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Up to Four Distinct Polypeptides Are Produced from the γ34.5 Open Reading Frame of Herpes Simplex Virus 2

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ABSTRACT

The herpes simplex virus 1 (HSV-1) ICP34.5 protein strongly influences neurovirulence and regulates several cellular antiviral responses. Despite the clinical importance of HSV-2, relatively little is known about its ICP34.5 ortholog. We found that HSV-2 produces up to four distinct forms of ICP34.5 in infected cells: a full-length protein, one shorter form sharing the N terminus, and two shorter forms sharing the C terminus. These forms appeared with similar kinetics and accumulated in cells over much of the replication cycle. We confirmed that the N-terminal form is translated from the primary unspliced transcript to a stop codon within the intron unique to HSV-2 γ34.5. We found that the N-terminal form was produced in a variety of cell types and by 9 of 10 clinical isolates. ICP27 influenced but was not required for expression of the N-terminal form. Western blotting and reverse transcription-PCR indicated the C-terminal forms did not contain the N terminus and were not products of alternative splicing or internal transcript initiation. Expression plasmids encoding methionine at amino acids 56 and 70 generated products that comigrated in SDS-PAGE with the C1 and C2 forms, respectively, and mutation of these sites abolished C1 and C2. Using a recombinant HSV-2 encoding hemagglutinin (HA)-tagged ICP34.5, we demonstrated that the C-terminal forms were also produced during infection of many human and mouse cell types but were not detectable in mouse primary neurons. The protein diversity generated from the HSV-2 γ34.5 open reading frame implies additional layers of cellular regulation through potential independent activities associated with the various forms of ICP34.5.

IMPORTANCE

The herpes simplex virus 1 (HSV-1) protein ICP34.5, encoded by the γ34.5 gene, interferes with several host defense mechanisms by binding cellular proteins that would otherwise stimulate the cell's autophagic, translational-arrest, and type I interferon responses to virus infection. ICP34.5 also plays a crucial role in determining the severity of nervous system infections with HSV-1 and HSV-2. The HSV-2 γ34.5 gene contains an intron not present in HSV-1 γ34.5. A shorter N-terminal form of HSV-2 ICP34.5 can be translated from the unspliced γ34.5 mRNA. Here, we show that two additional forms consisting of the C-terminal portion of ICP34.5 are generated in infected cells. Production of these N- and C-terminal forms is highly conserved among HSV-2 strains, including many clinical isolates, and they are broadly expressed in several cell types, but not mouse primary neurons. Multiple ICP34.5 polypeptides add additional complexity to potential functional interactions influencing HSV-2 neurovirulence.

Human alphaherpesviruses share the capacity to invade and establish latency in the nervous system. Herpes simplex virus 1 (HSV-1) and HSV-2, the most similar members of this group, typically infect mucosal surfaces after direct interpersonal contact. Replication in the mucosa precedes retrograde transport of virus to sensory nerve ganglia and often the central nervous system (CNS). From their site of latency in the ganglia, HSV-1 and HSV-2 periodically reactivate to cause recurrent shedding and mucosal disease (1). HSV-2 infects primarily the anogenital epithelium of nearly one in five adults in the United States (2) and up to 75% of adults worldwide (3, 4). HSV-2 also causes serious and sometimes fatal neurologic disease in babies born to women experiencing peripartum primary or recurrent infection (5).

HSV-1 and HSV-2 have colinear genomes and possess an important neurovirulence factor mapped to the γ34.5 (RL1) gene (6–8). Both viruses contain two copies of γ34.5 located within the inverted-repeat regions of the genome. γ34.5 is transcribed as a leaky late (γ1) gene (9). It encodes infected cell protein 34.5 (ICP34.5) (10), whose expression is detected as early as 2 to 3 h postinfection (11–14). Truncation or stop codon insertion mutants of HSV-1 and HSV-2 γ34.5 retain the capacity to replicate efficiently in many actively dividing cell types (14–16) and in footpad tissue of mice (17). However, these mutants replicate poorly in some confluent cell types *in vitro* (15) and show dramatically reduced lethality after peripheral (14, 17, 18) or intracerebral (6, 8, 14, 16, 17, 19) routes of infection. Thus, γ34.5 plays a critical role in HSV pathogenesis, and because of the markedly reduced capacity of HSV-1 γ34.5 null mutants to productively infect the nervous system, γ34.5 disruption has become an important element of HSV vectors for cancer therapy and gene therapy in the nervous system (20, 21).

ICP34.5 controls several additional aspects of the virus replication cycle and the virus' capacity to counter cellular antiviral responses. The amino (N)-terminal portion of HSV-1 ICP34.5 influences intracellular localization (22) and facilitates virus rep-

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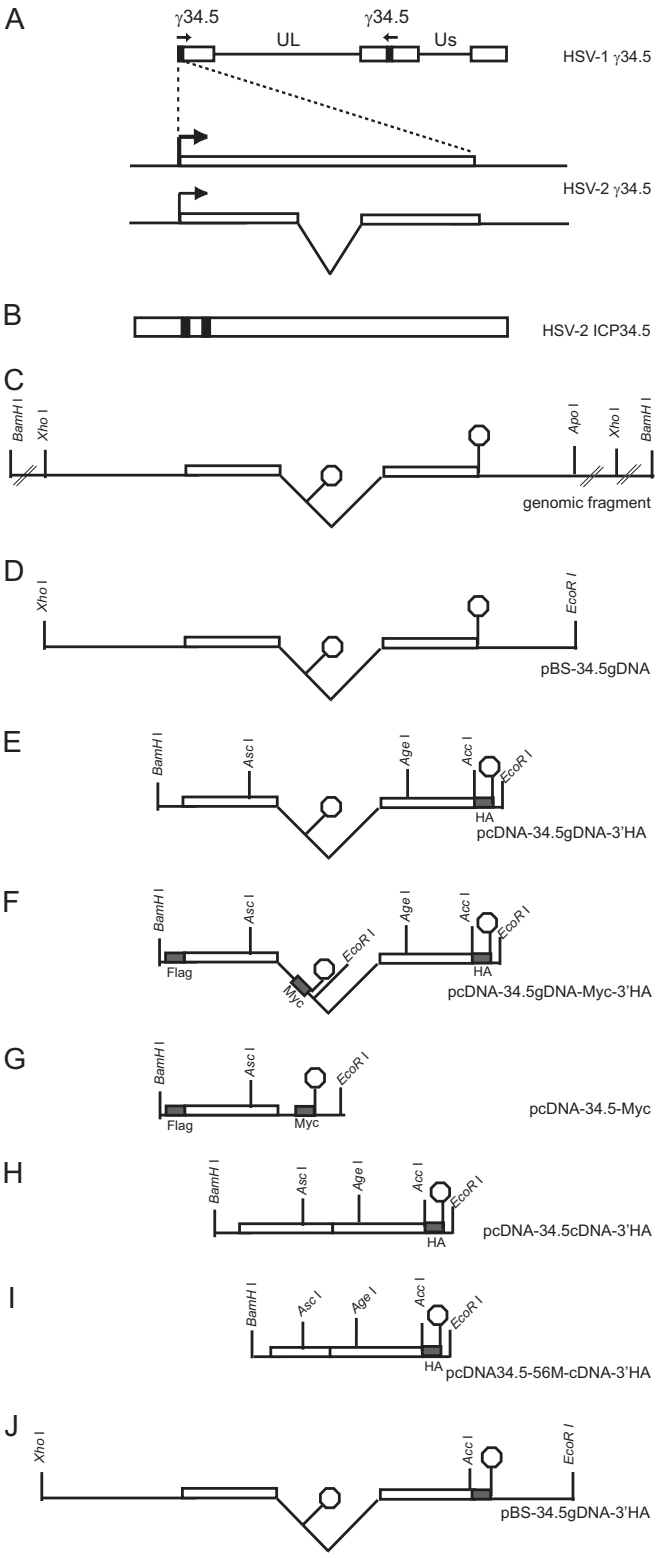

FIG 1 HSV-1 and HSV-2 γ34.5 open reading frames and expression constructs generated. (A) Two copies of the γ34.5 gene are located in the repeat regions of the HSV-1 and HSV-2 genomes. The HSV-1 γ34.5 gene consists of a single exon, and the HSV-2 γ34.5 gene contains an intron. Us, unique short region; UL, unique long region. (B) The HSV-2 ICP34.5 polypeptide translated from the mature spliced γ34.5 mRNA, with locations of peptide epitopes (solid boxes) used to generate rabbit antiserum. (C) BamHI genomic fragment of HSV-2 333 DNA containing the γ34.5 gene. (D) HSV-2 γ34.5 gene subcloned into pBS(+). (E) HSV-2 γ34.5 gene subcloned into pcDNA3.1(+) and fused in frame with a 3′ HA tag. (F) 34.5gDNA-3′HA construct mutated to encode Flag and Myc tags. (G) 34.5gDNA-Myc-3′HA construct truncated after the Myc tag. (H) 34.5cDNA construct with 3′ HA tag. A vertical line indicates the exon boundary. (I) Example of an N-terminal truncation mutant constructed from the cDNA plasmid. (J) Plasmid containing the HSV-2 γ34.5 gene with a 3′ HA tag used to produce the recombinant HSV-2 strain 34.5gDNA-3′HA.

lication (23) and virion maturation and egress (24, 25). ICP34.5 also binds TBK1 via an N-terminal domain to prevent its interaction with and activation of IRF3 (26), thus helping HSV-1 thwart the type I interferon (IFN) response. A beclin-1 binding domain overlaps the TBK1 binding domain in the N-terminal half of HSV-1 ICP34.5 (27) and confers the capacity to inhibit autophagy (28). HSV-1 ICP34.5 also binds protein phosphatase 1α (PP1α) via a carboxy (C)-terminal motif conserved in HSV-2 (29), guiding it to antagonize phosphorylation of eukaryotic initiation factor 2α (eIF2α) (30–32) mediated by the stress-induced kinase PKR (30, 33). Countering PKR activity is crucial to ICP34.5's capacity to inhibit type I IFN signaling (34), and ICP34.5 antagonism of PKR ultimately facilitates *in vivo* replication of HSV-1 (35, 36). The central portion of HSV-1 ICP34.5 contains tandem alanine-threonine-proline repeats (9) associated with neuroinvasiveness (37) and virus egress (38), but these repeats are not found in the HSV-2 ortholog. Thus, ICP34.5 lies at the nexus of several cellular antiviral responses that influence HSV neurovirulence.

