

# Efficient herpes simplex virus type 1 (HSV-1) capsid formation directed by the varicella-zoster virus scaffolding protein requires the carboxy-terminal sequences from the HSV-1 homologue

V. G. Preston,<sup>1</sup> J. Kennard,<sup>1</sup> F. J. Rixon,<sup>1</sup> A. J. Logan,<sup>2</sup> R. W. Mansfield<sup>2</sup> and I. M. McDougall<sup>1</sup>

<sup>1</sup> MRC Virology Unit, Institute of Virology, Church St, Glasgow G11 5JR, UK

<sup>2</sup> Department of Molecular Sciences, Pfizer Central Research, Sandwich, Kent CT13 9NJ, UK

The scaffolding protein and associated protease of the human herpesvirus varicella-zoster virus (VZV), encoded by genes 33·5 and 33 respectively, were synthesized in insect cells using a baculovirus expression system. The expressed 33·5 product formed numerous long, flexible, hollow rods, and in this respect differed from the herpes simplex virus type 1 (HSV-1) homologue which forms large aggregates consisting mainly of fibrous material interspersed with scaffold-like particles. Removal of 27 amino acids from the carboxy terminus of the VZV scaffolding protein by the gene 33 protease or expression of the cleaved product did not result in any discernible change in the morphology of the scaffolding protein. Again, this was in marked contrast to the situation in HSV-1 where removal of the 25 carboxy-terminal amino acids from the

scaffolding protein by the associated protease or expression of VP22a results in the formation of large numbers of scaffold-like particles. Despite these differences, when cells were multiply infected with baculoviruses expressing the HSV-1 capsid shell proteins and the VZV scaffolding protein complete capsids were observed, suggesting that the VZV protein could act as a scaffold for the assembly of the HSV-1 capsid shell. The efficiency of capsid assembly was increased substantially by exchanging the 23 carboxy-terminal amino acids of the VZV scaffolding protein for the corresponding 22 carboxy-terminal amino acids of the HSV-1 homologue, supporting previous work which showed that this region was critical for the formation of intact capsids.

## Introduction

The herpesvirus capsid assembles around an internal scaffold which is subsequently lost during packaging of the viral genome. The scaffolding proteins are specified by two overlapping, in frame, 3'-coterminal genes in herpes simplex virus type 1 (HSV-1) and this organization is conserved in all herpesviruses that have been examined so far (Baer *et al.*, 1984; Davison & Scott, 1986; Chee *et al.*, 1990; Griffin, 1990; Liu & Roizman, 1991*a*; Welch *et al.*, 1991; Albrecht *et al.*, 1992; Telford *et al.*, 1992, 1995; Gompels *et al.*, 1995; Haanes *et al.*, 1995; Steffy *et al.*, 1995; Camacho & Tabares, 1996; Nicholas, 1996) (refer to Fig. 1*a*). The larger gene encodes a serine protease which cleaves itself at two positions, generating a small capsid protein containing the catalytic domain of the

protease and a minor scaffolding protein (Davison *et al.*, 1992; Dilanni *et al.*, 1993*a*, 1994; Liu & Roizman, 1993). The product of the smaller gene, the major component of the scaffold, is also processed by the protease (Liu & Roizman, 1991*b*; Preston *et al.*, 1992). The carboxy-terminal amino acids of the uncleaved scaffolding proteins bind to the major capsid protein VP5 and this interaction is essential for formation of the capsid (Kennard *et al.*, 1995; Thomsen *et al.*, 1995; Hong *et al.*, 1996; Desai & Person, 1996). Cleavage of the scaffold by the protease leads to the removal of the carboxy-terminal amino acids from the scaffolding proteins. This process, which weakens the association between the scaffold and the major capsid protein, is essential for the exit of the scaffold from the capsid and subsequent packaging of the virus genome (Preston *et al.*, 1983; Gao *et al.*, 1994).

