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Identification and Characterization of the Herpes Simplex Virus Type 2 Gene Encoding the Essential Capsid Protein ICP32/VP19c

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We describe the characterization of the herpes simplex virus type 2 (HSV-2) gene encoding infected cell protein 32 (ICP32) and virion protein 19c (VP19c). We also demonstrate that the HSV-1 UL38/ORF.553 open reading frame (ORF), which has been shown to specify a viral protein essential for capsid formation (B. Pertuiset, M. Boccara, J. Cebrian, N. Berthelot, S. Chousterman, F. Puvian-Dutilleul, J. Sisman, and P. Sheldrick, J. Virol. 63:2169–2179, 1989), must encode the cognate HSV type 1 (HSV-1) ICP32/VP19c protein. The region of the HSV-2 genome deduced to contain the gene specifying ICP32/VP19c was isolated and subcloned, and the nucleotide sequence of 2,158 base pairs of HSV-2 DNA mapping immediately upstream of the gene encoding the large subunit of the viral ribonucleotide reductase was determined. This region of the HSV-2 genome contains a large ORF capable of encoding two related 50,538- and 49,472-molecular-weight polypeptides. Direct evidence that this ORF encodes HSV-2 ICP32/VP19c was provided by immunoblotting experiments that utilized antisera directed against synthetic oligopeptides corresponding to internal portions of the predicted polypeptides encoded by the HSV-2 ORF or antisera directed against a TrpE/HSV-2 ORF fusion protein. The type-common immunoreactivity of the two antisera and comparison of the primary amino acid sequences of the predicted products of the HSV-2 ORF and the equivalent genomic region of HSV-1 provided evidence that the HSV-1 UL38 ORF encodes the HSV-1 ICP32/VP19c. Analysis of the expression of the HSV-1 and HSV-2 ICP32/VP19c cognate proteins indicated that there may be differences in their modes of synthesis. Comparison of the predicted structure of the HSV-2 ICP32/VP19c protein with the structures of related proteins encoded by other herpesviruses suggested that the internal capsid architecture of the herpes family of viruses varies substantially.

The herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) are closely related with respect to virus structure, biochemistry, and biology (62). The virion morphologies of the viruses are virtually indistinguishable (70). The genetic arrangement of the two viral genomes is colinear for the most part, and the viruses display significant nucleotide sequence identity (>50%) (40, 62). Comparisons of the primary amino acid sequences of analogous HSV-1 and HSV-2 proteins predicted from DNA sequence analyses have demonstrated that the structures of many cognate proteins are conserved to a great extent (11, 26, 37, 74). The HSVs share some similar biological properties including the ability to establish and reactivate from latent infection in neural tissues of the infected host, a broad host range in cultured cells, and a number of features of viral pathogenesis (62). However, the viruses differ with respect to the particulars of viral gene regulation (32, 56), the structures of specific genes and proteins (47, 48, 52, 63), their abilities to transform cells in culture (60), and their pathogenic potential in certain hosts and tissues (16).

The complete nucleotide (nt) sequence of one HSV-1 isolate (strain 17) has been determined (51). This virus has a genome of 152,260 nt which contains at least 72 probable protein-encoding open reading frames (ORFs). Additional ORFs have been identified in other HSV-1 strains (12, 78), and more are likely to be discovered. Still, it is probable that the total number of ORFs discerned will remain in general agreement with reported estimates of less than 100 viral proteins specified in infected cells (62). The viral proteins encoded by many of the HSV-1 ORFs have been identified, and their basic roles in the coordinately regulated α, β, γ cascade of viral gene expression have been determined (33–35, 51). This is particularly true of the viral α *trans*-acting proteins and of the viral β proteins that are involved in viral DNA replication and nt pool synthesis. However, many of the viral γ structural proteins have yet to be assigned to specific genes, and their roles in virion assembly or structure remain to be elucidated (62).

One such protein, infected cell protein 32 (ICP32), has been shown to correspond to virion protein 19c (VP19c) (8). This identification was based on characteristic differences in electrophoretic mobilities between the HSV-1 and HSV-2 ICP32/VP19c proteins and on the extremely tight genetic cosegregation of ICP32 and VP19c in studies of HSV-1/HSV-2 intertypic recombinants (4, 41). These genetic studies mapped the HSV-1 and HSV-2 ICP32/VP19c genes to a region of the viral genome very close to the gene encoding ICP6, the large subunit of the viral ribonucleotide reductase.

VP19c is a component of type A capsids, but the role of the protein in capsid structure or assembly is currently unclear (25, 28, 66). Type A capsids are found predominantly in the nucleus and have also been termed empty capsids because their centers are electron translucent and thus

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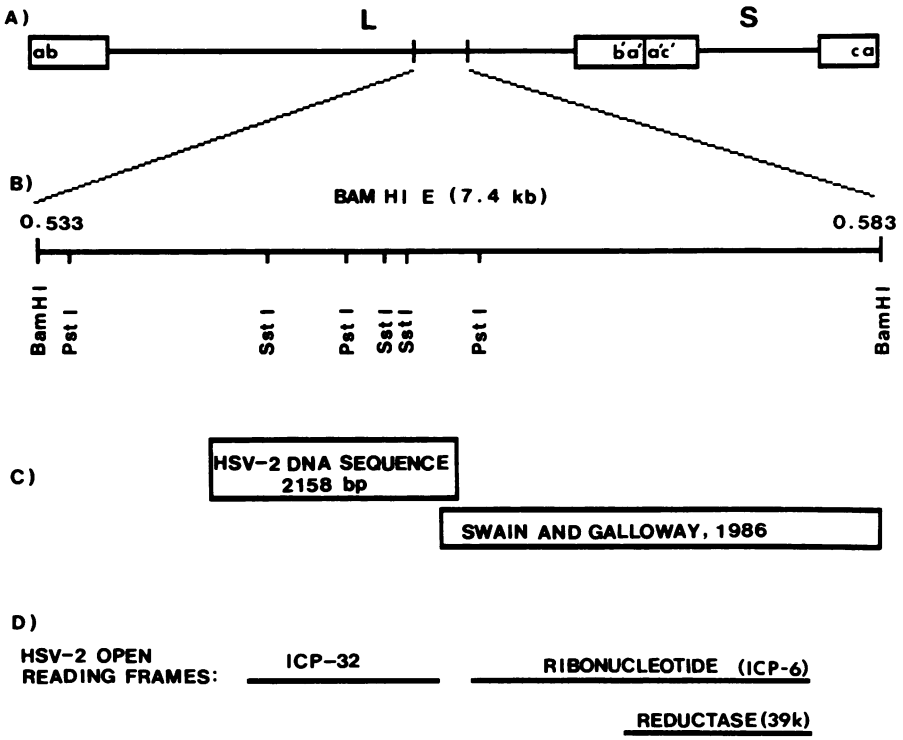