HSV-2 γ34.5 and its ICP34.5 product are dissimilar to their HSV-1 counterparts in some respects despite conservation of the neurovirulence property. The HSV-2 γ34.5 gene contains a 154-bp intron consisting largely of tandem repeats that is not found in HSV-1 γ34.5 (7, 39). The N-terminal portion of HSV-1 ICP34.5 is only 41% identical in amino acid sequence to the first exon of HSV-2 ICP34.5, and insertions appear to disrupt the corresponding HSV-1 beclin-1 and TBK1 binding domains (40). One HSV-1 ICP34.5 polypeptide of 39 to 43 kDa is consistently observed in Western blots (10, 25, 27), although an isoform with faster mobility has occasionally been reported (12, 23). In contrast, polyclonal antiserum directed to the N terminus of HSV-2 ICP34.5 detects two bands by Western blotting, one of 38 kDa and another of 28 kDa (41). Both bands represent authentic ICP34.5 (41, 42) and were recently identified as the full-length form and a shorter N-terminal form generated by alternative splicing, respectively (40). Our preliminary studies had yielded evidence of this N-terminal form and additional forms of HSV-2 ICP34.5. Given the significance of ICP34.5 as a neurovirulence factor and its manipulation in the development of cancer therapeutics, we investigated further the translation products of the HSV-2 γ34.5 gene and their expression in a variety of cell types and viral strains.

MATERIALS AND METHODS

Plasmids and cloning. Genomic DNA from HSV-2 strain 333 was digested with BamHI and subjected to electrophoresis, and a fragment of appropriate size to contain the γ34.5 gene was excised. Sequencing confirmed the presence of the complete γ34.5 gene. The fragment was trimmed by digestion with XhoI and ApoI and cloned into pBS-KS(+) between the XhoI and EcoRI sites, creating plasmid pBS-34.5gDNA (Fig. 1D). To generate an expression vector for synthesis of hemagglutinin (HA)-tagged ICP34.5, the γ34.5 open reading frame (ORF) was PCR amplified using CsCl-purified genomic DNA (gDNA) of HSV-2 strain 333 as

a template; AccuPrime Pfx DNA polymerase (Invitrogen); a forward primer containing a BamHI site (5′-CGCGGATCCCGCCCCGACGCGCTCCGGCTCCGGGCCTACGCCGAGCCCAGCCGCCCGCCATG-3′); and a reverse primer containing sequences specifying an HA tag, a stop codon, and an EcoRI site (5′-CCGGAATTCTCAAGCGTAGTCCGGTACGTCGTATGGGTAGACCGCCCGACGGCCCGG-3′). Alteration of the original stop codon to generate the HA tag also created an AccI site. The PCR product was cloned into pcDNA3.1(+) between the BamHI and EcoRI sites to create plasmid pcDNA-34.5gDNA-3′HA (Fig. 1E).

Cloning of HSV-1 γ34.5 was accomplished by PCR amplification using CsCl-purified genomic DNA of HSV-1 strain F as a template, AccuPrime Pfx DNA polymerase, a forward primer containing the BamHI site (Fwd 5′-TCGCGGATCCGGCACGCTCTCTGTCTCCATGGCCCGCCGCCGCCGCCATC-3′), and a reverse primer altering the stop codon and including the HA tag plus an EcoRI site (Rev 5′-CCGGAATTCTCAAGCGTAGTCCGGTACGTCGTATGGGTAGACCGAGTTCGCCGGGCCGGCTCCGC-3′). After BamHI-EcoRI digestion, the DNA fragment was ligated into pcDNA3.1(+), resulting in pcDNA-34.5gDNA-3′HA(1).

The γ34.5 genes from several clinical isolates of HSV-2 were also cloned with a C-terminal HA tag. Viral DNA was purified, using standard phenol-chloroform extraction, from viral cultures that were passaged in Vero cells only once. The γ34.5 ORF was PCR amplified from the viral DNA template using AccuPrime Pfx DNA polymerase; a forward primer containing a BamHI site; and a reverse primer containing sequences specifying an HA tag, a stop codon, and an EcoRI site, as described above. The PCR products were cloned into pcDNA3.1(+) between the BamHI and EcoRI sites to create plasmids pcDNA-34.5gDNA-#1-3′HA, pcDNA-34.5gDNA-#2-3′HA, pcDNA-34.5gDNA-#7-3′HA, and pcDNA-34.5gDNA-#10-3′HA.

To generate an expression plasmid containing both an N-terminal Flag tag and the C-terminal HA tag, a portion of pcDNA-34.5gDNA-3′HA was PCR amplified using the forward primer Fwd 5′-Flag (5′-CGCGGATCCATGGACTACAAAGACGATGACGACAAGCTTATGTCCCGCCGGCGTCCCCCGCCGCCGGGGTCCCCGGCGCC-3′) and the reverse primer indicated above. The PCR product was cloned into pcDNA3.1(+) between the BamHI and EcoRI sites to create plasmid pcDNA-5′Flag-34.5gDNA-3′HA.

An expression plasmid encoding a Myc tag in place of the original stop codon in the HSV-2 γ34.5 intron was created using a custom-synthesized double-stranded DNA (dsDNA) product (Integrated DNA Technologies). This gene fragment included a portion of γ34.5 starting at the AscI site in exon 1 and extending through the AgeI site in exon 2. The intron sequence was altered to specify a Myc tag in place of the original stop codon, followed by a new stop codon and an EcoRI site, thus preserving the length of the intron. This gene fragment was ligated into pcDNA-5′Flag-34.5gDNA-3′HA that had been digested with AscI and AgeI, creating plasmid pcDNA-34.5gDNA-Myc-3′HA (Fig. 1F). To generate an expression vector including only the Myc-tagged N-terminal portion of γ34.5, pcDNA-34.5gDNA-Myc-3′HA was digested with EcoRI, and after gel purification, the approximately 6,000-bp fragment was religated to create plasmid pcDNA-34.5-Myc (Fig. 1G).

To generate an HA-tagged γ34.5 cDNA, nested-PCR amplification of the reverse-transcribed, spliced γ34.5 mRNA (see "RNA isolation and RT-PCR" below) was accomplished using a forward primer containing a BamHI site and a reverse primer specifying an HA tag and containing an EcoRI site. The PCR product (845 bp) was ligated into the BamHI-EcoRI sites in pcDNA3.1(+) to create plasmid pcDNA-34.5cDNA-3′HA (Fig. 1H). Alternatively, a forward primer containing a BamHI site and a Flag tag was used to generate a Flag-tagged γ34.5 cDNA construct.

To generate a construct carrying mutations in the γ34.5 gene that abrogate protein expression, the plasmid pcDNA-34.5gDNA-3′HA was modified in two steps. First, a stop codon was inserted at amino acid 13 by means of a short oligonucleotide linker cloned into the SanDI restriction site. Oligonucleotides (Fwd, 5′-GTCCCCGTTAACGCCGCCGGG-3′, and Rev, 5′-GACCCGGCGGCGTTAACGGG-3′) were suspended in STE buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA) at a 100 mM concentration. The two strands were mixed in equimolar amounts, heated to 95°C for 5 min, and allowed to gradually cool. The resulting double-stranded DNA fragment was added at a 10 mM concentration to a ligation reaction mixture also containing plasmid pcDNA-34.5gDNA-3′HA that had been digested with SanDI. The resulting plasmid, pcDNA-34.5gDNA-HpaI-3′HA, was verified by restriction digestion and sequencing. Second, two PCR fragments were generated from the pcDNA-34.5gDNA-HpaI-3′HA plasmid to introduce an M35A mutation in the γ34.5 gene and to add a KpnI restriction site. The first amplicon was generated using primers Fwd, 5′-CGCGGATCCCGCCCCGACGCGCTCCGGCTCCGGGCCTACGCCGAGCCCAGCCGCCCGCCATG-3′, and Rev, 5′-CGCGGTACCCGAGTCGTACGCAGGGACCGCTTGGGAGTCTGCGGTTGGGAGC-3′. The three nucleotide substitutions resulted in an amino acid change from Met to Ala and a unique KpnI site. The second amplicon was generated using primers Fwd, 5′-GACTCGGGTACCGCGGTCGAGAGCGCGCCGGCCGCGTCCTCGCTC-3′, and Rev, 5′-CCGGAATTCTCAAGCGTAGTCCGGTACGTCGTATGGGTAGACCGCCCGACGGCCCGG-3′. The first PCR amplicon was digested with BamHI and HpaI, and the second amplicon was digested with HpaI and EcoRI. A three-way ligation was performed with the amplicons and BamHI-EcoRI-digested pcDNA plasmid. The resulting construct, pcDNA-34.5gDNA-HpaI/M35A-3′HA, contained a stop codon at amino acid 13 and an M35A mutation that prevented expression of full-length ICP34.5. Clones were screened by HpaI and KpnI digestion and were verified by sequencing.

