persistently infected DI-3 cells (Fig. 7E). It should be noted that the presence of PAA throughout infection did not influence the synthesis of the UL1 protein or affect the levels detected during infection (Fig. 7F). Also, mock-infected HE cells incubated with the TrpE-UL1 antiserum and EHV-1-infected cells incubated with preimmune serum did not yield any level of fluorescence above background (Fig. 7A and B, respectively).

Time course and stability of the UL1 polypeptide. Western blot analysis was utilized to follow the synthesis of the UL1 polypeptide at various times p.i. Murine L-M cells were infected with DIP-passed-64-enriched EHV-1 (MOI, 2.0 PFU per cell), and cell extracts were harvested at 2, 4, 6, 8, 10, and 12 h p.i. (Fig. 8A). The 33-kDa UL1 protein was readily detected at 6 h p.i. (Fig. 8A, lane 3) and continued to increase in abundance up to 12 h p.i. (lane 6). The level of sensitivity of this experiment was not sufficient to detect the UL1 protein at 4 h p.i., although it was shown to be present at early times of infection by immunofluorescence assays.

The stability of the UL1 polypeptide was analyzed by pulse-chase analysis (Fig. 8B). Murine L-M cells infected with DIP passage 64 (MOI, 2.0 PFU per cell) were pulse-labeled for 30 min with [³⁵S]methionine at 5 h p.i. and then

chased with cold methionine (100,000-fold excess) for the times indicated (Fig. 8B). Cell extracts containing equal amounts of protein were incubated with either the TrpE-UL1 antiserum or preimmune serum, and the immunoprecipitated proteins were analyzed by SDS-PAGE. The 33-kDa UL1 protein was immunoprecipitated throughout the chase period of 48 h (Fig. 8B, lanes 3 to 7). Although a slight decrease in the abundance of the UL1 protein was observed as the time of chase increased (compare lanes 5 and 7), these results suggest that the UL1 protein is not rapidly degraded and does not undergo extensive posttranslational processing. The UL1 protein was not detected in mock-infected cell extracts (lane 2) or with preimmune serum (lane 8).

The UL1 protein in purified EHV-1 virions. Since the UL1 protein increased in abundance late in infection and since many myristylated viral proteins are also virion structural proteins (for a review, see reference 47), mature EHV-1 virions were assayed by Western blot analyses for the presence of the UL1 polypeptide. Purified EHV-1 virions were solubilized in 1 $\times$ Laemmli sample buffer (25) and fractionated by SDS-PAGE (Fig. 9A). The blotted virion proteins were reacted with either preimmune serum (lane 1) or the TrpE-UL1 antiserum (lane 2) at a dilution of 1:15,000.

FIG. 6. Detection of the UL1 protein in standard EHV-1-infected HE cells. EHV-1-infected cells (MOI of 10 PFU/ml) were fixed at 3 (A), 4 (B), 6 (C), and 7 (D) h p.i. and reacted with TrpE-UL1 antiserum and FITC-goat anti-rabbit immunoglobulin G as described in Materials and Methods.

Indeed, a protein of approximately 33 kDa was detected by using the TrpE-UL1 antiserum (lane 2) but was not detected with preimmune serum (lane 1). An additional protein migrating at approximately 62 kDa also observed with the TrpE-UL1 antiserum (lane 2) may represent a cross-reactive virion protein or an aggregate (dimer) of the UL1 polypeptide.

To localize further the UL1 polypeptide in mature virions, purified virus was incubated at 37°C in a solution of PBS containing 1.0% Nonidet P-40 and 1.0% deoxycholate to separate the viral envelope from the nucleocapsid-tegument regions. The viral proteins in the envelope fraction (supernatant) and those in the nucleocapsid-tegument fraction (pellet) were fractionated by SDS-PAGE and analyzed by Western blot analysis (Fig. 9B). As a negative control, both the supernatant and pellet fractions were reacted with preimmune serum (lanes 1 and 2). As a positive control, both the supernatant and pellet fractions were reacted with a peptide-specific antiserum to EHV-1 glycoprotein D (lanes 5 and 6) (10). As expected, the majority of glycoprotein D (55 kDa) was detected in the supernatant or envelope fraction (lane 6), while only a small amount was observed in the pellet fraction (lane 5). In contrast, essentially all of the UL1 protein was observed in the pellet (nucleocapsid-tegument) fraction (lane 3), as no UL1 protein was detected in the supernatant (envelope) fraction (lane 4).