FIG. 1. (A) Position on the HSV-2 (G) genome of the DNA sequenced in this study. (B) Map locations of relevant restriction endonuclease sites. The *Bam*HI E fragment which maps from approximately 0.533 to 0.583 HSV-2 map units was subcloned as three *Sst*I fragments and two *Pst*I fragments. (C) Map position of the 2,104-base-pair (bp) region of HSV-2 DNA sequenced in these studies and its relation to published HSV-2 DNA sequences (74). (D) Location of the HSV-2 large ORF shown in this study to encode ICP32/VP19c is depicted relative to the gene encoding the large subunit of the viral ribonucleotide reductase (74).

appear to lack any internal core structure (25, 67). In addition to VP19c, type A capsids also contain four other proteins, VP5, VP23, VP24, and a small 12-kilodalton (kDa) protein (15, 17, 25, 27). VP5 composes the hexameric capsomers and appears to be linked to VP19c by a disulfide bond (19, 22, 73, 83). VP23 is exposed to the exterior of the virion and probably serves to connect the hexameric capsomers to one another (9). VP24 and the small 12-kDa protein have been assigned to the capsid interior, but their structural roles in the capsid remain obscure (9, 66). One report suggested that VP19c composes the 12 pentameric capsomers of the viral capsid (77). However, other reports have adduced VP19c to be an internal capsid protein (8, 9, 25, 67). One clue to the function of VP19c may lie in the observation that the HSV-1 and HSV-2 ICP32/VP19c proteins (after denaturation and partial renaturation) have a strong affinity for DNA (8). Whether this property of the ICP32/VP19c molecule(s) reflects the structural role of the protein(s) in the capsid or is functionally important to the processes of capsid assembly or viral DNA packaging remains to be determined.

Recently, Pertuiset et al. (58) reported on the physical mapping and nt sequence of an HSV-1 gene essential for capsid assembly. These investigators observed that a mutation in an ORF mapping from 0.553 to 0.565 HSV-1 map units (ORF.553), corresponding to the HSV-1 (strain 17) UL38 ORF, affects capsid assembly in a temperature-sensitive manner. However, the protein encoded by ORF.553 was not identified in these studies, which made it difficult for the authors to postulate a role for the ORF.553 gene product in capsid morphogenesis.

In this account, we report the nt sequence of a large ORF present in the HSV-2 genome that corresponds to the HSV-1 ORF.553 and UL38 ORFs. We demonstrate that this HSV-2 ORF encodes ICP32/VP19c, and we provide evidence that the cognate HSV-1 ICP32/VP19c molecule is the protein essential for capsid formation that is encoded by the UL38/ORF.533 HSV-1 ORF. In addition, the data demonstrate that there are type-specific differences in the regulation or processing or both of the HSV-1 and HSV-2 ICP32/VP19c molecule(s). The results of these studies resolve the location of the gene encoding ICP32/VP19c on the viral genome and provide detailed molecular information on which to base future studies of the structural and functional roles of ICP32/VP19c in capsid assembly and virion morphogenesis.

MATERIALS AND METHODS

Viruses and cells. The origin and properties of the HSV-1 (Justin) and HSV-2 (G) isolates used in these studies have been described previously (20, 21). Virus stocks were prepared and their titers were determined on HEp-2 cells (64). Crude cell lysates were prepared by infecting HEp-2 cells with 5 to 10 PFU per cell, and cultures were incubated at 37°C (5, 56).

DNA sequencing. The 7.4-kilobase (kb) *Bam*HI E fragment, map coordinates 0.553 to 0.583, of HSV-2 (G) DNA isolated from virions was cloned into pUC19 for DNA sequencing (57) (Fig. 1). Three *Sst*I fragments of 2, 1, and 0.3 kb and two *Pst*I fragments of 2.4 and 1.4 kb were subcloned into the polylinker region of M13mp20 and M13mp21 (Inter-

national Biotechnologies, Inc., New Haven, Conn.). Single-stranded and replicative-form DNAs were prepared as described previously (54).

Both strands of the HSV-2 DNA inserts were sequenced by the dideoxy-chain termination method (65). The first sequencing reactions were done with the M13 universal primer (International Biotechnologies). Subsequent reactions were done with custom primers (17 nt) derived from sequence generated in previous sequencing experiments. The oligonucleotide primers, all purified by high-pressure liquid chromatography, were purchased from Operon Technologies, Inc. (Alamita, Calif.). Both strands of all HSV-2 DNA inserts were completely sequenced, and computer methods were used to compile and analyze the data.

In initial experiments, considerable difficulty was experienced in obtaining readable sequence. This was because the secondary structures which formed in the G+C-rich DNA resulted in blocks to the Klenow polymerase as well as in compressions in the gel. Improved results were obtained with deoxy-7-deaza-GTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in place of dGTP (55) and strongly denaturing gels (7 M urea, 40% formamide) (49). The best results were obtained by using dIPT in place of dGTP (75), *Escherichia coli* single-stranded-DNA-binding protein (10), and Sequenase (modified bacteriophage T4 DNA polymerase) (United States Biochemical Corp., Cleveland, Ohio). It was not necessary to use formamide in the gels with the latter method. [^{35}S]dATP (Dupont, NEN Research Products, Boston, Mass.) was used as the radioactive label. The gels were fixed in 12% acetic acid–10% methanol, dried, and autoradiographed for approximately 15 h (7).

Generation of antipeptide antisera. The choice of oligopeptides was based on regions of amino acid identity conserved between the predicted primary amino acid sequence encoded by the HSV-2 ICP32/VP19c ORF and the predicted polypeptide product of the varicella-zoster virus type 20 (VZV-20) ORF (18). Predicted regional hydropathicity and structural mobility were also considered (13, 14, 36, 45, 61). Oligopeptides (12 to 15 amino acids long) were obtained from Biosearch (Palo Alto, Calif.). Peptides were coupled to keyhole limpet hemocyanin, and antisera were prepared essentially as described previously (1). Three rabbits were immunized for each peptide, and hyperimmune antisera were obtained (three bleedings per rabbit).