In order to eliminate the splice acceptor site in γ34.5, a 293-bp synthetic dsDNA fragment from GENEART* (Life Technologies) and pcDNA-34.5gDNA-3′HA were digested with AscI and AgeI, and gel-purified segments were ligated. The resulting construct, pcDNA-34.5gDNA-V163/165A-3′HA, had the 5′ splice acceptor site sequence changed from CGCAGGTGTGC to CTTAAGTGTGC. In addition, four nucleic acid substitutions downstream from the site resulted in amino acid changes V163A and V165A and the introduction of an EcoRV restriction site. The clones were screened for the unique EcoRV site and verified by sequencing.

Plasmids expressing N-terminal truncation mutants of HSV-2 ICP34.5 were generated by PCR using pcDNA-34.5cDNA-3′HA as a template, forward primers containing codons specifying a methionine residue in place of the existing codon (Fwd-56M, 5′-CTCGGATCCATGCGCTGGCTGCTGGTGCCCCAGG-3′; Fwd-61M, 5′-CTCGGATCCATGCCCCAGGCGGACGACAGCGACGAC-3′; or Fwd-70M, 5′CTCGGATCCATGGACTACGCCGGCAACGACGACGCAGAGTG-3), and the reverse primer containing sequences specifying an HA tag described above. The PCR products were cloned into BamHI-EcoRI sites of pcDNA3.1(+). The clones yielded pcDNA-56M-cDNA-3′HA (Fig. 1I), pcDNA-61M-cDNA-3′HA, and pcDNA-70M-cDNA-3′HA, respectively.

Plasmids containing internal substitutions were constructed with PCR-generated DNA products and a three-way ligation strategy. To substitute amino acids Leu-Arg-Arg at positions 55-56-57, pcDNA-34.5 cDNA-3′HA was used as a template. The first PCR product was made using the primers Fwd, 5′-CGCGGATCCCGCCCCGACGCGCTCCGGCTCCGGGCCTACGCCGAGCCCAGCCGCCCGCCATG-3′, and Rev, 5′-AAAACTGCAGCGAGCGAGGACGCGGCCGGCGCGCTCTCGAC-3′, and digested with BamHI and PstI. The second PCR product was made using the primers Fwd, 5′-AAAACTGCAGCTTGGCTGCTGGTGCCCCAGGCGGACGAC-3′, and Rev, 5′-CCGGAATTCTCAAGCGTAGTCCGGTACGTCGTATGGGTAGACCGCCCGACGGCCCGG-3′, and digested with EcoRI and PstI. A three-way ligation was performed with BamHI-EcoRI-digested pcDNA3.1(+), resulting in pcDNA-55-56-57-AAA-cDNA-3′HA.

To mutate amino acids Asp-Ala-Asp at positions 69-70-71 to Ala-Ala-Ala, a plasmid was constructed using the same three-way ligation strategy described above. Primers Fwd, 5′-CGCTCGGATCCCGCCCCGACGCGCTCCGGCTCCGGGCCTACGCCGAGCCCAGCCGCCCGCCATG-3′, and Rev, 5′-AAAACTGCAGCGTCGCTGTCGTCCGCCTGGGGCACCAG-3′, were used to generate the first segment; primers Fwd, 5′-AAAACTGCAGCTTACGCCGGCAACGACGACGCAGAGTG-3′, and

the Rev primer described above carrying an HA tag were used to generate the second PCR segment. After BamHI and PstI digestion of segment 1 and EcoRI and PstI digestion of segment 2, a three-way ligation was performed with BamHI-EcoRI-digested pcDNA3.1(+), yielding pcDNA-69-70-71-AAA-cDNA-3′HA.

To generate a plasmid containing the HA-tagged gDNA with additional flanking sequences suitable for recombinant-virus isolation, a three-way ligation was performed. First, an AgeI-AccI fragment was excised from pcDNA-34.5gDNA-3′HA. Second, a segment of pBS-34.5gDNA was amplified by PCR using AccuPrime Pfx DNA polymerase; a forward primer specifying an AccI site, an HA tag, and a stop codon (5′-GGCGGTCTACCCATACGACGTACCGGACTACGCTTGACGGC GGAGCCCCGGAGCTCCGAAGGTCTG-3′); and a reverse primer positioned at the junction of viral genomic DNA and the EcoRI site of the vector (5′-CCCGGGCTGCAGGAATTCATTAGCATACTAGGAAGCC CAGG-3′). This fragment was digested with AccI and EcoRI. Third, pBS-34.5gDNA was digested with AgeI and EcoRI, and the remaining vector was ligated with both fragments described above to create pBS-34.5gDNA-3′HA (Fig. 1J).

Plasmids pC27 (encoding HSV-1 ICP27 behind a minimal human cytomegalovirus [HCMV] promoter in pUHD10-3) (43) and pBH27 (encoding HSV-1 ICP27 behind its native promoter in pUC19) (44) were kindly provided by Steve Rice, as was pT2-27 (encoding HSV-2 ICP27 behind its native promoter in pCR2.1-TOPO) (45).

The translated HSV-2 γ34.5 sequence was analyzed using ExPASy PeptideCutter software (Bioinformatics Resource Portal; Swiss Institute of Bioinformatics [SIB] [http://www.expasy.org]).

Cells and viruses. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3% newborn calf serum and 3% bovine growth serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (1× PS). 293 cell lines, provided by Daniel Hawiger and Bill Wold, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, and 1× PS. Primary human foreskin fibroblasts (HFFs) derived from neonatal foreskins were provided by David Sibley and John Tavis. They were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1× PS and used at passages 15 to 18. The human glioblastoma lines SW1088, U118, and U373 were provided by Karoly Toth and cultured in DMEM supplemented with 10% FBS, 1× HEPES, 1× nonessential amino acids, and 1× PS. The BE(2)-C human neuroblastoma line, provided by David Davido, was maintained in 1:1 minimal essential medium (MEM)–F-12 supplemented with 10% FBS, 1× PS, 2 mM L-glutamine. The Neuro-2a mouse neuroblastoma line, provided by David Davido, was cultured in MEM supplemented with 10% FBS and 1× PS. Primary C57BL/6 mouse embryonic fibroblasts (MEFs) and cortical neurons were prepared from embryos at 13 to 15 days gestation. MEFs were cultured in DMEM supplemented with 10% bovine growth serum and 1× PS and used through passage 4. Cortical neurons were isolated as previously described (46, 47) and cultured in neurobasal medium (Invitrogen) containing 2 mM L-glutamine, 1× B27 supplement (Gibco), and 1× PS. Primary C57BL/6 mouse astrocytes were prepared from pups at 1 to 3 days of age and were cultured in DMEM supplemented with 10% FBS, 1× HEPES, and 1× PS and used through passage 18.

HSV-2 333JS4 is a plaque isolate of strain 333 (48). A virus expressing HA-tagged ICP34.5 was generated by homologous recombination. Vero cells were cotransfected with HSV-2 333JS4 DNA and plasmid pBS-34.5gDNA-3′HA using an Amaxa nucleofector (Lonza). Plaque isolates were screened by PCR, and a recombinant virus was plaque purified to homogeneity and named HSV-2 34.5gDNA-3′HA. HSV-2 strains 1, 2, 7, and 10 are primary clinical isolates passaged once in cell culture. Cell lysate stocks of HSV-2 strains and HSV-1 strain F (49) were prepared on Vero cell monolayers as previously described (50). Cell lysate stocks of HSV-1 strains *d*301 (51) and *dl*27lacZ1 (52) were prepared on complementing S2 (51) or V27 (53) cells. Titers of the virus stocks were determined by titration on Vero or V27 monolayers as previously described (54).

Animals. The New Zealand White rabbit used for antiserum production and the C57BL/6 mice used for primary cell isolation were handled in strict accordance with good animal practice as defined by institutional and Public Health Service guidelines, with work approved by the Saint Louis University Institutional Animal Care and Use Committee. The animals were housed in the Department of Comparative Medicine, Saint Louis University School of Medicine, St. Louis, MO.

Antisera. Rabbit polyclonal antiserum to HSV-2 ICP34.5 was raised against synthetic peptides PGAPAVPRPGA and SAPAASSLLRRWLLV, corresponding to amino acids 17 to 27 and 47 to 61, respectively, in the N terminus of HSV-2 ICP34.5 exon 1. A cysteine residue was added to one end of each peptide to facilitate coupling to maleimide-activated keyhole limpet hemocyanin (KLH) (Thermo Scientific). Rabbit polyclonal antiserum to the amino terminus of the HSV-1 ICP34.5 protein (33) was the kind gift of Ian Mohr.

Western blotting. Cells were mock infected or infected at a multiplicity of infection (MOI) of 5 with HSV-2 strain 333, HSV-2 34.5gDNA-3′HA, or HSV-1 strain F. For transfections, Lipofectamine (Vero) or Lipofectamine LTX (other cell types) and Plus reagent (Invitrogen) were used. 293 cells were alternatively transfected with Lipofectamine 2000. Monolayers were collected at various times postinfection or -transfection, as indicated in the figure legends. Cells were lysed in RIPA buffer, mixed with 2× Laemmli buffer, heated at 95°C for 5 min, and then subjected to SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked in Tris-buffered saline–Tween 20 (TBST) containing 5% nonfat dry milk. Incubations were carried out in TBST using rabbit antiserum to HSV-2 ICP34.5, rat monoclonal anti-HA antibody (Roche), rabbit anti-Myc antibody (Sigma), rabbit anti-eIF2α (Santa Cruz), and mouse anti-VP5 (East Coast Biotech). Anti-rabbit, anti-rat, or anti-mouse alkaline phosphatase-conjugated antibodies (Promega) were used for detection. All primary and secondary antibody incubations were carried out in TBST. Bands were visualized using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Promega), according to the manufacturer's instructions.