Recently, HSV-1 immature capsids have been assembled in insect cells multiply infected with recombinant baculoviruses expressing HSV-1 capsid proteins. Of the four outer shell

**Author for correspondence:** Valerie Preston.

Fax +44 141 337 2236. e-mail V.PRESTON@VIR.GLA.AC.UK

proteins, VP5, which forms the hexameric and pentameric capsomers of the icosahedral capsid, and the two proteins, VP19C and VP23, which form intercapsomeric connections (Schrage *et al.*, 1989; Booy *et al.*, 1991; Newcomb *et al.*, 1993), are essential for capsid formation using the baculovirus system (Tatman *et al.*, 1994; Thomsen *et al.*, 1994). The remaining capsid shell protein, VP26, which is found on top of each hexon, is dispensable (Booy *et al.*, 1994; Tatman *et al.*, 1994; Thomsen *et al.*, 1994; Zhou *et al.*, 1994). The major scaffolding protein (the UL26·5 product), in the absence of the protease, can act as a scaffold and, similarly, the protease (the UL26 product) can substitute for the major scaffolding protein although capsid assembly was much less efficient due to the rapid cleavage of the carboxy terminus of the minor scaffolding protein (Tatman *et al.*, 1994; Thomsen *et al.*, 1994). These findings have been confirmed and extended using HSV-1 mutants (Gao *et al.*, 1994; Matusick-Kumar *et al.*, 1994).

Varicella-zoster virus (VZV), like HSV-1, is a human herpesvirus belonging to the subfamily *Alphaherpesvirinae*. Its morphology is very similar to that of other herpesviruses and a major component of the nucleocapsid, thought to be analogous to the HSV-1 UL26·5 product, has been described (Friedrichs & Grose, 1986). In this paper, we have extended the early observations on the scaffolding proteins by Friedrichs & Grose (1986) and compared the proteins with those of HSV-1. The aim of the work is to provide information on the requirements for, and constraints on, capsid assembly and to help identify key regions in the scaffolding protein.

## Methods

■ **Cells and viruses.** SF21-AE cells and BHK-21 clone 13 cells were cultured as described previously (Kennard *et al.*, 1995). The parental virus used to construct the recombinant baculoviruses was AcPAK6. Baculoviruses containing the HSV-1 genes UL26, UL26·5, UL18, UL19, UL38 and a mutant form of UL26·5, expressing VP22a, have been described previously (Preston *et al.*, 1994; Tatman *et al.*, 1994; Kennard *et al.*, 1995). CV-1 cells infected with the Dumas strain of VZV were generously provided by D. Stevenson (Dept. of Molecular and Cell Biology, Univ. Glasgow).

### ■ Construction of plasmids

**VZV constructs.** The large *Hind*III-*Kpn*I *Hind* fragment from a plasmid containing the VZV genomic fragment *Kpn*I d (Davison & Scott, 1983) was ligated to pUC18 cleaved with *Hind*III and *Kpn*I. *Xba*I sites were introduced on either side of the protease gene 33 within the VZV *Hind*III-*Kpn*I fragment by inserting a 12 bp *Xba*I oligonucleotide linker into the unique *Bsp*EI and *Dra*III sites, made blunt ended with *Escherichia coli* DNA polymerase large fragment. The 33·5 gene, flanked by *Xba*I sites, was constructed in two cloning steps from the resulting plasmid pJP15. This plasmid was cleaved with *Xba*I and *Spl*I and the fragment containing the pUC18 vector sequences was ligated to a PCR product, specific for the 5' end of the VZV 33·5 gene, which had been digested with *Xba*I and *Spl*I. This PCR product spanned VZV sequences 60962–61230 (Davison & Scott, 1986) and had an *Xba*I site upstream of the ATG start codon. The plasmid generated was referred to as pJP17. The large *Spl*I fragment from pJP15, containing the 3' end of the 33·5 gene, was cloned into the unique *Spl*I site in pJP17 to