Preparation of 58,700-kDa TrpE fusion protein and generation of antiserum. The pATH10 plasmid was obtained from T. J. Koerner. This plasmid makes a 37,000-molecular-weight fragment of the TrpE protein under control of the *trp* promoter. The 1.4-kb *Pst*I fragment of HSV-2 DNA (Fig. 1) was ligated into the *Pst*I site at the 3′ end of the *trp* gene in pATH10 to produce a fusion gene and cloned by transformation of *E. coli* RR1 cells. The fusion protein synthesized by this recombinant clone should have 197 amino acids of the HSV-2 protein (amino acids 260 to 466) and a molecular weight of 58,700. The fusion protein was induced with 20 μg of 3-β-indoleacrylic acid (Sigma Chemical Co., St. Louis, Mo.) per ml and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fusion protein band was excised from the SDS-polyacrylamide gel, and 25 to 50 μg of purified protein was used to immunize rabbits (76).

PAA inhibition of viral DNA synthesis. Cells were treated with phosphonoacetic acid (PAA) at a concentration of 300 μg/ml of culture medium 4 h before infection, and this level was maintained throughout infection (82).

Radiolabeling of proteins and SDS-PAGE. Mock- and virus-infected cultures were radiolabeled with [^{35}S]methionine (>1,000 Ci/mmol; Dupont) at 20 μCi/ml in methionine-free medium. Polypeptides in cell lysates were electrophoretically separated on a 9.25% *N*,*N*′-diallyltartardiamide-cross-linked SDS-polyacrylamide gel (56).

Immunoblotting. Polypeptides separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (8). For autoradiography, blots were dried at 37°C for several hours before being exposed to Kodak XRP1 X-ray film at −70°C. For immunoblotting, membrane filters were blocked by incubation for 2 h in phosphate-buffered saline containing 3% nonfat milk powder and then reacted with rabbit antipeptide serum diluted in phosphate-buffered saline containing 1% gelatin for 2 to 12 h at room temperature. After being washed with phosphate-buffered saline containing 0.05% Tween 20, blots were reacted with biotin-conjugated goat anti-rabbit immunoglobulin G for 1 to 2 h at room temperature, followed by incubation for 30 min at room temperature with avidin-peroxidase complex (VectaStain; Vector Laboratories). Bound antibody was visualized by developing in horseradish peroxidase color development substrate solution (Bio-Rad Laboratories, Richmond, Calif.).

Computer analysis. Alignment of the predicted primary amino acid sequence of the HSV-2 ICP32/VP19c polypeptide employed several protein alignment programs (46, 72, 81), and minor adjustments were done by hand. The alignment of the predicted product of the Epstein-Barr virus (EBV) BORF1 ORF in regions that showed little homology with the other predicted proteins was arbitrarily biased toward maximizing conservative substitutions with little consideration of gap penalties. Protein hydropathicity indexes were predicted by the algorithms of Hopp and Woods (36) and Kyte and Doolittle (45).

RESULTS

DNA sequence analysis. To determine the nucleotide sequence of the portion of HSV-2 DNA deduced to encode the ICP32/VP19c protein, we sequenced the region of the viral genome mapping from approximately 0.553 to 0.565. *Sst*I and *Pst*I subfragments of the *Bam*HI E fragment were cloned into appropriate M13 vectors, and primer walking techniques were used to determine the DNA sequence. The organization of the region of the viral genome sequenced, including locations of pertinent restriction endonuclease sites, orientation with respect to previously published HSV-2 DNA sequences, and the provenance of the DNA sequences encoding the ICP32/VP19c and ICP6 (large subunit of ribonucleotide reductase) proteins is illustrated in Fig. 1.

The nucleotide sequence of the noncoding strand of the HSV-2 ICP32/VP19c gene is shown in Fig. 2. Only one 1,222-base-pair ORF (from nt 541 to 1938) large enough to encode ICP32/VP19c was identified in the sequenced region. This ORF contains two ATG codons located near the 5′ end, at nt 541 and 571, which could serve as translation start sites (42–44), and the ORF extends to the TGA stop codon beginning at nt 1939. Translation initiation and termination at these codons would result in polypeptides of 50,538 and 49,472 Da, both of which are in close agreement to previous size estimates for HSV-2 ICP32/VP19c (56). The predicted sequence of the larger polypeptide is shown in Fig. 2. The DNA sequences 5′ to the ORF contain several possible transcriptional regulatory elements. These are elucidated in the Discussion and are illustrated in Fig. 5. The consensus polyadenylation signals AATAAA and TGTGTTG (53, 59) appear in the sequence 121 and 150 nt 3′ to the ORF.

FIG. 2. Nucleotide sequence of the HSV-2 ICP32/VP19c gene. The nucleotide sequence of the ORF is depicted. The amino acid sequence of the predicted polypeptide is presented starting at the first ATG, which represents the first of two possible translation initiation sites (see text). The DNA sequence presented in this figure begins with the probable TATA box which occurs at nucleotide position 361 of the 2,158-base-pair sequence determined in this study. HSV-2 DNA sequences 5′ to this position are presented in Fig. 5. The consensus polyadenylation signals AATAAA and TGTGTTG associated with the ORF are underlined. The locations of the *Sst*I and *Pst*I restriction sites are indicated by asterisks (*).

Identification of ICP32/VP19c as the product of the HSV-2 ORF. (i) Immunoblotting experiments. The polypeptide product of the HSV-2 ORF was identified as ICP32/VP19c by immunoblotting experiments. These experiments (Fig. 3 and 4) utilized two types of antisera: (i) antisera directed against synthetic oligopeptides corresponding to portions of the predicted 50,538-molecular-weight polypeptide encoded by the ORF, and (ii) antisera directed against a TrpE/HSV-2 ORF fusion protein overexpressed and isolated from *E. coli*. Synthetic oligopeptides corresponding to regions of the predicted polypeptide encoded by the HSV-2 ORF that share sequence or structural identity with the predicted product of the VZV 20 ORF were synthesized and used to raise antisera in rabbits. The peptides synthesized and their locations in the predicted molecule were as follows: LTRQVTLTDLCQPNA (number 1), residues 110 to 124; ERAGALLLALRHPTD (number 2), residues 125 to 139; and TAVCSGPQEATHT (number 3), residues 264 to 276. All the antisera reacted with both the HSV-1 and HSV-2 ICP32/VP19c proteins, although antisera to peptides 1 and 2 showed some nonspecific binding to both cellular and other viral proteins (data not shown). The results (Fig. 3B) demonstrate that the peptide 2 antiserum reacts specifically with both the HSV-1 and HSV-2 ICP32/VP19c molecules in immunoblotting experiments. Antiserum was also prepared from rabbits inoculated with a TrpE/HSV-2 ORF fusion protein specifying the carboxy-terminal 197 amino acids encoded by the ORF. The immunoblotting experiments in Fig. 3C indicate that this antiserum directed against the fusion protein also displayed a high affinity for both the HSV-1 and HSV-2 ICP32/VP19c molecules. These results strongly reinforce the conclusions of the antipeptide immunoblotting experiments and again demonstrate that the product of the HSV-2 ORF must be ICP32/VP19c.