The effect of ICP27 on HSV-2 ICP34.5 expression was determined by transfection of HSV-2 pBS-34.5gDNA-3′HA and the HSV-1 ICP27-expressing plasmid pC27 or pBH27 or the HSV-2 ICP27-expressing plasmid pT2-27. Cell lysates were prepared 24 h posttransfection. Alternatively, cells transfected with pBS-34.5gDNA-3′HA 14 h previously were superinfected with HSV-2 34.5gDNA-3′HA or HSV-1 *d*301 or *dl*27lacZ at an MOI of 5. Cell lysates were prepared 10 h postinfection.

RNA isolation and RT-PCR. Vero cell monolayers were mock infected or infected at an MOI of 5 with HSV-2 strain 333. At 6 h postinfection, the cells were trypsinized and collected by centrifugation. The nuclei were separated from the cytoplasm by incubation of the cells in lysis buffer (50 mM Tris-Cl, pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% [vol/vol] Nonidet P-40, and 1,000 U/ml RNaseOut [Invitrogen]) for 5 min on ice, followed by centrifugation at 4°C for 2 min at 300 × *g*. Cytoplasmic mRNAs in the supernatant were purified using TRI Reagent (Sigma) following the manufacturer's instructions. After DNase digestion using a Turbo DNA-free kit (Ambion), 500 ng of each RNA sample was reverse transcribed using anchored oligo$(dT)_{18}$ primers and a Transcriptor First Strand cDNA synthesis kit (Roche) in a 20-μl volume according to the manufacturer's instructions. To search for transcripts generated in the region of γ34.5, the same forward primer (Fwd 34.5-5′UTR, 5′-TAGGC AAGCACGGACTGGCGGTTACAC-3′) was used with a series of reverse primers located stepwise across the region. The reverse primers were Rev 34.5-in-Exon-1, 5′-TGTTCGCCCACTCTGCGT-3′; Rev 34.5-in-Exon-2, 5′-TCTCCCAGGCCACCAGATAG-3′; Rev 34.5-in-mid-Exon-2, 5′-TCCAGGCACGGTCCGATGA-3′; and Rev 34.5-in-Stop, 5′-TCGCCGGTTCAACCCTAGAC-3′. Using primers complementary to sequences in the 5′ and 3′ untranslated regions (UTRs), two PCR products were isolated by gel electrophoresis, excised, and sequenced. The smaller product, corresponding to the spliced γ34.5 mRNA, was ampli-

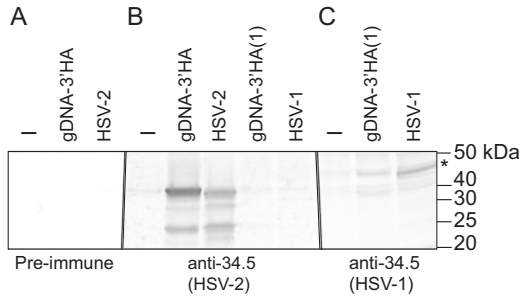

FIG 2 Two ICP34.5 isoforms are detected with antiserum to the N terminus. Lysates were prepared from uninfected cells (−), cells infected with HSV-1 or HSV-2, or cells transfected with plasmids expressing HSV-1 or HSV-2 34.5gDNA. The membrane was divided and probed with rabbit preimmune serum (A), rabbit antiserum to the N terminus of HSV-2 ICP34.5 (B), or rabbit antiserum to the N terminus of HSV-1 ICP34.5 (C). The asterisk indicates the HSV-1 polypeptide.

fied by nested PCR using primers containing BamHI and EcoRI sites and cloned into pcDNA3.1(+) to generate pcDNA-34.5cDNA-3′HA, as described above.

Nucleotide sequence accession number. The sequence of the HSV-2 γ34.5 gene was submitted to GenBank under accession number KJ796642.

RESULTS

To investigate the polypeptides generated from the γ34.5 open reading frame, we elicited rabbit antiserum to HSV-2 ICP34.5 by immunization with two peptides in the N-terminal portion of the protein (Fig. 1B) (41). In Western blots, preimmune serum did not react with HSV-2 proteins in lysates of HSV-2-infected Vero cells (Fig. 2A) or cells transfected with a plasmid containing the full-length γ34.5 open reading frame fused in frame to an HA tag (34.5gDNA-3′HA). The rabbit anti-peptide antiserum detected two bands with mobilities of approximately 38 and 27 kDa in p34.5gDNA-3′HA-transfected cells and cells infected with HSV-2 strain 333 (Fig. 2B). The antiserum did not react with HSV-1 γ34.5-transfected or HSV-1-infected cell lysates (Fig. 2B). Probing with antiserum specific for HSV-1 ICP34.5, we observed only a single band of 40 kDa in lysates of cells transfected with the HSV-1 expression vector p34.5gDNA-3′HA(1) or in HSV-1-infected cells (Fig. 2C), as was previously shown (33). Thus, the N-terminally directed antiserum is specific to HSV-2 ICP34.5 and detects two forms of HSV-2 ICP34.5 in infected or p34.5gDNA-3′HA-transfected cells: one full-length form and a shorter in-frame polypeptide that contains the N terminus.

To confirm the nature of the 38-kDa and 27-kDa polypeptides as full-length and N-terminally truncated forms of HSV-2 ICP34.5, respectively, we prepared Western blots from lysates of cells transfected with the HSV-2 construct p34.5-cDNA-3′HA or p34.5-gDNA-3′HA. Mock-transfected cells or cells transfected with the HSV-1 construct p34.5gDNA-3′HA(1) were specificity controls. The N-terminally directed antiserum revealed the expected 38-kDa and 27-kDa polypeptides in lysate of HSV-2 p34.5gDNA-3′HA-transfected cells (Fig. 3A), but the 27-kDa form was not detected in lysate of p34.5cDNA-3′HA-transfected cells, confirming the 27-kDa form as a translation product of the unspliced mRNA that terminates at a stop codon in the intron. Anti-HA antibody similarly revealed a band of 38 kDa, confirming this form as full-length ICP34.5 (Fig. 3A). Unexpectedly, anti-HA antibody also detected two bands with higher mobilities (approximately 27 and 26 kDa) in lysates of cells transfected with the HSV-2 cDNA or gDNA construct (Fig. 3A). We suspected that two additional polypeptide forms that contain C-terminal but not N-terminal epitopes were generated in p34.5gDNA-3′HA-transfected cells. Therefore, we prepared a Western blot of lysate from cells transfected with p34.5gDNA-3′HA, divided the membrane, and probed one half with antiserum to the N terminus and the other half with antibody to the C-terminal HA tag (Fig. 3B). The anti-34.5 antiserum yielded the expected bands at 38 kDa and 27 kDa, and the anti-HA antibody yielded bands at 27 kDa and 26 kDa. Thus, two additional polypeptides that contain the C terminus of HSV-2 ICP34.5, but not the N terminus, were revealed. They were designated C1 and C2.

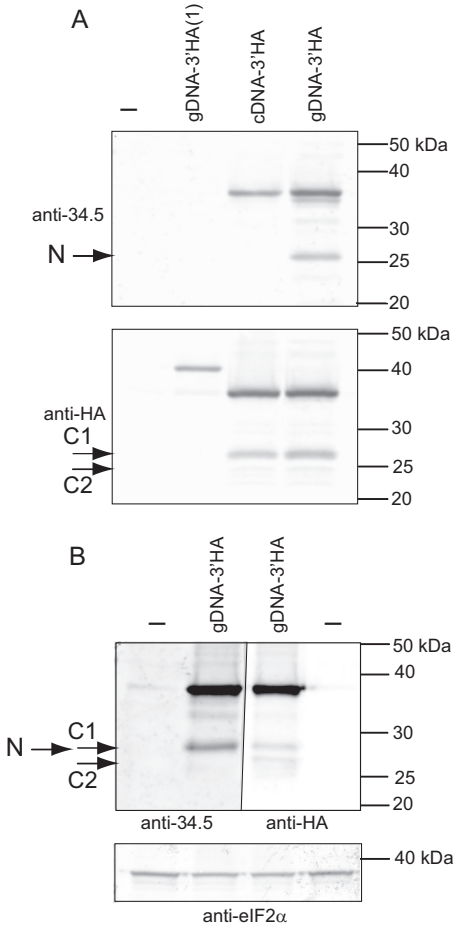

FIG 3 Several polypeptides are generated from the HSV-2 γ34.5 open reading frame. Vero cells were mock transfected (−) or transfected with an expression construct containing the HSV-1 or HSV-2 γ34.5 open reading frame fused to a C-terminal HA tag. Lysates were prepared for Western blotting at 24 h post-transfection. The membranes were directly probed (A) or divided and probed (B) with anti-34.5 antiserum or anti-HA antibody.