recreate the full-length 33·5 gene flanked by *Xba*I sites. The resulting plasmid was referred to as pJP19. A gene encoding amino acid sequences 1–342, present in the 33·5 product cleaved by the VZV gene 33 protease, was generated from this plasmid. A PCR fragment reconstructing the 3' end of the gene (VZV sequences 60405–60513), with a stop codon TAA followed by an *Xba*I site after the codon for alanine residue 342 in the 33·5 gene, was cleaved with *Bam*HI and *Kpn*I and ligated to pJP19 cleaved with *Bam*HI and *Kpn*I to create the plasmid pJK90. *Xba*I fragments containing the 33 and 33·5 genes, and the gene encoding the cleaved form of the 33·5 product, lacking the 27 carboxy-terminal amino acids, were cloned into the unique *Xba*I site within baculovirus transfer vector pACL29.1 (Livingstone & Jones, 1989) and the resulting plasmids recombined with the virus genome of AcPAK6. *Xba*I fragments containing genes 33 and 33·5 were also cloned into the unique *Xba*I site of the mammalian expression vector pCMV10, which contains the human cytomegalovirus (HCMV) immediate early (IE) promoter (Kennard *et al.*, 1995). A chimeric scaffolding gene was constructed by digesting pJP19 (contains the 33·5 gene) with *Sty*I (cleaves the 33·5 gene at a site just downstream of sequences specifying the maturation cleavage site) and *Kpn*I, and ligating the larger fragment to a small *Sty*I-*Kpn*I fragment formed by annealing complementary oligonucleotides. The small fragment contained HSV-1 carboxy-terminal sequences (HSV-1 sequences 52646–52714) followed by an *Xba*I site (McGeoch *et al.*, 1988). All the above cloned PCR fragments were sequenced to ensure that no adventitious mutations were present. The VZV homologue of HSV-1 VP5 (gene 40) was derived from a cosmid, constructed by A. J. Davison, containing VZV sequences encompassing this gene. A PCR product, spanning the amino-terminal end of gene 40 (VZV sequences 71542–71816) and incorporating the HCMV pp65 epitope tag from UL83 at the 5' end of the gene, was made (McLauchlan *et al.*, 1994). The product contained a *Kpn*I site at the 5' end of the gene, followed by a *Bgl*II site, a methionine codon, the epitope tag sequence and the 5' end of gene 40 starting from the second codon. The PCR fragment was digested with *Xba*I (cleaves near the 3' end of the gene 40 fragment) and *Kpn*I and ligated to pLitmus (New England Biolabs) cleaved with the same enzymes. The resulting plasmid was digested with *Xba*I and *Eco*RV and the full-length gene 40 reconstructed by ligating the digested plasmid to the *Xba*I-*Eag*I fragment of gene 40 containing the carboxy-terminal sequences of the gene. The modified gene 40 was subcloned as a *Bgl*II fragment into the *Bgl*II site of pCMV10Bgl (Nicholson *et al.*, 1994).

**Baculovirus expression vector containing HSV-1 genes UL18, UL19 and UL38.** The baculovirus expression vector pAcAB3 (Belyaev & Roy, 1993) was cleaved with *Xba*I and *Stu*I and the larger fragment ligated to an *Xba*I-*Hinc*II fragment, containing the UL38 gene derived from pBJ382 (Tatman *et al.*, 1994). The resulting plasmid was referred to as pAcAB3.1. The *Sal*I site in pT318, containing the UL18 gene (Tatman *et al.*, 1994), was converted to a *Bam*HI site to enable the UL18 gene to be cloned as a *Bam*HI fragment into the *Bgl*II site of pAcAB3.1. The UL19 gene from pJN6 (Nicholson *et al.*, 1994) was cloned as a *Bgl*II fragment into the unique *Bam*HI site of the baculovirus expression vector pAcAB3.11, containing both UL18 and UL38 genes, to form pAcAB3.7.