(ii) Kinetic class of ICP32/VP19c. The HSV-1 and HSV-2 ICP32 proteins have been classified as γ_2 proteins and as such depend on viral DNA synthesis for expression (29, 81). To ascertain the kinetic class of the HSV-1 and HSV-2 proteins reacting with the antisera, we performed immunoblot analyses on HSV-1 and HSV-2 ICPs produced in infected cultures treated with PAA. Figure 4A shows SDS-PAGE-separated ^{35}S-labeled polypeptides (18 to 20 h postinfection) extracted from untreated or PAA-treated, mock-infected HSV-1-infected, or HSV-2-infected HEp-2 cells. A comparison of the protein profiles of the untreated (lanes 1 to 3) and PAA-treated (lanes 4 to 6) infected cultures demonstrates that PAA-mediated inhibition of viral DNA synthesis dramatically decreased the amounts of both the HSV-1 and HSV-2 ICP32 molecules synthesized in infected cultures. The results of immunoblotting of the proteins produced in untreated and PAA-treated infected cultures (Fig. 4B) demonstrated that the HSV-1 and HSV-2 polypeptides that are immunoreactive with the antipeptide sera depend on viral DNA synthesis for expression.

Taken together, the results of these immunoblotting experiments demonstrate that ICP32/VP19c is the polypeptide

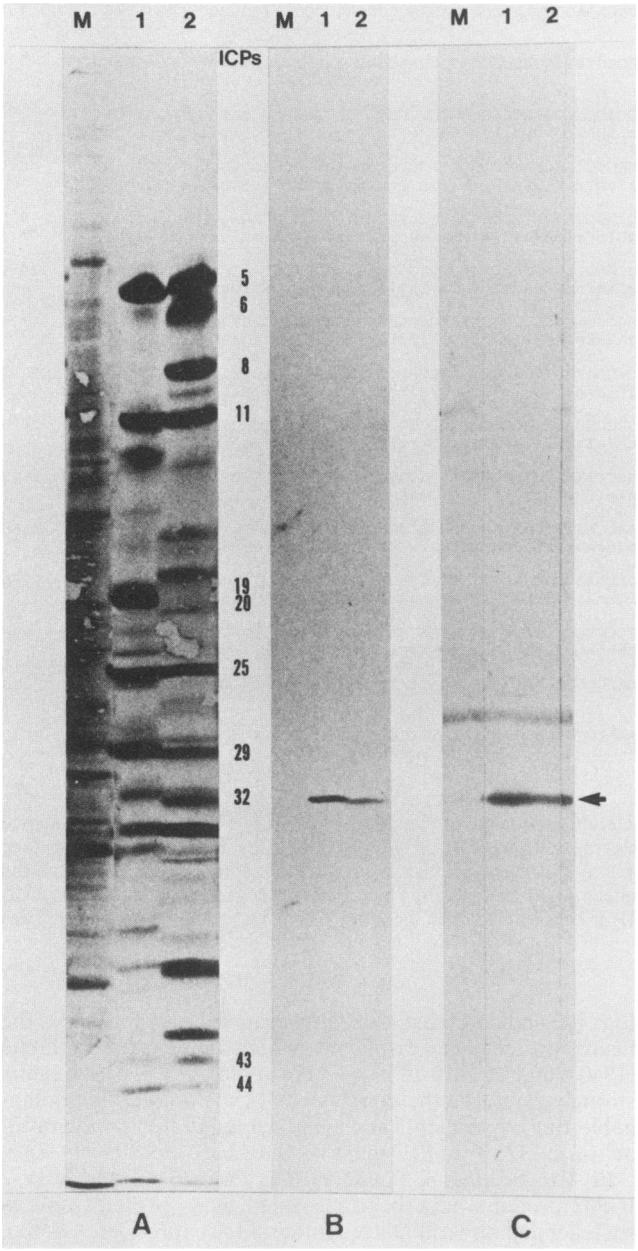

FIG. 3. Western immunoblots of HSV-1- and HSV-2-infected cell polypeptides. (A) ^{35}S-labeled (18 to 20 h postinfection) polypeptides produced in mock-infected (lane M), HSV-1-infected (lane 1), and HSV-2-infected (lane 2) cells, separated by electrophoresis through a 9.25% *N,N'*-diallyltartardiamide-cross-linked SDS-polyacrylamide gel, and transferred to nitrocellulose paper (see Materials and Methods). (B) Western blot of polypeptides produced in mock-infected (lane M), HSV-1-infected (lane 1), and HSV-2-infected (lane 2) cells with antiserum directed against the ERAGAL LLALRHPTD oligopeptide. (C) Western immunoblot of proteins produced in mock-infected (lane M), HSV-1-infected (lane 1), and HSV-2-infected (lane 2) cells with antiserum against the TrpE/HSV-2 ICP32/VP19c fusion protein. The arrow indicates the position of the HSV-1 and HSV-2 ICP32/VP19c bands.

product of the HSV-2 large ORF mapping immediately upstream of the gene encoding the large subunit of the viral ribonucleotide reductase. These studies also provide strong evidence that the HSV-1 UL38/ORF.553 ORF (51, 58) must encode the cognate HSV-1 ICP32/VP19c.

DISCUSSION

In this report, we characterized the HSV-2 gene encoding infected cell protein 32 (ICP32) and virion protein 19c (VP19c). We also demonstrated that the HSV-1 UL38 and ORF.553 ORFs, which have been shown to specify a protein essential for capsid formation (58), must encode the cognate HSV-1 ICP32/VP19c protein.