Since the UL1 protein was present in mature virions,

neutralization assays were performed to determine whether the TrpE-UL1 antiserum could block EHV-1 infection of murine L-M cells. The results of these studies indicated that the TrpE-UL1 antiserum was unable to neutralize the virus and prevent virus infection (data not shown). These data suggest that the UL1 protein is a component of the mature EHV-1 virion and that UL1 does not appear to be localized within the viral envelope as is glycoprotein D.

The TrpE-UL1 antiserum cross-reacts with a BHV-1 virion protein. Since the UL1 gene exhibits homology, albeit limited homology, to the recently identified circ gene of BHV-1 (11, 41), purified BHV-1 virions were assayed by Western blot analyses for BHV-1 proteins that react with the TrpE-UL1 antiserum. Various amounts (0, 1, and 2 μ l) of purified BHV-1 virions and a fixed amount of purified EHV-1 virions (10 μ l) were fractionated by SDS-PAGE, and the blotted virion proteins were reacted with either preimmune serum (Fig. 10A, lanes 1 to 4) or the TrpE-UL1 antiserum (Fig. 10B, lanes 5 to 8). Both EHV-1 and BHV-1 virion proteins that were incubated with preimmune serum yielded no signal (Fig. 10A, lanes 1 to 4), whereas the TrpE-UL1 antiserum recognized the 33-kDa EHV-1 UL1 protein (Fig. 10B, lane 5) as well as a 35-kDa BHV-1 virion protein (Fig. 10B, lanes 6 and 7). Whether this 35-kDa BHV-1 virion protein represents the product of the BHV-1 circ gene remains to be determined, since the circ protein has yet to be identified and characterized.

FIG. 7. Indirect immunofluorescence showing the distribution of the UL1 protein in standard EHV-1-infected HE cells in the presence or absence of 200 μ g of PAA per ml, DIP-enriched EHV-1-infected HE cells, and persistently infected DI-3 cells. (A) Mock-infected HE cells reacted with TrpE-UL1 antiserum; (B) DI-3 cells (passage 83) reacted with preimmune rabbit serum; (C) standard EHV-1-infected HE cells (4 h p.i.) reacted with TrpE-UL1 antiserum; (D) DIP-enriched EHV-1-infected HE cells (4 h p.i.) reacted with TrpE-UL1 antiserum; (E) DI-3 cells (passage 83) reacted with the TrpE-UL1 antiserum; (F) PAA-treated standard EHV-1-infected HE cells (6 h p.i.) reacted with TrpE-UL1 antiserum.

gDISCUSSION

EHV-1 genes conserved in the genome of DIPs are of interest, since their gene products may function in persistent EHV-1 infection. The UL1 gene of EHV-1 is included within sequences at the L terminus that are conserved in the DIP genome (1, 2, 49, 50). In this report, the polypeptide encoded by the UL1 gene has been identified and characterized with regard to its time of synthesis, stability, intracellular localization, and posttranslational modification.

UL1-specific antiserum was used to identify the 33-kDa UL1 protein in cells infected with standard EHV-1 or with DIP-enriched preparations of EHV-1. The UL1 protein synthesized in cells infected with various passages of DIP-

enriched preparations of EHV-1 was more abundant than that synthesized in cells infected with standard EHV-1 as judged by Western blot, immunoprecipitation, and indirect immunofluorescence analyses. These observations parallel those observed for the UL2 protein, which is also overexpressed in DIP-enriched infection as the UL2 gene is also conserved in the DIP genome (18). It should be noted that cloned copies of both the UL1 and UL2 genes were utilized in transient cotransfection experiments to determine whether the UL1 or UL2 protein, in conjunction with the EHV-1 immediate-early protein, could regulate expression of chloramphenicol acetyltransferase (CAT) from various EHV-1 promoter-CAT constructs (44). The results from

FIG. 8. Time course and pulse-chase analyses of UL1 polypeptide in L-M cells infected with DIP-passage-64-enriched EHV-1. Protein concentrations were determined by using the bicinchoninic acid protein assay reagent (Pierce), and equal amounts of protein were analyzed in each lane. (A) DIP-passage-64-enriched EHV-1 infected cell extracts were harvested at 2, 4, 6, 8, 10, and 12 h p.i., and the filter containing the blotted proteins was reacted with TrpE-UL1 antiserum at a dilution of 1:15,000. (B) Pulse-chase analysis of the UL1 protein synthesized in DIP-passage-64-enriched EHV-1-infected cells. Cells were pulsed at 5 h p.i. for 30 min with [35 S]methionine. The duration of infection and the hours of chase are indicated. Cells were labeled at 5 h p.i. and harvested at 6, 12, 24, and 48 h p.i. The [35 S]methionine-labeled proteins were immunoprecipitated with either TrpE-UL1 antiserum (lanes 2 to 7) or preimmune serum (lane 8). Lane 2 represents mock-infected cells. Molecular weight standards (M, lane 1) (in thousands) are indicated.