We investigated mRNA species transcribed from the γ34.5 open reading frame to better understand the origin of the 27-kDa and 26-kDa C-terminal forms. Using primers specific to the N and C termini of HSV-2 γ34.5, two products could be amplified by PCR after reverse transcription (RT) of total mRNA extracted from cells (data not shown). Cloning and sequencing verified that these products corresponded to full-length spliced and unspliced HSV-2 γ34.5 mRNAs. Of note, the sequence of the HSV-2 333

γ34.5 gene was similar to what had been previously reported in the GenBank database (accession number DQ149924.2) but contained an extra 12 nucleotides near the N terminus, adding 3 Arg residues and 1 Pro residue. To determine whether additional splice variants or internally initiated transcripts could account for the smaller C-terminal forms we had detected, cytoplasmic and nuclear mRNAs were isolated from cells 6 h postinfection, when robust expression of C1 and C2 is observed. The mRNAs were reverse transcribed, and a PCR forward primer in the 5′ untranslated region was paired with a series of reverse primers to walk along the entire γ34.5 gene. Only one species was amplified by RT-PCR from both the cytoplasmic and nuclear fractions using primers positioned upstream of the intron. Only two species were obtained from both the cytoplasmic and nuclear fractions using any reverse primer positioned downstream of the intron. Sequencing of the products cloned using a reverse primer in the 3′ untranslated region revealed that the larger species corresponded to unspliced γ34.5 mRNA and the smaller species represented the mature, spliced γ34.5 mRNA (cDNA). These results confirm that full-length, unspliced, and polyadenylated γ34.5 mRNA is found in the cytoplasm and provide evidence that only one spliced mRNA is produced and no other transcripts or splice variants are detected. Thus, the C-terminal forms of HSV-2 ICP34.5 do not appear to be generated by alternative splicing or transcript initiation at the internal ATG (codon 35) or noncanonical start codons.

To rigorously verify the natures of the four forms of HSV-2 ICP34.5, we generated three constructs: a 3′-HA-tagged gDNA construct containing a Myc tag at the original stop codon in the intron (p34.5gDNA-Myc-3′HA) (Fig. 1F), a construct consisting of sequences from the 5′ end of γ34.5 to a Myc tag at the original stop codon in the intron (p34.5-Myc) (Fig. 1G), and a 3′-HA-tagged cDNA construct (p34.5cDNA-3′HA) (Fig. 1H). Each of the constructs also contained a 5′ Flag tag. Infected or transfected cell lysates were prepared for Western blotting, and the divided membrane was probed with N-terminus-specific anti-ICP34.5 antiserum or with anti-Myc antibody. As expected, the anti-34.5 antiserum detected bands of 38 and 27 kDa in the lysate of HSV-2-infected cells, and in transfected-cell lysates, these bands had slightly lower mobilities due to the terminal tags (Fig. 4A). Three observations confirm and extend the recent conclusion of Tang et al. (40) that the 27-kDa band detected with N-terminus-specific antisera is a translation product of the unspliced mRNA that terminates at the stop codon in the intron. First, only full-length ICP34.5 was detected with anti-34.5 antiserum in the lysate of cells transfected with p34.5cDNA-3′HA (Fig. 4A). Second, the gDNA clone with a Myc tag placed at the original stop codon in the intron (p34.5gDNA-Myc-3′HA) yielded a product with mobility similar to that of the product of p34.5-Myc-3′HA, as detected using anti-34.5 antiserum (Fig. 4A). Third, this product was also detected by anti-Myc antibody used to probe the other half of a divided membrane (Fig. 4B). These observations provide definitive proof that the N-terminal form is generated by translation of the unspliced γ34.5 mRNA to the stop codon in the intron. Next, another membrane was divided, and the halves were probed with anti-34.5 antiserum (Fig. 4C) or antibody to the HA tag (Fig. 4D). The anti-HA immunoblot revealed full-length ICP34.5 and two faster-migrating isoforms in cells transfected with either the gDNA or the cDNA construct (Fig. 4D). In addition, while migration of the N-terminal form generated from the gDNA construct was retarded in the gel compared with the HSV-2-infected sample due to

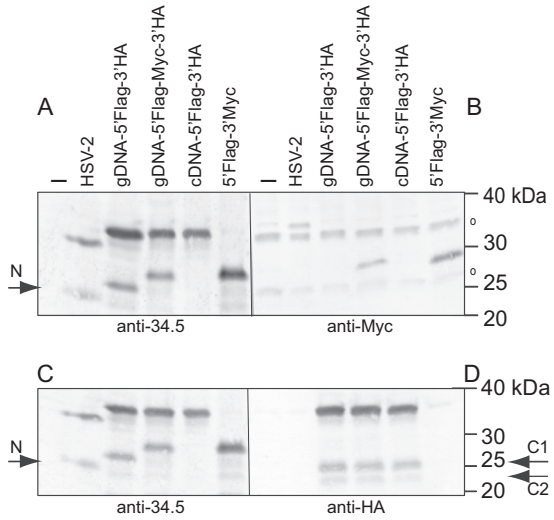

FIG 4 Generation of the ICP34.5 C-terminal forms is independent of the N-terminal form. Vero cells were infected with HSV-2 at an MOI of 5, transfected with empty vector, or transfected with plasmids expressing HSV-2 γ34.5 gDNA or cDNA with the indicated tags. The cells were lysed 16 h postinfection or 48 h posttransfection and prepared for Western blotting. The membranes were divided and probed using anti-ICP34.5 antiserum (A), anti-Myc antibody (B), anti-34.5 antiserum (C), or anti-HA antibody (D). The circles indicate background cellular Myc bands.

the Flag and Myc tags, migration of the C-terminal forms remained constant (Fig. 4D), ruling out the possibility that the anti-HA antibody cross-reacts with the N-terminal 27-kDa protein. These results indicate that the C-terminal forms of ICP34.5 do not contain N-terminal sequences and that generation of the C-terminal forms of ICP34.5 does not depend on sequences within the intron. Thus, although only two γ34.5 mRNA transcripts can be detected in infected cells, at least 4 forms of ICP34.5 are produced in frame from the HSV-2 γ34.5 ORF: one full-length form, one N-terminal form translated from the unspliced message, and two C-terminal forms generated from the spliced message by a mechanism that does not appear to involve alternative splicing or internal initiation.

A previous report had indicated the N-terminal form is synthesized only in HSV-2-infected and not in gDNA-transfected 293 cells (40); however, we observed the N-terminal form in Vero cells infected with HSV-2 and also to a lesser extent when the cells were transfected with p34.5gDNA-3′HA (Fig. 2). This apparent difference between Vero and 293 cells led us to examine 293 cells, and again, we detected the N-terminal form of ICP34.5 after p34.5gDNA-3′HA transfection in approximately the same ratio to full-length protein as in Vero cells (Fig. 5A). A second, independent lineage of 293 cells also produced the N-terminal form after p34.5gDNA-3′HA transfection, as well as HSV-2 infection (data not shown). Interestingly, after HSV-2 infection, more of the N-terminal form than of the full-length protein was produced in 293 cells than in Vero cells (Fig. 5A). We therefore surveyed other cell types to determine the extent to which production of the N-terminal form is suppressed by virus infection compared with p34.5gDNA-3′HA transfection. In infected mouse Neuro-2A and human SW1088 glioblastoma cells, the full-length and N-terminal forms accumulated in approximately equal proportions, but full-length ICP34.5 predominated over the N-terminal form after

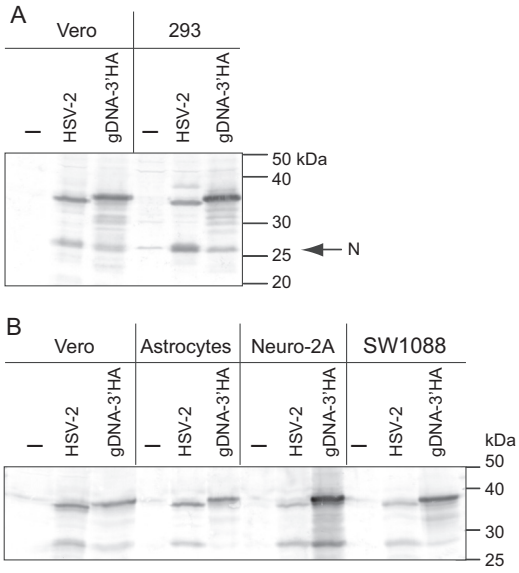

FIG 5 The N-terminal form of ICP34.5 is translated in a variety of cell types but to differing extents. The indicated cells were left untreated (−), infected with HSV-2 333 at an MOI of 5, or transfected with p34.5gDNA-3′HA. At 18 h postinfection or 24 h posttransfection, replicate monolayers were collected and processed for Western blotting. Proteins were detected using anti-34.5 antiserum in Vero and 293 cells (A) or Vero cells and the indicated mouse and human cell types (B).

p34.5gDNA-3′HA transfection (Fig. 5B). In contrast, mouse primary astrocytes readily produced the N-terminal form after infection, but very little accumulated in p34.5gDNA-3′HA-transfected cells (Fig. 5B). Importantly, these results demonstrate that while infection generally increases accumulation of the N-terminal form relative to full-length ICP34.5, all cell types tested, including 293 cells, also produced the N-terminal form from transfected p34.5gDNA-3′HA. Thus, suppression of splicing by a viral factor contributes to but is not required for expression of the N-terminal form.