Comparison of ORFs encoding HSV-2 and HSV-1 ICP32/VP19c proteins. Comparison of the HSV-1 and HSV-2 DNA sequences encoding their respective ICP32/VP19c molecules indicates that the overall organization of the two genes is quite similar. However, there are a number of differences between them. The two DNA sequences display 66% (413 of 622 nt) overall identity, with 164 mismatched and 45 unmatched bases. Conserved features include the putative translation start sites for the genes encoding ICP32/VP19c and ICP10 (51), two TATA box-like structures which occur close to the transcription initiation sites for the 3.6-kb (ICP10) and 1.9-kb (ICP32) mRNAs previously mapped in this region of the HSV-1 genome (58), and a conserved SP1 transcription factor-binding site (HSV-2 position 344). A major difference is the presence in the HSV-2 sequence of several small oligonucleotide stretches that have no counterparts in the HSV-1 sequence. These occur in the region between the conserved TATA boxes that probably represents truly intergenic DNA sequences. Other dissimilarities include the presence in the HSV-2 ICP32/VP19c ORF of a second potential translation initiation site (ATG) and the occurrence of possible internal transcription factor-binding sites within the HSV-1 ORFs predicted to encode ICP32/VP19c and ICP10 (Fig. 5).

To date, most of the studies of HSV late gene expression have focused on the regulation of three HSV-1 γ_2 genes, US11 (39), glycoprotein C (2, 30, 31), and a gene encoding a 70-kDa regulatory protein (50, 69). These studies have shown that γ_2 gene expression depends on viral DNA replication and can be authentically examined only in the context of the viral genome (2, 69). Within the virus genome, the promoter-regulatory sequences important to the expression of the glycoprotein C gene map between −34 and −19 nt relative to the transcription start site (30, 31). In addition, analyses of glycoprotein C genes resident in cells have shown that sequences downstream of +22 are needed for γ_2-specific regulation (2), suggesting that a complex array of *cis*-acting elements is involved in γ_2 gene regulation. The HSV-1 and HSV-2 DNA sequences display approximately 76% nucleotide identity in the 5′ regions of the ORFs.

Nucleotide sequence variability in the promoter-regulatory elements of the HSV-1 and HSV-2 genes may be reflected in the fact that the synthesis of the HSV-1 and HSV-2 proteins is affected differently by PAA. After PAA treatment of infected cells, a small amount of HSV-1 ICP32 was detected on immunoblots with the antipeptide sera, while no HSV-2 ICP32 was detected under the same conditions (Fig. 4). While it is possible that these viruses vary in their sensitivity to DNA synthesis inhibitors, studies of late gene expression in other virus systems (80) suggest that the sequence variation observed in the promoter-regulatory regions of the HSV-1 and HSV-2 ICP32/VP19c genes is sufficient to generate minor differences in the levels of expression of the two genes.

The occurrence of a second possible translation initiation site in the HSV-2 ICP32/VP19c ORF may account for the observed differences in the synthesis or processing or both of the proteins. Thus, the HSV-1 and HSV-2 ICP32/VP19c

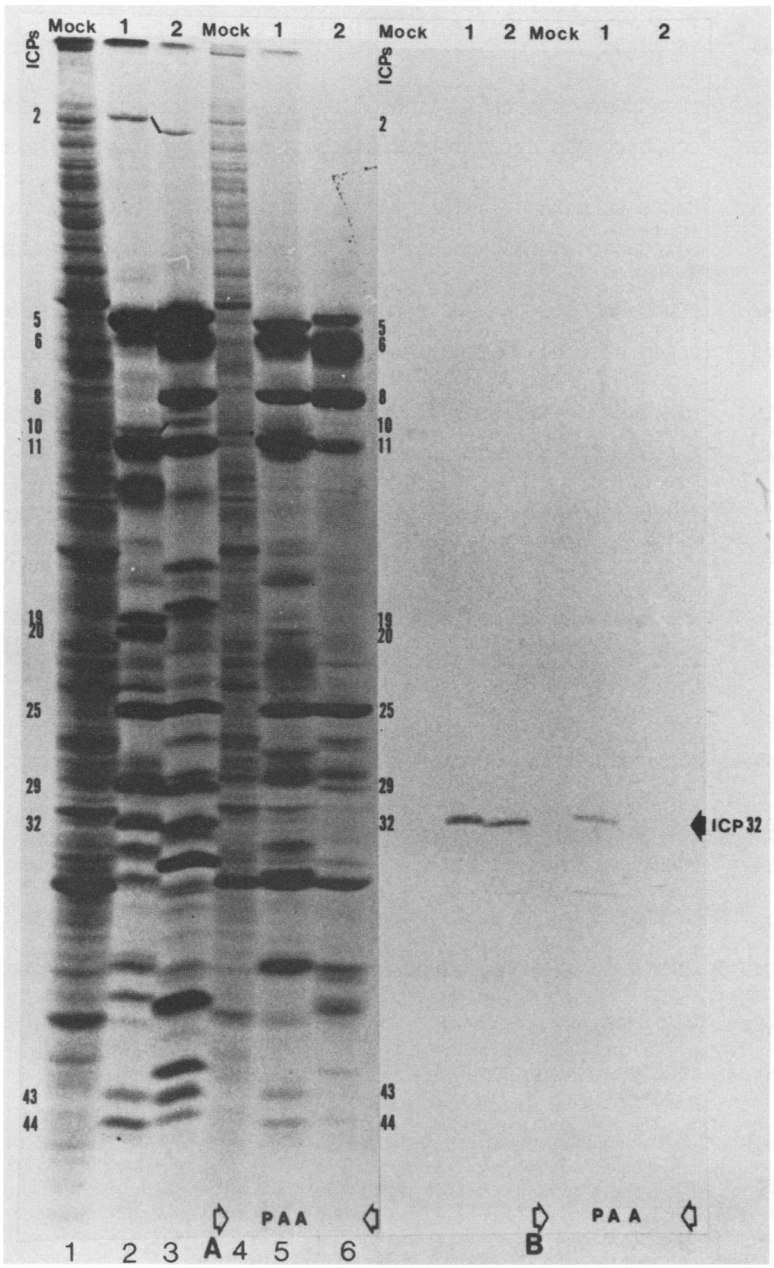

FIG. 4. Effect of PAA on Western immunoblots of HSV-1- and HSV-2-infected cell polypeptides. (A) Polypeptide profiles obtained after SDS-PAGE (9.5% *N,N'*-diallyltartardiamide) of ^{35}S-labeled (18 to 20 h postinfection) proteins synthesized in mock-infected (lane M), HSV-1-infected (lane 1), and HSV-2-infected (lane 2) cultures (lanes 1 to 3) or in identically processed cultures treated with 300 μg of PAA per ml throughout infection (lanes 4 to 6). (B) Western blot of polypeptides produced in untreated and PAA-treated mock-infected (lane M), HSV-1-infected (lane 1), and HSV-2-infected (lane 2) cultures after reaction with rabbit antiserum directed against the ERA oligopeptide.