these CAT assays indicated that neither the UL1 nor UL2 protein could significantly transactivate or transrepress CAT expression from EHV-1 immediate-early, early, or late promoter-CAT constructs (data not shown).

The amino acid sequence of UL1 was reported previously (52), and numerous potential sites of posttranslational modification within the UL1 polypeptide were noted (19). One potential site of modification included a myristylation site at the N terminus of the UL1 polypeptide (19). Indeed, results from biochemical and immunoprecipitation analyses suggested that the UL1 polypeptide was modified by myristic acid in cells infected with standard or DIP-enriched EHV-1. As further evidence of myristylation, the radiolabel ([3 H]myristic acid) on the UL1 protein was not released following incubation of the immunoprecipitated UL1 protein in a 1.0 M solution of potassium hydroxide (data not shown). These data suggest that the radiolabel is associated with an

amide-linked myristic acid moiety rather than an ester-linked palmitic acid moiety (42). Although this is the first EHV-1-myristylated protein identified, a plethora of myristylated viral proteins have been described in other viral systems (for a review, see reference 47), including p60^{v-src} of Rous sarcoma virus (40), the gag proteins of many retroviruses including human immunodeficiency virus (36), pre-S1 protein of hepatitis B (35), VP2 of SV40 (45), VP4 of poliovirus (6), and the UL11 gene product of HSV-1 (26). The UL11 gene of HSV-1 was shown to encode a family of myristylated proteins that are also structural components of the mature virion (26). Moreover, all of the viral myristylated proteins mentioned above represent virion structural proteins as well. Similarly, the UL1 protein of EHV-1 was shown to be associated with mature virions and appears not to be localized within the virion envelope. Lastly, it should be noted that the TrpE-UL1 antiserum also recognized a 35-kDa

FIG. 9. Western blot analyses of EHV-1 virions with TrpE-UL1 antiserum. (A) Purified EHV-1 virions were suspended in Laemmli sample buffer, boiled, and fractionated by SDS-PAGE. The blotted virion proteins were reacted with either preimmune serum (Pi; lane 1) or TrpE-UL1 antiserum (UL1; lane 2) at a 1:15,000 dilution. (B) Purified EHV-1 virions were incubated in a detergent buffer (see Materials and Methods) to separate the envelope from the nucleocapsid-tegment. Proteins within the envelope fraction (S) and within the nucleocapsid-tegment fraction (P) were analyzed by SDS-PAGE, and the blotted proteins were reacted with preimmune serum (1:15,000, lanes 1 and 2), TrpE-UL1 antiserum (1:15,000, lanes 3 and 4), or anti-peptide antiserum (1:800) raised against EHV-1 glycoprotein D (gD, lanes 5 and 6 [10]).

FIG. 10. Western blot analyses of purified EHV-1 and BHV-1 virions. Purified EHV-1 virions (E; lane 1) and 2 μ l (B²; lane 2), 1 μ l (B¹; lane 3), or 0 μ l (B⁰; lane 4) of BHV-1 virions were fractionated by SDS-PAGE following disruption in RIPA buffer and boiling for 5 min. The filter containing the blotted virion proteins was reacted with either preimmune serum at a 1:2,000 dilution (A) or TrpE-UL1 antiserum at a 1:10,000 dilution (B).

BHV-1 virion protein that may represent the BHV-1 circ gene product, a homolog of UL1 of EHV-1 (11, 41).