The N-terminal form of HSV-2 ICP34.5 derives from translation of the unspliced mRNA, and coexpression of ICP27 has been implicated in suppression of HSV-2 γ34.5 mRNA splicing in 293 cells (40). To investigate the effects of ICP27 on production of the N-terminal form in infected cells, Vero cells were transfected with p34.5gDNA-3′HA and either superinfected with HSV-1 strains expressing or lacking ICP27 or cotransfected with plasmids expressing ICP27 (Fig. 6). In cells transfected with p34.5gDNA-3′HA, full-length and N-terminal forms of HSV-2 ICP34.5 were detected by Western blotting using HSV-2 ICP34.5-specific antiserum, and the full-length form predominated. Superinfection with *d*301, a replication-defective HSV-1 strain that expresses ICP27, caused greater accumulation of the ICP34.5 N-terminal form expressed from the plasmid (Fig. 6). In contrast, superinfection with *dl*27lacZ1, a replication-defective HSV-1 strain that does not produce ICP27, had no effect on accumulation of the N-terminal form expressed by the plasmid. Thus, infection with virus expressing ICP27 favors the generation of the N-terminal form of ICP34.5. Similarly, cotransfection of p34.5gDNA-3′HA and plasmids expressing HSV-1 or HSV-2 ICP27 enhanced production of the ICP34.5 N-terminal form (Fig. 6). The results were similar when the transfection was performed with Lipofectamine 2000

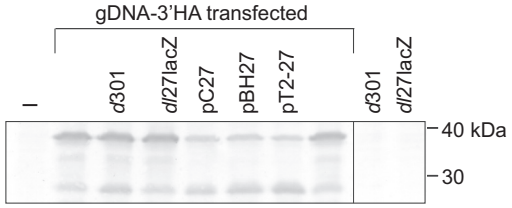

FIG 6 ICP27 is not required for expression of the ICP34.5 N-terminal form. Vero cells were mock transfected (−) or transfected with p34.5gDNA-3′HA and were either cotransfected with plasmids expressing ICP27 or subsequently infected at an MOI of 10 with the indicated virus. Additional mock-transfected cells were infected with HSV-1 strains as a negative control. At 24 h posttransfection (10 h after infection), lysates were prepared and subjected to Western blotting using anti-HA antiserum.

instead of Lipofectamine and Plus reagent (data not shown) or when the experiment was performed in 293 cells (data not shown). Thus, expression of ICP27 increased the ratio of N-terminal to full-length ICP34.5, but in all cases, expression of the N-terminal form was not dependent on ICP27.

Cells infected with the HSV-2 laboratory strain 333 synthesize the N-terminal form of ICP34.5 (Fig. 7), and Western blotting of infected cell lysates revealed that this N-terminal form is also expressed by HSV-2 laboratory strains 186 (shown in Fig. 8A) and G (data not shown). In addition, 9 out of 10 primary clinical isolates of HSV-2 expressed detectable amounts of the N-terminal form (Fig. 7). The amount of N-terminal form relative to full-length ICP34.5 varied between strains, as did the mobility of the N-terminal form. We confirmed the presence of a stop codon in the γ34.5 introns of all strains tested, and sequence information revealed that variation in the distance from the splice donor site to the stop codon likely accounts for strain-dependent differences in mobility. Thus, expression of the N-terminal form is highly conserved among HSV-2 strains.

We tested whether generation of the C-terminal forms of ICP34.5 is also conserved among HSV-2 strains. The γ34.5 genes from four of the HSV-2 clinical isolates and the laboratory strain 186 were cloned by PCR amplification with a C-terminal HA tag. These constructs were transfected into Vero cells, and lysates were prepared for Western blotting. Anti-34.5 antiserum again revealed full-length and N-terminal forms generated by each construct (Fig. 8A), including isolate 10, which had not produced a detectable N-terminal form during infection (Fig. 7). Anti-HA antibody revealed full-length ICP34.5, and also the C-terminal forms, in cells transfected with 34.5gDNA-3′HA clones generated from all four clinical isolates tested and HSV-2 laboratory strains

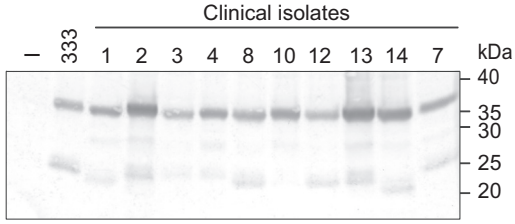

FIG 7 Synthesis of the ICP34.5 N-terminal form is ubiquitous among HSV-2 strains. Vero cells were infected for 18 h with the indicated HSV-2 laboratory strain 333 or primary clinical isolates at an MOI of 5, and the lysates were subjected to Western blotting using anti-34.5 antiserum.

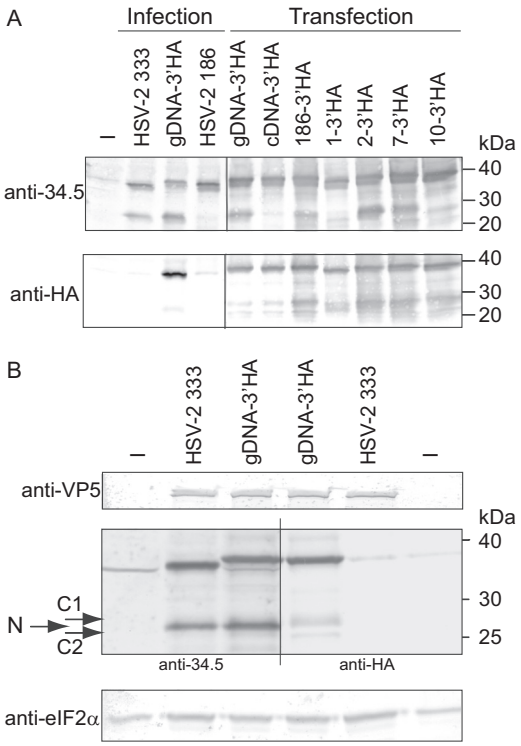

FIG 8 The C-terminal forms are generated from the γ34.5 genes of HSV-2 clinical isolates and also during natural infection. (A) Vero cells were infected at an MOI of 5 for 16 h with the indicated HSV-2 strains or transfected with plasmids containing γ34.5 cloned from HSV-2 laboratory strains or primary clinical isolates, each with a C-terminal HA tag. Western blotting was performed on lysates collected 24 h posttransfection using anti-34.5 antiserum or anti-HA antibody. (B) Vero cells were infected at an MOI of 5 for 16 h with HSV-2 strain 333 or recombinant virus 34.5gDNA-3′HA expressing HA-tagged ICP34.5, and the membrane was divided in two before Western blotting. Proteins from a second gel loaded in identical fashion were transferred to a membrane and probed with anti-VP5 and anti-eIF2α as virion and cell equivalency controls, respectively.

333 and 186 (Fig. 8A) and strain G (data not shown). Lysates of cells infected with HSV-2 333 or 186 were negative controls. Interestingly, the mobilities of the C-terminal forms varied slightly from strain to strain (Fig. 8A), likely due to small differences in the lengths and amino acid compositions of their sequences near the C terminus (M. Korom, unpublished data).

To determine whether the C-terminal forms are made during natural HSV-2 infection, we constructed a virus that encodes ICP34.5 fused to a C-terminal HA tag. A plasmid containing the HSV-2 333 γ34.5 gene flanked by portions of the 5′ and 3′ UTRs was altered to encode a C-terminal HA tag (pBS-34.5-3′HA) (Fig. 1I). This plasmid was cotransfected into Vero cells with full-length DNA from HSV-2 strain 333. A recombinant virus in which both γ34.5 genes had been replaced was identified by PCR and plaque purified to homogeneity. Multistep growth assays revealed that the virus, HSV-2 34.5gDNA-3′HA, replicates equivalently to the 333 parental strain (data not shown). To clearly define the identities and mobilities of the various HSV-2 ICP34.5 forms produced during natural infection, lysates of Vero cells were prepared 16 h after infection with HSV-2 333 or the 34.5gDNA-3′HA virus, and Western blots were performed on a divided membrane (Fig. 8B). As expected, anti-34.5 antiserum detected full-length ICP34.5 in both viruses, with mobility slightly retarded in the 34.5gDNA-3′HA lysate due to the HA tag. The N-terminal form of ICP34.5 was also detected at 27 kDa in lysates from both viruses. In cells infected with 34.5gDNA-3′HA, anti-HA antibody detected full-length ICP34.5, and also the C-terminal form C1 at 27.5 kDa and a less prominent C2 band at 26 kDa. Because the C1 and C2 forms generated by 34.5gDNA-3′HA infection contain an HA tag, their true mobilities likely would be slightly higher when produced by wild-type virus. Thus, the C-terminal forms are produced during natural HSV-2 infection.

Because we had observed cell-type-dependent differences in the amounts of the N-terminal form detected after transfection versus infection (Fig. 5 and 6), we surveyed accumulation of the N- and C-terminal forms of ICP34.5 in various cell types during the course of natural infection with HSV-2 34.5gDNA-3′HA (Fig. 9). Cell lysates were prepared at 3-h intervals from 3 to 24 h postinfection. In infected Vero cells, the full-length and N-terminal forms were present in equivalent proportions at 3 h postinfection, with accumulation increasing up to 9 h postinfection and remaining stable thereafter (Fig. 9A). The C1 and C2 forms were first detected at 6 h postinfection, and their accumulation peaked slightly later than that of the full-length and N-terminal forms. In 293 cells, the ratio of N-terminal to full-length forms was greater than in Vero cells, and C1 and C2 were detected later in the course of infection and accumulated only in small amounts (Fig. 9B). Next, we tested cell lines and primary cell types from tissues naturally targeted by HSV-2. In primary HFFs, accumulation of the N-terminal and both C-terminal forms was detectable by 6 h postinfection but waned after 9 h postinfection (Fig. 9C). The proportion of N-terminal to full-length forms was lower than in Vero cells. The human glioblastoma line SW1088 (Fig. 9D) and 2 other glioblastoma lines (data not shown) showed accumulation of N-terminal and C-terminal forms in a pattern that closely resembled Vero cells. Interestingly, in primary MEFs, accumulation of all forms appeared accelerated relative to other cell types, with expression detected by 3 h postinfection and then declining after 9 h postinfection (Fig. 9E). Accumulation of ICP34.5 forms in mouse primary astrocytes resembled what had been observed in the human glioma cell line, with much less accumulation of the C1 form than of the full-length form and virtual absence of the C2 form over most of the time course (Fig. 9F). In mouse primary cortical neurons, the full-length and N-terminal forms accumulated more slowly than other cell types, beginning 6 h postinfection and reaching a peak at 12 to 15 h postinfection. In addition, and in contrast to other mouse and human cell types tested, neither the C1 nor the C2 form could be detected (Fig. 9G).