proteins differ with respect to electrophoretic mobilities (Fig. 3 and 4) even though the HSV-1 UL38 ORF is predicted to encode a 465-amino-acid polypeptide of 50,269 Da and the HSV-2 ORF is predicted to encode a similarly sized 466-amino-acid polypeptide of 50,538 Da. These electrophoretic differences were previously noted by Morse et al. (56), who estimated the size of HSV-1 ICP32 at 51,500 Da and that of HSV-2 ICP32 at 50,500 Da. A straightforward manner of reconciling the predicted sizes of the HSV-1 and HSV-2 ICP32 proteins with their experimentally determined sizes would be to assume that the HSV-1 protein is more extensively modified than the HSV-2 protein by posttranslational phosphorylation or other additions. However, early in infection, the HSV-2 protein displays an electrophoretic mobility equivalent to that of HSV-1 ICP32, but as infection progresses, the mobility of the HSV-2 protein appears to increase while that of the HSV-1 protein remains constant (data not shown). In addition, two to three radiolabeled bands were detected in infected cell extracts after SDS-PAGE that seem to be intermediates between the faster and slower forms of HSV-2 ICP32. It is important that only the smallest of these seemingly related forms of the HSV-2

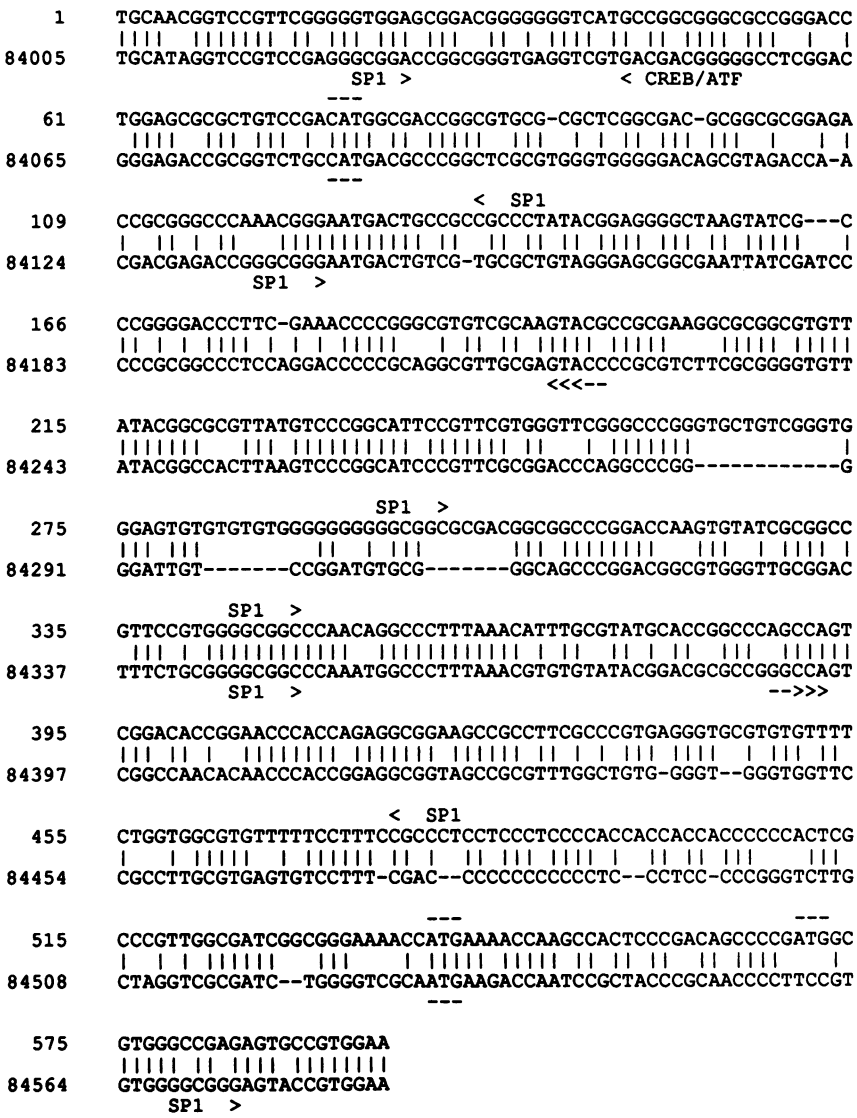

FIG. 5. Comparison of the HSV-2 DNA sequences upstream of the ICP32/VP19c gene with the HSV-1 (strain 17) UL37-UL38 intragenic region (51). The HSV-2 DNA sequences upstream of the gene encoding ICP32/VP19c are aligned with nt 84005 to 84585 of the HSV-1 (strain 17) DNA sequence. This region of HSV-1 DNA contains portions of the UL37 and UL38 ORFs together with intergenic DNA sequences. Translational start sites for the HSV-1 and HSV-2 ICP32- and ICP10-encoding ORFs are indicated by the underscored and overscored ATGs and CATs, respectively. A second possible translation start site occurring in the HSV-2 ICP32 ORF is also indicated. Transcription initiation sites and orientation of transcripts mapped for the HSV-1 1.9-kb (ICP32) and 3.6-kb (ICP10) mRNAs (58) are denoted by triple-headed arrows. The locations of potential transcription factor-binding sites identified by a computer search for 44 such sites are denoted by the triple-headed arrows.

ICP32/VP19c protein has been identified as a constituent of the capsid (8). There are a number of possible mechanisms that could generate multiple forms of HSV-2 ICP32 at different times postinfection. The most plausible would appear to be a switch to an alternate site of translational initiation late in virus infection (24). Posttranslational additions to the resultant smaller polypeptide might then give rise to the intermediate-size bands. In support of this interpretation, the HSV-2 ICP32/VP19c ORF does contain a second ATG codon 30 nt downstream of the first, within a Kozak milieu (42–44), which might function as an alternate translation initiation site. The HSV-1 ICP32/VP19c ORF lacks such a second ATG. Alternatively, the different-sized HSV-2 ICP32/VP19c proteins might arise from proteolytic processing of a larger precursor into progressively smaller forms of the protein. However, the cognate HSV-1 protein does not appear to be subject to such processing, and computer analysis of the predicted HSV-1 and HSV-2 proteins with respect to known protease cleavage sites failed to reveal any substantial differences in expected protease sensitivities between the two molecules. Also, the predicted HSV-2 polypeptide does not contain cleavage sites for known proteases that might generate cleavage products similar in size to the observed HSV-2 ICP32/VP19c molecules.