Myristylation is thought to occur either cotranslationally or immediately following translation of the protein (47). The cellular enzyme responsible for myristylation, *N*-myristyl transferase, was shown to behave as a peripheral membrane protein in cultured murine cells and may exist as part of the ribosome complex (47). Several proposed functions for the myristic acid moiety include (i) facilitating membrane association or fusion events, (ii) enhancing protein-protein interactions, (iii) ensuring the correct folding of the protein, and (iv) localizing the protein in cellular membranes or juxtapositioning of the myristylated protein with other membrane-associated proteins (47). The importance and relevance of myristylation have been made clear recently. For example, the myristate-protein interactions of VP4 of poliovirus were shown to be critical for stability and proper capsid conformation of mature virions (30). Secondly, myristylation of the alpha subunit of the G-binding protein was shown to be absolutely essential for its proper signaling and transformation functions (12). Lastly, myristylation of viral oncoproteins (v-Src and v-Abl) was shown to be required for their fibroblast-transforming functions (3, 7, 37). Moreover, myristylation of these oncoproteins appears to be necessary but not sufficient for localization to cellular membranes (7, 37).

The results from indirect immunofluorescence analyses indicated that the myristylated UL1 protein is associated with cellular membranes in EHV-1-infected cells and confirmed that the UL1 gene product is overexpressed in cells infected with DIP-enriched EHV-1 preparations compared with cells infected with standard EHV-1. Since the UL1 protein was also detected in mature virions, it may play a role in the assembly of the virus. Although the UL1 protein was not observed in the nucleus by indirect immunofluorescence, it did appear to be associated at the perinuclear region (rough endoplasmic reticulum-Golgi apparatus) during early stages of infection and may be associated with the nuclear membrane at late stages of infection. Further studies, including cellular fractionation, will be necessary to determine whether UL1 is present in the nuclei of infected cells late in infection. It is tempting to speculate that if the UL1 protein functions in viral assembly, the DIPs of EHV-1 may conserve this gene since overexpression of the UL1 gene product may help to ensure that the DIP genome is packaged and/or that DIPs are assembled and perhaps enriched during

infection. However, elucidation of the role of the UL1 protein in EHV-1 cytolytic and persistent infections and its precise location within the EHV-1 virion awaits further investigations.

ACKNOWLEDGMENTS

We thank Suzanne Zavec and Scarlett Flowers for excellent technical support, Vikram Misra for the generous gift of purified BHV-1 virions, and Alan Jenkins and Yeini Thompson for assistance with the immunofluorescence assays. We also thank David McNabb for fruitful discussions and C. Clay Flowers for the use of gD antiserum.

This investigation was supported by Public Health Research grant AI 22001 to D.J.O. and AI 22894 to G.B.C. from the National Institutes of Health, a Grayson-Jockey Club Research Foundation Inc. research grant to D.J.O., and grants 89-37266-4735 and 92-37204-8040 from the U.S. Department of Agriculture Animal Molecular Biology Program to D.J.O.

REFERENCES

- Baumann, R. P., S. A. Dauenhauer, G. B. Caughman, J. Staczek, and D. J. O'Callaghan. 1984. Structure and genetic complexity of the genomes of herpesvirus defective interfering particles associated with oncogenic transformation and persistent infection. *J. Virol.* **50**:13-21.
- Baumann, R. P., J. Staczek, and D. J. O'Callaghan. 1986. Cloning and fine mapping the DNA of equine herpesvirus type 1 defective interfering particles. *Virology* **153**:188-200.
- Buss, J. E., C. J. Der, and P. A. Soliski. 1988. The six amino-terminal amino acids of p60^{src} are sufficient to cause myristylation of p21^{v-ras}. *Mol. Cell. Biol.* **8**:3960-3963.
- Caughman, G. B., A. T. Robertson, W. L. Gray, D. C. Sullivan, and D. J. O'Callaghan. 1988. Characterization of equine herpesvirus type 1 immediate-early proteins. *Virology* **163**:563-571.
- Caughman, G. B., J. Staczek, and D. J. O'Callaghan. 1985. Equine herpesvirus type 1 infected cell polypeptides: evidence for immediate-early/early/late regulation of viral gene expression. *Virology* **145**:49-61.
- Chow, M., J. F. E. Newman, D. Filman, D. M. Hogle, D. J. Rowlands, and F. Brown. 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. *Nature (London)* **327**:482-486.
- Daley, G. Q., R. A. Van Etten, P. K. Jackson, A. Bernards, and D. Baltimore. 1992. Nonmyristoylated Abl proteins transform a factor-dependent hematopoietic cell line. *Mol. Cell. Biol.* **12**:1864-1871.
- Dauenhauer, S. A., R. A. Robinson, and D. J. O'Callaghan. 1982. Chronic production of defective-interfering particles by hamster embryo cultures of herpesvirus persistently infected and oncogenically transformed cells. *J. Gen. Virol.* **60**:1-14.
- Davison, A. J., and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* **67**:597-611.
- Flowers, C. C., and D. J. O'Callaghan. 1992. Equine herpesvirus 1 glycoprotein D: mapping of the transcript and neutralization epitope. *J. Virol.* **66**:6451-6460.
- Fraefel, C., B. Vogt, and M. Schwyzer. 1992. Program Abstr. 17th International Herpesvirus Workshop, abstr. 90.
- Gallego, C., S. K. Gupta, S. Winitz, B. J. Einfeld, and G. L. Johnson. 1992. Myristylation of the G-alpha 2 polypeptide, a G protein alpha subunit, is required for its signaling and transformation functions. *Proc. Natl. Acad. Sci. USA* **89**:9695-9699.
- Gray, W. L., R. P. Baumann, A. T. Robertson, G. B. Caughman, D. J. O'Callaghan, and J. Staczek. 1987. Regulation of equine herpesvirus type 1 gene expression: characterization of immediate-early, early, and late transcription. *Virology* **158**:79-87.
- Gray, W. L., R. P. Baumann, A. T. Robertson, D. J. O'Callaghan, and J. Staczek. 1987. Characterization and mapping of equine herpesvirus type 1 immediate-early, early, and late transcripts. *Virus Res.* **8**:233-244.
- Harlow, E., and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