Production of the C-terminal forms was conserved across most cell and tissue types, but the mechanism of their generation was unclear. In some cell types, the C-terminal forms appeared to arise slightly later than the full-length protein, suggesting they could be derived by proteolytic cleavage of full-length ICP34.5. We attempted to perform a pulse-chase experiment; however, very little HA-tagged protein could be isolated. Furthermore, the labeled N-terminal form of ICP34.5 coimmunoprecipitated with HA-tagged polypeptides and could not be conclusively distinguished from the C1 form. Therefore, we transfected cells with constructs containing mutations that alter expression of the full-length protein and assessed the effects on C1 and C2. Anti-34.5 antiserum revealed the expected full-length and N-terminal forms in lysates of cells transfected with the p34.5gDNA-3′HA construct, but only

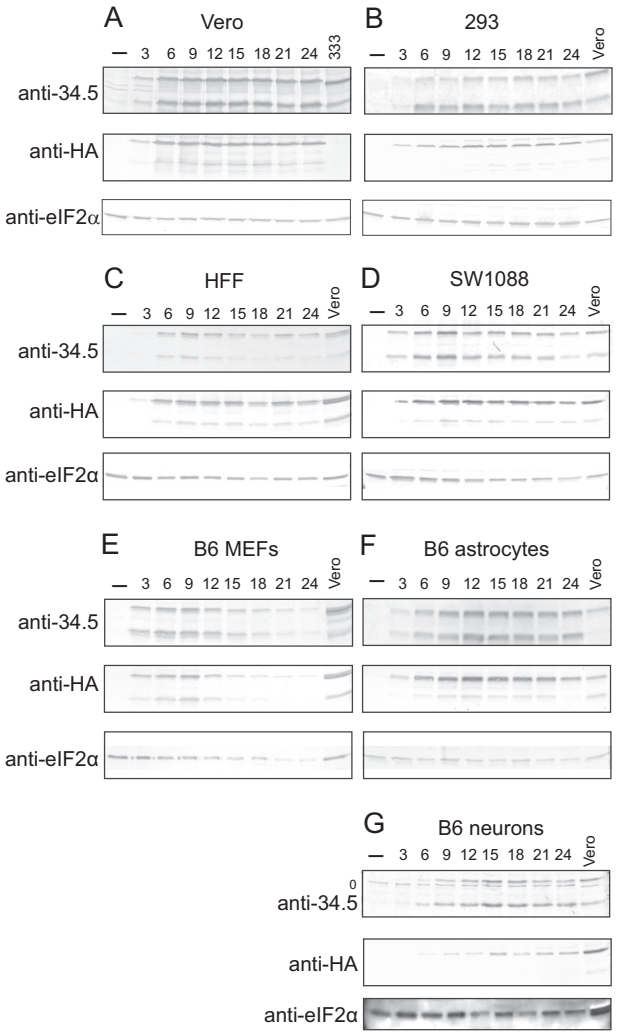

FIG 9 The C-terminal forms are produced in many cell types but not in some of CNS origin. The indicated cell types were mock infected (−) or infected at an MOI of 5 with recombinant HSV-2 expressing HA-tagged ICP34.5 (34.5gDNA-3′HA). At incremental times postinfection, cell lysates were prepared from replicate monolayers for Western blotting using anti-34.5 antiserum, anti-HA antibody, or anti-eIF2α as a loading control. Lysate of HSV-2 333- or 34.5gDNA-3′HA-infected Vero cells is shown as a positive control for expression of various forms of ICP34.5. (A and B) Primate Vero (A) and human 293 (B) cell lines. (C) Primary HFFs. (D) Human glioblastoma cell line SW1088. (E) Primary MEFs. (F) Mouse primary astrocytes. (G) Mouse primary neurons. The open circle indicates a nonspecific band. Infected Vero cell lysate is shown for comparison.

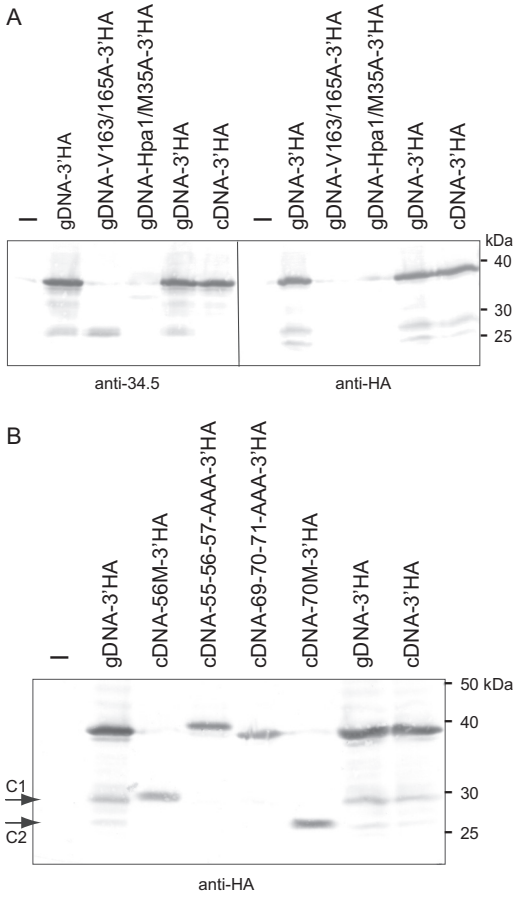

FIG 10 The C1 and C2 forms originate from full-length ICP34.5 at or near amino acid residues 56 and 70, respectively. (A) Vero cells were transfected with p34.5gDNA-3′HA or p34.5gDNA-3′HA containing the indicated mutations. p34.5cDNA-3′HA is shown as a splicing control. (B) Vero cells were transfected with p34.5cDNA-3′HA or the indicated N-terminal truncation mutants. Cell lysates were prepared 24 h posttransfection and subjected to Western blotting using anti-34.5 antiserum or anti-HA antibody, as indicated.

the N-terminal form was visible when p34.5gDNA-3′HA containing a mutation at the splice acceptor site was used (Fig. 10A). Anti-HA Western blotting of the same lysates revealed that C1 and C2 were generated from the gDNA construct, but not from the construct that ablates splicing. C1 and C2 also were not detected in lysates of cells transfected with p34.5gDNA-3′HA containing a stop codon at amino acid 13 and an M35A mutation that prevented expression of full-length ICP34.5 (Fig. 10A). Therefore, C1 and C2 generation requires expression of full-length ICP34.5. To identify the approximate start sites of the C1 and C2 forms and thus potential protease cleavage sites, a set of N-terminal deletions was generated from p34.5cDNA-3′HA so that various codons specifying internal amino acids were mutated to encode methionine. The mobilities of the expression products of these plasmids in SDS-PAGE were compared with those of C1 and C2 derived from cells transfected with the parent p34.5cDNA-3′HA. HA-tagged polypeptides detected in cells transfected with p56M-cDNA-3′HA and p70M-cDNA-3′HA migrated similarly to C1 and C2 (Fig. 10B). The product of an additional construct, p61M-cDNA-3′HA, showed intermediate mobility (data not shown). In addition, cells transfected with a plasmid expressing a triplet of alanine mutations at amino acids 55 to 57 produced the full-length, but not the C1 or C2, form (Fig. 10B). Similarly, a triplet of alanine mutations at amino acids 69 to 71 allowed expression of full-length ICP34.5 but ablated production of C1 and C2. These results provide further evidence that the C1 form likely originates within amino acids 55 to 57 and that the C2 start site likely lies within amino acids 69 to 71.

DISCUSSION

Minor translation products and proteolytic cleavage fragments are often generated by viruses, including the herpesviruses, particularly late in the course of infection. We have observed that up

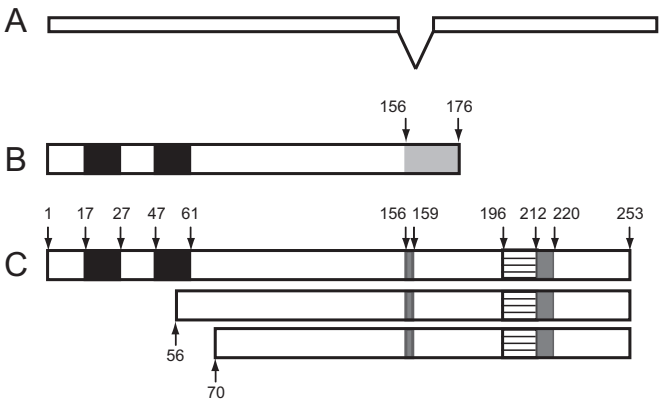

FIG 11 Summary diagram of HSV-2 ICP34.5 forms. (A) HSV-2 γ34.5 pre-mRNA. (B) N-terminal form of ICP34.5. The light-gray shading indicates translation extended along the pre-mRNA from the intron junction to the intron stop codon. (C) Full-length ICP34.5 and C1 and C2 forms. The black boxes indicate peptide sequences used to generate anti-34.5 antiserum. Residues conserved with HSV-1 ICP34.5 regions identified as PP1α binding and effector domains (dark-gray boxes) and the eIF2α binding domain (hatched box) are shown. Regions in HSV-2 ICP34.5 analogous to the HSV-1 ICP34.5 binding domains for beclin-1 (amino acids 60 to 85) and TBK1 (amino acids 63 to 102) are poorly conserved and therefore are not depicted.

to four forms of ICP34.5 are generated in HSV-2-infected and γ34.5-transfected cells: the full-length protein, an N-terminal form, and two C-terminal forms (Fig. 11). Importantly, the N-terminal form is robustly expressed in infected and transfected cells of a variety of types and species origins. The C-terminal forms, although they accumulate to a lesser extent, are detectable early as well as late in infection and are produced in a variety of mouse and human cells. The nearly absolute conservation of these forms among numerous HSV-2 strains tested and their accumulation over the course of the infection cycle in multiple cell types suggest the additional forms of ICP34.5 confer a functional advantage on the virus.