The HSV-2 DNA and predicted protein sequences determined in this study were analyzed with respect to significant

A

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                10        20        30        40        50        60        70        80        90       100
HSV2        MK----TKPLPTAPMAWAESAVE----TTTGPRELAG---HAPLRRV---LRPP-IARRD---GPVLLGDRA-PRRT--ASTMWLLGIDPAESSPGTRAT
HSV1 UL38   MK----TNPLPATPSVWGGSTVE-L-PPTT--RDTAGQ---GLLRRV---LRPP-ISRRD---GPGLPRGSG-PRRA--ASTLWLLGLDGTDAPPGALTP
VZV  20     MGSQP-TNSHFTLNEQTLCGTNISLLGNN---RFI--QIGNGLHMTYAPGFFGNWS--RDLTIGPRFGGLNKQPIHVPPKRTETASIQVTPRSIVINRMN
EBV BORF1                                                                                              MKVQGSV
CONSENSUS

               110       120       130       140       150       160       170       180       190       200
HSV2        LDDTEQAVDK-ILRG-ARRAGGLTVPGAPRY-----HLTRQVT-LTDLCQPNAERAGALLLALRHPTDLPHLAR-----H---RAPPGRQTERLAEA-WG
HSV1 UL38   NDDTEQALDK-ILRG-TMR-GGALI-GSPRH-----HLTRQVI-LTDLCQPNADRAGTLLLALRHPADLPHLA------HQ--RAPPGRQTERLGEA-WG
VZV  20     NI---QINPTSIGNP-Q--VTIRLPLNNFKS---TTQLIQQVS-LTDFFRPDIEHAGSIVLILRHPSDMIGEA---NTLTQAGRDPDV-LLEGLRNL-FN
EBV BORF1   DRRRLQRRIAGLLPPPARRLNISRGSEFTRDVRGLVEEHAQASSLSAAAVW---RAG--LLAPGEVAVAGGGSGGGSFSWSGWR-PPVFGDFLIHASSFN
CONSENSUS        Q     *                 *          Q   L*        *AG  *L     *     *          R P*       *

               210       220       230       240       250       260       270       280       290       300
HSV2        QLLEASALGSGRAESGCARAGLVSFNFLVAAC-AAAYDARD-AA--EAVRAHI--TTN---YGG--------TRAGARLDR-FSECLRAMVHTHVFPHEV
HSV1 UL38   QLMEATALGSGRAESGCTRAGLVSFNFLVAAC-AASYDARD-AA--DAVRAHV--TAN---YRG--------TRVGARLDR-FSECLRAMVHTHVFPHEV
VZV  20     -AC--TAPWTV-GEGGGLRAYVTSLSF-IAACRAEEYTDKQ-AA--DANR-----TAIVSAYGC--------SRMETRLIR-FSECLRAMVQCHVFPHRF
EBV BORF1   NA-EATGTPLFQFKQSDPFSG-VDAVFTPLSLFILMNHGRGVAARVEAGGGLTRM-ANLL-YDSPATLADLVPDFG-RL----------VADRRF-HNF
CONSENSUS        **           *      F   **       *  AA  *A       *    Y              RL           V  * F H

               310       320       330       340       350       360       370       380       390       400
HSV2        MRFFGGLVSWVTQDELASVTAVCSGPQEATHTGHPGRPRSAV--TIPACAFVDLDAELCL-GGPGAA---FLYLVFTYR----QCREDQ-ELCCVYVVKS
HSV1 UL38   MRFFGGLVSWVTQDELASVTAVCAGPQEAAHTGHPGRPRSAV--ILPACAFVDLDAELGL-GGPGAA---FLYLVFTYR----QRR-DQ-ELCCVYVIKS
VZV  20     ISFFGSLLEYTIQDNLCNLTAVAKGPQEAARTDKTSTRRVTA--NIPACVFWDVDKDLHLSAD-GL-KHVFL--VFVYTCF--QRR--QREGVRLHLALS
EBV BORF1   ITPVGPLVENIKSTYLNKITTVVHGPVVS----KAI-PRSTVKVTVPQEAFVDLDA--WLSGGAGGGGGV----CFVGGLGLGQPCPAD--A-RLYVAL-
CONSENSUS   *   G L*          *T*V  GP  *    *   PR *    *P   F D*D    L *G G          *       Q        * *

               410       420       430       440       450       460       470       480       490       500
HSV2        QLPPRGLEAALERLFGRLRITNTIHGAEDMT-PPPPNRNVDFPLAVLARASSQSPR--CSASQVTNPQF-V--DRLYRWQPDLRGRPTARTCTYAAFAEL
HSV1 UL38   QLPPRGLEPALERLFGRLRITNTIHGTEDMT-PPAPNRNPDFPLAGLA-ANPQTPR--CSAGQVTNPQF-A--DRLYRWQPDLRGRPTARTCTYAAFAEL
VZV  20     QLNEQCFGRGIGFLLGRIRAENAAWGTEGVANTHQPYNTRALPLVQLS-NDPTSPR--CSIGEITGVNWNLARQRLYQWTGDFRGLPTQLSCMYAAYTLI
EBV BORF1   ----TYEEAGPRFTFF-----------------------------------QSSRGHCQIMNI---------LRIYYS-PSI-------MHRYAVV---
CONSENSUS           *                                          * R  C    *          R*Y                YA