16. Harty, R. N., M. Chen, V. R. Holden, Y. Zhao, and D. J. O'Callaghan. Unpublished data.
17. Harty, R. N., W. L. Gray, and D. J. O'Callaghan. Unpublished data.
18. Harty, R. N., V. R. Holden, and D. J. O'Callaghan. 1993. Transcriptional and translational analyses of the UL2 gene of equine herpesvirus 1: a homolog of UL55 of herpes simplex virus type 1 that is maintained in the genome of defective interfering particles. *J. Virol.* **67**:2255–2265.
19. Harty, R. N., and D. J. O'Callaghan. 1992. Identification and expression of the UL1 gene product of equine herpesvirus 1. *Virus Res.* **25**:105–116.
20. Harty, R. N., R. R. Yalamanchili, and D. J. O'Callaghan. 1991. Transcriptional analysis of the UL1 gene of equine herpesvirus 1: a gene conserved in the genome of defective interfering particles. *Virology* **183**:830–833.
21. Henry, B. E., W. W. Newcomb, and D. J. O'Callaghan. 1979. Biological and biochemical properties of defective interfering particles of equine herpesvirus type 1. *Virology* **92**:495–506.
22. Henry, B. E., R. A. Robinson, S. A. Dauenhauer, S. S. Atherton, G. S. Hayward, and D. J. O'Callaghan. 1981. Structure of the genome of equine herpesvirus type 1. *Virology* **115**:97–114.
23. Holden, V. R., R. R. Yalamanchili, R. N. Harty, and D. J. O'Callaghan. 1992. ICP22 homolog of equine herpesvirus 1: expression from early and late promoters. *J. Virol.* **66**:664–673.
24. Koerner, T. J., J. E. Hill, A. M. Myers, and A. Tzagoloff. 1991. High-expression vectors with multiple cloning sites for construction of *trpE* fusion genes: pATH vectors. *Methods Enzymol.* **194**:477–490.
25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
26. MacLean, C. A., B. Clark, and D. J. McGeoch. 1989. Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristylated. *J. Gen. Virol.* **70**:3147–3157.
27. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region of the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69**:1531–1574.
29. McNabb, D. S., and R. J. Courtney. 1992. Identification and characterization of the herpes simplex virus type 1 virion protein encoded by the UL35 open reading frame. *J. Virol.* **66**:2653–2663.
30. Moscufo, N., and M. Chow. 1992. Myristate-protein interactions in poliovirus: interactions of VP4 threonine 28 contribute to the structural conformation of assembly intermediates and the stability of assembled virions. *J. Virol.* **66**:6849–6857.
31. O'Callaghan, D. J., and R. N. Harty. General features of the equine herpesviruses. In R. G. Webster and A. Granoff (ed.), *Encyclopedia of virology*, in press. Saunders Scientific Publications, London.
32. O'Callaghan, D. J., B. E. Henry, J. H. Wharton, S. A. Dauenhauer, R. B. Vance, J. Staczek, and R. A. Robinson. 1981. Equine herpesvirus: biochemical studies on genomic structure, DI particles, oncogenic transformation and persistent infection, p. 387–418. In Y. Becker (ed.), *Developments in molecular virology*, vol. 1. Herpesvirus DNA. Martinus Nijhoff, The Hague, The Netherlands.
33. O'Callaghan, D. J., J. M. Hyde, G. A. Gentry, and C. C. Randall. 1968. Kinetics of viral deoxyribonucleic acid, protein, and infectious particle production and alterations in host macromolecular synthesis in equine abortion (herpes) virus-infected cells. *J. Virol.* **2**:793–804.