■ **Antibodies.** Mouse monoclonal antibodies used were 406 (Serotec) and 5010 (Rixon *et al.*, 1988), specific for sequences within HSV-1 VP22a, LP12, which recognizes HSV-1 major capsid protein VP5, and 9220 (DuPont), which recognizes a 10 amino acid sequence (pp65 epitope) from the HCMV UL83 product (McLauchlan *et al.*, 1994). Rabbit antiserum 64 was raised against the peptide GEPTYITHRRRRVSPS (amino acid residues 471–486 of the VZV 33 ORF) coupled to the carrier protein keyhole limpet haemocyanin via the serine residue. Rabbit

antisera 108, raised against the peptide ADDADMFANQMMVGRC (amino acid residues 590–605 of the VZV 33 ORF), was also coupled to keyhole limpet haemocyanin but via the cysteine residue. Rabbit antibody 20999, specific for the 14 amino acids adjacent to the carboxy-terminal residue of the HSV-1 UL26 and UL26·5 product, has been described elsewhere (Preston *et al.*, 1992). In indirect immunofluorescence experiments primary mouse and rabbit antibodies bound to antigen were detected using fluorescein isothiocyanate-conjugated goat anti-mouse IgG (FITC–GAM) and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (TRITC–GAR) (Sigma) respectively. In immunoelectron microscopy, goat anti-rabbit IgG coupled to 10 nm colloidal gold particles (British Biocell International) was used as a secondary antibody.

**Immunofluorescence and immunoblotting.** Immunofluorescence studies were carried out as described by Nicholson *et al.* (1994) and the procedure for immunoblotting was performed as outlined by Preston *et al.* (1994), using colloidal gold particles bound to protein A followed by silver enhancement to detect VZV and HSV-1 proteins.

**Electron microscopy.** Thin sections of baculovirus-infected cells embedded in Epon 812 resin were prepared as described by Addison *et al.* (1984). Virus-infected cell samples for immunoelectron microscopy were embedded in Unicryl (British Bioscience International) using essentially the same method as for Lowacryl KM4 resin (Rixon *et al.*, 1988) except that after dehydration the samples were infiltrated with 100% Unicryl for 1 h, followed by further incubation for 1 h with fresh resin. The samples were then incubated overnight in fresh resin and the following day exposed to short-wavelength UV light for 3 days at  $-20^{\circ}\text{C}$ . The procedure for immunolocalization was essentially as described by Rixon *et al.* (1988) except thin sections were incubated for 2 h in diluted primary antibody instead of 45 min.

**Plasmid transfections.** Plasmid DNA (0·5  $\mu\text{g}$ ) in 25  $\mu\text{l}$  20 mM HEPES pH 7·4, 150 mM NaCl was mixed with 25  $\mu\text{l}$  LipofectACE (Gibco BRL) diluted in OptiMEM (Gibco BRL). After 15 min at room temperature, 450  $\mu\text{l}$  OptiMEM was added to the sample and the contents of the tube transferred on to a BHK cell monolayer ( $1 \times 10^5$  cells), previously washed twice with OptiMEM. The cells were incubated at  $37^{\circ}\text{C}$  for 5 h, then overlaid with 450  $\mu\text{l}$  Eagle's medium containing 10% newborn calf serum and incubation continued for a further 15 h at  $37^{\circ}\text{C}$ .

**Radioactive labelling of baculovirus-infected cell polypeptides.** Baculovirus-infected cells were radiolabelled with [ $^{35}\text{S}$ ]methionine as described by Kennard *et al.* (1995).

**Purification of capsids containing an HSV-1 shell from baculovirus-infected cells.** Capsids were isolated as described previously (Kennard *et al.*, 1995). Scaffold-like particles were purified using the same protocol.