               510       520       530       540       550       560
HSV2        GVMPDDSPRCLHRTE-RFGAVGVPVVI-LEGVVW--RPGGWRACA
HSV1 UL38   GMMPEDSPRCLHRTE-RFGAVSVPVVI-LEGVVW--RPGEWRACA
VZV  20     GTIPSESVRYTRRME-RFGGYNVPT-IWLEGVVWGGTNT-WNECYY
EBV BORF1   --------QPLHIEELTFGAVACLGT-FSATDGWRRSAFNYRGSSLPVVEIDSFYSNVSDWEVIL
CONSENSUS           * E  FG*  *       *  W      *
```

B

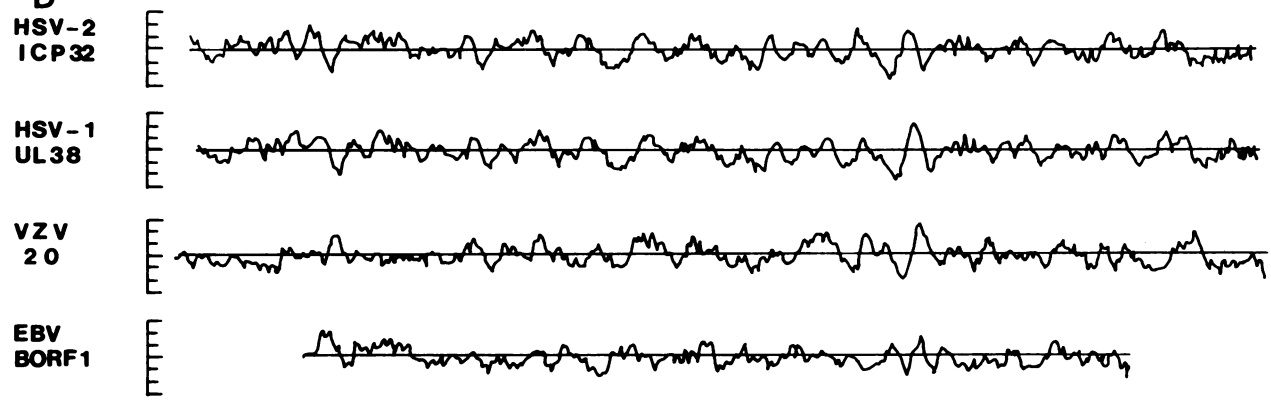

FIG. 6. (A) Comparison of the predicted amino acid sequence of the HSV-2 ICP32/VP19c protein with related gene products from other herpesviruses. The predicted amino acid sequence of the HSV-2 ICP32/VP19c molecule is aligned with the products of the HSV-1 UL38 (51), VZV 20 (18), and EBV BORF1 (3) ORFs. Residue assignments are given in the single-letter amino acid code. The consensus sequence indicates positions in which all four predicted proteins display amino acid identity or conservative substitution (*). (B) Comparison of the hydropathicity plots of the VP19c-related herpesvirus proteins. This comparative alignment of hydropathicity plots of the predicted products of the ORFs encoding the related herpesvirus proteins is based on the results of primary sequence alignment presented in panel A.

homologies to known oncogenes or tumor suppressor protein-binding motifs because this ORF, which encodes a DNA-binding protein, maps to a region of the viral genome (mtr-III) implicated in cellular transformation (6, 23, 38, 68). No significant homologies were found.

Structure and functions of ICP32/VP19c-related herpesvirus proteins. Several reports have noted that the sequences of the predicted proteins of the HSV-1 UL38/ORF.553 (51, 58), VZV 20 (18), and EBV BORF1 (3) ORFs are significantly related. Computer analysis of the predicted HSV-2 ICP32/VP19c sequence indicated that it also is a member of this family of viral proteins (Fig. 6A). The HSV-2 protein displays significant amino acid identity with the HSV-1 (78%, 466-amino-acid overlap), VZV (29%, 362-amino-acid

overlap), and EBV (20.2%, 119-amino-acid overlap) proteins. Much of the sequence variation in this group of proteins occurs in the region equivalent to the amino-terminal 100 to 120 amino acids of the HSV-2 ICP32 protein. Such amino-terminal variation has been noted before in other comparisons of related groups of herpesvirus proteins (11, 26, 74). However, the observation that the predicted EBV BORF1 product appears to be completely missing an equivalent protein domain, coupled with the hypothesis that the HSV-2 protein may exhibit alterations in this region due to alternative translational initiation late in infection, suggests that this region of the molecules is not essential for protein function. Analysis of the primary amino acid sequences of this family of viral proteins failed to reveal any obvious structural motifs that might explain the previously reported DNA-binding properties of the HSV-1 and HSV-2 VP19c molecules (9).

Comparative hydropathicity analysis (45) (Fig. 6B) of the ICP32/VP19c family of herpesvirus proteins illustrates two important points. First, with respect to hydropathicity, the HSV-1, HSV-2, and VZV proteins appear remarkably similar beyond the first 100 or so amino acids. Most of the amino acid substitutions that have occurred among these three viral proteins have been very conservative. The physical and biochemical properties of these proteins might also be expected to be conserved. Second, while the EBV BORF1 protein sequence is significantly related to the other proteins, it has diverged considerably with respect to size and predicted physical properties. This variance is not untoward given the postulated evolutionary relationships between the alpha Herpesviridae (HSV-1, HSV-2, and VZV) and the gamma Herpesviridae (EBV) (32). However, the extent of variation is somewhat surprising in a gene that presumably encodes the EBV counterpart of the essential capsid structural protein VP19c. It will be of interest to examine the DNA sequences of the members of the related beta Herpesviridae to determine whether they also encode a protein related to VP19c.

The role of the VP19c molecule in capsid structure and virion assembly is currently unclear. The available evidence is as follows. (i) VP19c is an integral structural part of type A capsids (25, 66) and is essential for capsid formation (59). (ii) It may form a disulfide bond to VP5, the major capsid protein that makes up the hexameric capsomeres (19, 22, 83). (iii) Both HSV-1 and HSV-2 VP19cs after denaturation and partial renaturation display affinity for DNA (8). (iv) VP19c has been reported to be both a component of pentameric capsomeres (78) and an internal virion protein (8, 9, 25). If VP19c does compose the HSV pentameric capsomeres, it obviously has an essential role in capsid assembly, and its juxtaposition to the hexameric capsomeres might allow for disulfide bonding to VP5. However, the DNA-binding properties of the HSV-1 and HSV-2 VP19c molecules seem difficult to reconcile with a pentamer assignment. Also, if the predicted product of the EBV BORF1 gene has an analogous role in the structure of the EBV capsid, then it would appear that a rather different structural solution to pentamer assembly must occur in the EBV capsid compared with the HSV capsids. If, on the other hand, a significant portion of VP19c is located in the capsid interior, then it may contribute to the band of mass density shown by Schrag et al. (66) to lie directly beneath the hexameric capsomeres. VP19c might thus form part of an essential matrix holding the hexamers together, and it might easily form disulfide bonds to VP5 (66). In this position, VP19c might also be oriented in such a manner as to present a DNA-binding domain to the interior of the capsid, thus conferring a strong affinity for DNA to the interior surface of the empty type A capsid. Such a molecular arrangement could be important for the processes of viral DNA packaging or virion core formation or both. In addition, if all the VP19c-related herpesvirus proteins function similarly, then the divergent structure of the product of the EBV BORF1 gene, in particular, suggests that the internal capsid structures of the herpes family of viruses differ substantially. Such variations in internal capsid architecture may reflect or influence the size of the viral genomes packaged by each of the herpesviruses.

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