34. Perdue, M. L., M. C. Kemp, C. C. Randall, and D. J. O'Callaghan. 1974. Studies on the molecular anatomy of L-M cell strain of equine herpesvirus type 1. Proteins of the nucleocapsids and intact virions. *Virology* **50**:201–216.
35. Persing, D. H., H. E. Varmus, and D. Ganem. 1987. The preS1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. *J. Virol.* **61**:1672–1677.
36. Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature (London)* **313**:277–284.
37. Resh, M. D. 1989. Specific and saturable binding of pp60^{v-src} to plasma membranes: evidence for a myristyl-src receptor. *Cell* **58**:281–286.
38. Robinson, R. A., P. W. Tucker, S. A. Dauenhauer, and D. J. O'Callaghan. 1981. Molecular cloning of equine herpesvirus type 1 DNA: analysis of standard and defective viral genomes and viral sequences in oncogenically transformed cells. *Proc. Natl. Acad. Sci. USA* **78**:6684–6688.
39. Ruyechan, W. T., S. A. Dauenhauer, and D. J. O'Callaghan. 1982. Electron microscopic study of equine herpesvirus type 1 DNA. *J. Virol.* **42**:297–300.
40. Schultz, A. M., L. E. Henderson, S. Oroszlan, E. A. Garber, and H. Hanafusa. 1985. Amino terminal myristylation of the protein kinase p60^{src}, a retroviral transforming protein. *Science* **227**:427–429.
41. Schwyzer, M. (University of Zurich). 1992. Personal communication.
42. Sefton, B. M., I. S. Trowbridge, and J. A. Cooper. 1982. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus, and Abelson virus contain tightly bound lipid. *Cell* **31**:465–474.
43. Smith, R. H., G. B. Caughman, and D. J. O'Callaghan. 1992. Characterization of the regulatory functions of the equine herpesvirus 1 immediate-early gene product. *J. Virol.* **66**:936–945.
44. Smith, R. H., R. N. Harty, and D. J. O'Callaghan. Unpublished data.
45. Streuli, C. H., and B. E. Griffin. 1987. Myristic acid is coupled to a structural protein of polyoma virus and SV40. *Nature (London)* **326**:619–622.
46. Telford, E. R., M. S. Watson, K. McBride, and A. J. Davison. 1992. The DNA sequence of equine herpesvirus-1. *Virology* **189**:304–316.
47. Towler, D., J. I. Gordon, S. P. Adams, and L. Glaser. 1988. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* **57**:69–99.
48. Whalley, J. M., G. R. Robertson, and A. J. Davison. 1981. Analysis of the genome of equine herpesvirus type 1: arrangement of cleavage for restriction endonuclease *EcoRI*, *BglII*, and *BamHI*. *J. Gen. Virol.* **57**:307–323.
49. Yalamanchili, R. R., and D. J. O'Callaghan. 1990. Sequence and organization of the genomic termini of equine herpesvirus type 1. *Virus Res.* **15**:149–162.
50. Yalamanchili, R. R., and D. J. O'Callaghan. 1990. Organization and function of the ORIs sequence in the genome of EHV-1 DI particles. *Virology* **179**:867–870.
51. Yalamanchili, R. R., B. Raengsakulrach, R. P. Baumann, and D. J. O'Callaghan. 1990. Identification and nucleotide sequence analysis of the site of recombination that generates the defective genomes of equine herpesvirus type 1. *Virology* **175**:448–455.
52. Yalamanchili, R. R., B. Raengsakulrach, and D. J. O'Callaghan. 1990. Equine herpesvirus 1 sequence near the left terminus codes for two open reading frames. *Virus Res.* **18**:109–116.
53. Zhao, Y., V. R. Holden, R. N. Harty, and D. J. O'Callaghan. 1992. Identification and transcriptional analyses of the UL3 and UL4 genes of equine herpesvirus 1, homologs of the ICP27 and glycoprotein K genes of herpes simplex virus. *J. Virol.* **66**:5363–5372.