## Results

### Expression of VZV gene 33 and 33·5 products by recombinant baculoviruses

The VZV genes 33 and 33·5 were recombined into the baculovirus expression vector AcPAK6 and recombinant baculoviruses AcVZV33 and AcVZV33·5, which express the protease and the major scaffolding protein respectively, were isolated. Baculovirus-infected cell polypeptides were labelled with [ $^{35}\text{S}$ ]methionine and separated on an SDS–polyacrylamide

gel (Fig. 1*b*). Large amounts of the VZV 33·5 product were present in AcVZV33·5-infected cells and the VZV protein was visible on a Coomassie blue-stained SDS–polyacrylamide gel of the baculovirus-infected cell extract (data not shown). By contrast, the protease was expressed poorly and only just detectable in [ $^{35}\text{S}$ ]methionine-labelled virus-infected cell extracts (Fig. 1*b*, lane 4). As was the case with the HSV-1 protease, the AcVZV33-infected cell polypeptide profile contained two novel bands and these are analogous to the HSV-1 protease cleavage products VP24 and VP21 (Fig. 1*b*, lanes 2 and 4). The VZV homologue of VP21 had a similar apparent  $M_r$  to the VZV 33·5 product even though its size was calculated from DNA sequence analysis to be significantly greater than the VZV major scaffolding protein (Fig. 1, lanes 3 and 4). To confirm that viruses were expressing the VZV proteins and to compare their properties with those of HSV-1, immunoblot analysis was carried out using antibodies specific for VZV or HSV-1 scaffolding proteins. Mock- and virus-infected cell polypeptides were separated by SDS–PAGE, transferred on to nitrocellulose membranes and probed with primary antibodies. Antibody–antigen complexes were detected using Protein A conjugated to colloidal gold followed by incubation with a silver enhancing solution. When a blot of the samples was probed with VZV antibodies, which recognize sequences within the cleaved form of VZV 33·5 scaffolding protein, bands were detected in the AcVZV33·5-infected cell track which comigrated with the high  $M_r$  forms present in VZV-infected CV-1 cells (Fig. 2*a*, lanes 5 and 8). The lower  $M_r$  band detected in the AcVZV33·5-infected cell track is probably a breakdown product since a band of this mobility was not observed in the VZV-infected cell track. Lower  $M_r$  bands which comigrated with lower  $M_r$  bands present in the VZV-infected cell track, however, were present in cells coexpressing the 33·5 product and the VZV or HSV-1 protease, suggesting that both the HSV-1 and the VZV protease could cleave the VZV scaffolding protein (Fig. 2*a*, lanes 6, 7 and 8). These lower  $M_r$  forms were not detected when a blot of these samples was probed with antibody that recognized VZV carboxy-terminal sequences which were removed by the VZV 33 product, confirming that the proteases were cleaving the VZV scaffolding protein near its carboxy terminus (Fig. 2*b*, lanes 6, 7 and 8). Similarly, using HSV-1-specific antibodies which recognize the cleaved form of UL26·5 and those which recognize sequences within the 25 carboxy-terminal amino acids in immunoblot analysis, the HSV-1 major scaffolding protein, the UL26·5 product, was shown to be cleaved by both the HSV-1 and VZV proteases (Fig. 2*c*, *d*).

### Structure of the gene 33·5 product formed in baculovirus-infected cells

Cells infected with baculovirus expressing the HSV-1 UL26·5 product contain large aggregates of the protein and more rarely scaffold-like particles (Preston *et al.*, 1994). To