The N-terminal form of HSV-2 ICP34.5 was recently identified as a translation product of the unspliced mRNA, which terminates at a stop codon in the intron (termed ICP34.5β) (40). This conclusion was reached because the N-terminal form was not expressed from the cDNA clone of the mature spliced mRNA and because the protein product of a construct terminating at the intron stop codon showed the same mobility in SDS-PAGE as the 28-kDa band detected during HSV-2 infection. Our results corroborate and extend this finding by demonstrating that a Myc tag, placed at the location of the original intron stop codon in the gDNA clone, is detected in gDNA-transfected cells. Thus, we have conclusive evidence that the N-terminal form is translated from the primary unspliced mRNA and terminates at the stop codon in the intron.

All HSV-2 primary clinical isolates and three laboratory strains tested contained a stop codon in the γ34.5 intron, and 12 of 13 strains demonstrated the capacity to synthesize the N-terminal form of ICP34.5. This conservation is significant, considering that γ34.5 has the greatest sequence variability of any HSV gene (55). We also found that all cell types tested produced the N-terminal form of HSV-2 ICP34.5 during infection with 34.5gDNA-3′HA, often at levels nearly equivalent to those of the full-length protein. The N-terminal form typically was detected as early as 3 h postinfection, though initial detection was later in certain cell types. Peak accumulation occurred between 9 and 18 h postinfection and remained high through 24 h postinfection in all cell types tested except MEFs. Despite the conservation among HSV-2 strains and the high level of expression, the function of the N-terminal form remains enigmatic. Neurovirulence is a defining property of ICP34.5, but absence of the N-terminal form does not alter HSV-2 neurovirulence (reference 56 and K. Davis, unpublished data), a finding that may be related to the inability of this form to bind PP1α and eIF2α and thereby influence eIF2α phosphorylation (Fig. 11). In addition, binding sites for TBK1 and beclin-1 in the N-terminal portion of the HSV-1 ortholog are not well conserved. Further functional analysis of the HSV-2 ICP34.5 N-terminal form will be required.

We observed that some HSV-2 strains express a greater proportion of the N-terminal form than others (Fig. 7). ICP27 suppresses splicing of numerous cellular and some viral mRNAs (57–59), including splicing of the γ34.5 primary transcript in HSV-2-infected 293 cells, promoting synthesis of the N-terminal (ICP34.5β) form (40). In contrast to previous results (40), we observed expression of the N-terminal form in 293 cells after transfection of the gDNA construct in the absence of ICP27, and also in many other primate and murine cell types transfected with the gDNA construct. Thus, under a wide variety of conditions, production of the N-terminal form does not require ICP27-dependent silencing of γ34.5 mRNA splicing, though ICP27 does enhance its accumulation when expressed by transfection or infection (Fig. 6). Because the previous report and our current work utilized the same virus strain, cell type, and transfection reagent, we cannot speculate about the cause of the discrepancy. Variation in the amount of the N-terminal form expressed in different cell types could reflect several possible factors, including different rates of unspliced mRNA export to the cytoplasm. HSV-2 ICP27 does not confer leptomycin B resistance on HSV-1 (45), suggesting that other HSV-2 proteins may modulate nuclear export of viral proteins, and conceivably of viral mRNAs, as well.

The C-terminal forms of HSV-2 ICP34.5 are produced in both transfected and infected cells, and their production appears to be a feature conserved among the seven HSV-2 strains from which γ34.5 was cloned. C1 and C2 often accumulated in infected cells to a lesser extent than in transfected cells. This observation reinforces the contention that C1 and C2 derive from the full-length protein and thus are decreased in abundance when unspliced transcript predominates. A series of observations are consistent with this. (i) Preliminary mass spectrometry evidence indicated that the 5′ end of the C1 form lies upstream of the intron junction and has an amino acid sequence consistent with its generation from the spliced mRNA or the full-length protein product of the spliced mRNA (Korom, unpublished). (ii) ICP34.5 expressed from the intronless p34.5cDNA-3′HA construct produces the C-terminal forms (Fig. 3, 4, and 10). (iii) Mutation of the splice junction in γ34.5 abolishes expression of the full-length and C-terminal forms, suggesting the C-terminal forms are either translated from the mature mRNA or are cleavage products of the full-length protein (Fig. 10A). (iv) C1 and C2 are not generated if full-length protein cannot be synthesized (Fig. 10A). Gel mobilities of truncated ICP34.5 polypeptides expressed from N-terminal deletion mutants suggest a start site at approximately amino acids 55 to 57 for C1 and amino acids 69 to 71 for C2 (Fig. 10B). These regions are completely conserved among the HSV-2 laboratory strains

and clinical isolates we tested (Korom, unpublished). Additional transcripts and alternative splicing of the primary transcript are unlikely mechanisms of C1/C2 generation, because only two species of mRNA—the predicted spliced and unspliced forms—could be isolated from infected cells by us and others (40). The mRNA was analyzed 6 h postinfection, a time when the C-terminal forms were visible by Western blotting. We also searched rigorously for additional transcripts using primers located sequentially along the gene but found only the two predicted species. Generation of the C-terminal forms also is unlikely to represent internal translation initiation at noncanonical start sites (60–63), because potential alternative start codons in a strong Kozak context are positioned only near the N terminus and downstream of the splice junction in HSV-2 γ34.5. Therefore, we favor the hypothesis that the C-terminal forms are generated by proteolytic cleavage. A bioinformatics search for proteolytic cleavage sites identified the regions around amino acids 56 and 70 as containing a recognition motif for several cellular proteases but none with specificity solely for these sites, suggesting cleavage by a viral protease or virus-activated cellular protease with altered specificity or constraints due to protein secondary or tertiary structure. Blockade of proteasome function did not affect generation of C1 and C2 (data not shown). We did not detect C-terminal forms of HSV-1 ICP34.5 in cells transfected with the HSV-1 construct 34.5gDNA-3′HA(1) (Fig. 3A). Amino acid sequence alignment of HSV-1 and HSV-2 ICP34.5 revealed numerous differences in and around amino acids 55 to 57 and a gap in the HSV-1 sequence corresponding to amino acids 70 to 75 of HSV-2 ICP34.5, providing a likely explanation. Thus, the novel C1 and C2 forms of ICP34.5 appear to be unique to HSV-2. Unexpectedly, the 55-56-57 AAA and 69-70-71 AAA mutations each abolished generation of both C1 and C2 (Fig. 10A). The introduced mutations may have altered the conformation of this region of ICP34.5, rendering the neighboring intact site unsuitable for protease recognition. Indeed, the full-length products of the 55-56-57 AAA and 69-70-71 AAA constructs migrate with slightly different mobilities. Additional work will be needed to firmly establish the mechanism of C1 and C2 generation.

All cell types except mouse primary neurons produced the C1 form of ICP34.5. Similarly, the C2 form was not readily observed in mouse primary astrocytes (Fig. 9F), mouse primary neurons (Fig. 9G), or the human neuroblastoma line BE(2)C (data not shown). Thus, generation of the C-terminal forms of ICP34.5 appears to be partially cell type dependent. C1/C2 generation is not dependent on the cellular differentiation state, because it occurred in extraneural primary cells and in transformed cell lines. C1/C2 generation may, however, require an enzyme or other function associated with dividing cells, because primary neurons did not produce the C-terminal forms. Interestingly, relatively little C1 and C2 are produced in 293 cells, which may be of neural origin (64, 65).

Restraining ICP34.5 production in the nerve cell body is thought to be critical for suppression of neurovirulence and the capacity of HSV to establish latency in the neuron. Two microRNAs (miRNAs) processed from the HSV-1 and HSV-2 primary LAT transcript mediate this suppression (41, 42, 66). Functions associated with the C terminus of ICP34.5 include bridging phosphorylated eIF2α and PP1α to dephosphorylate the former and permit translation to proceed (30–32, 67). If the C-terminal forms function either independently of the full-length protein or in conjunction with it to prevent translational arrest, then lack of their production in neurons could further regulate virus replication and enhance establishment of latency. Although the C1 form is produced in mouse primary astrocytes, its relative abundance is low, and the C2 form is not produced, suggesting again that inefficient production of C-terminal forms in the nervous system disposes the virus toward regulated replication. Identification and ablation of the exact start sites of the C-terminal forms in HSV-2 γ34.5 will allow us to address the question of their contributions to HSV-2 virulence.

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