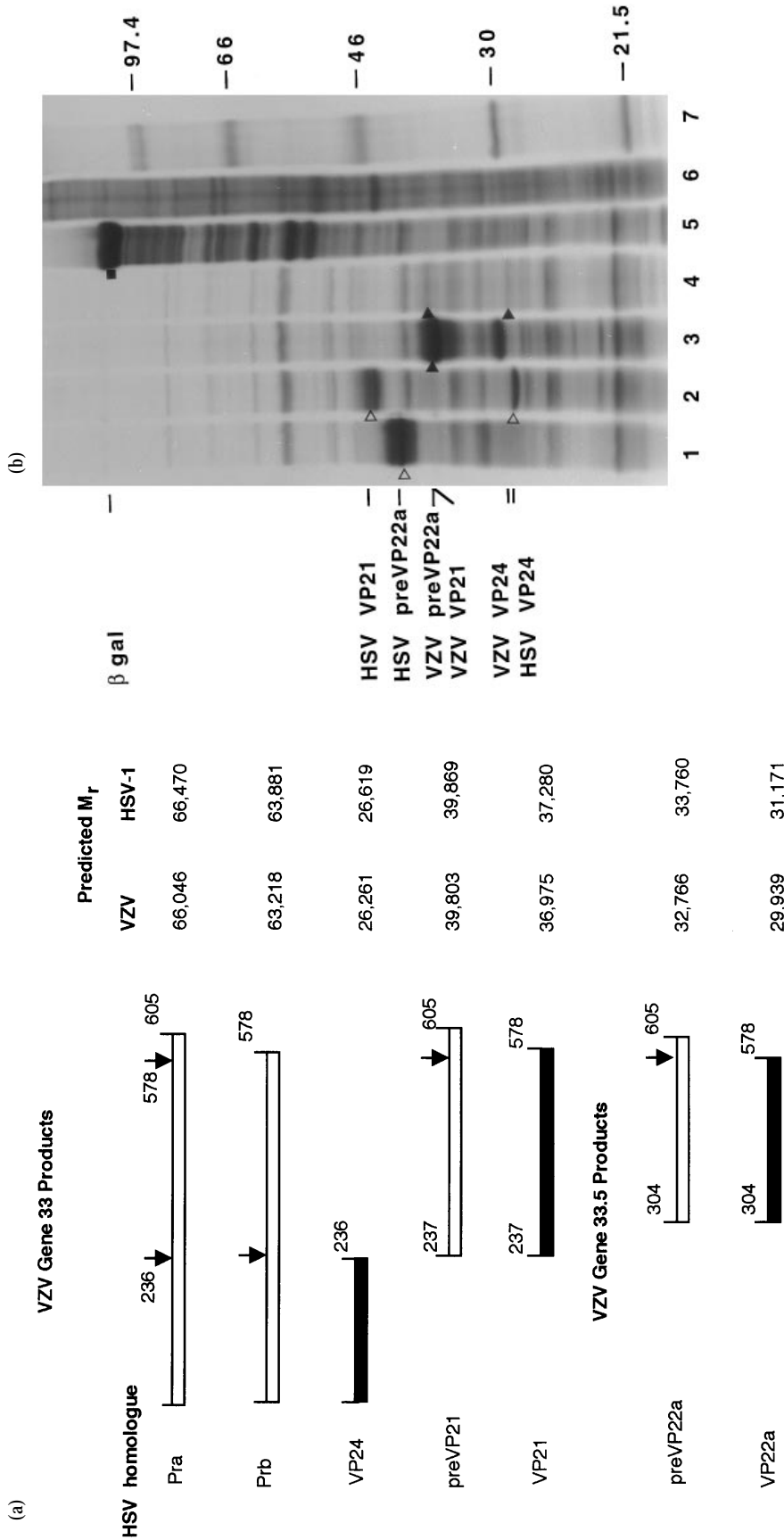


Fig. 1. (a) Organization of the VZV 33 and 33.5 gene products and comparison with HSV-1 homologues. The numbers associated with the boxes represent amino acid residues in gene 33 and the arrows indicate predicted cleavage sites. The number near each cleavage site refers to the alanine residue on the amino-terminal side of the scissile bond. The filled-in boxes represent the final cleavage products. (b) Expression of VZV gene 33 and 33.5 products from cells infected with recombinant baculoviruses and comparison with HSV-1 homologues. Mock-infected cells (lane 6) and cells infected with AcUL26 (lane 2), AcVZV33.5 (lane 3), AcVZV33 (lane 4) or AcPAK6 (lane 5) were labelled with [<sup>35</sup>S]methionine and the polypeptides separated under denaturing conditions on a 5.5–12.5% gradient polyacrylamide gel. ▲, VZV polypeptides; ▽, HSV-1 polypeptides; ■, β-galactosidase. M<sub>r</sub> standards are shown in lane 7.

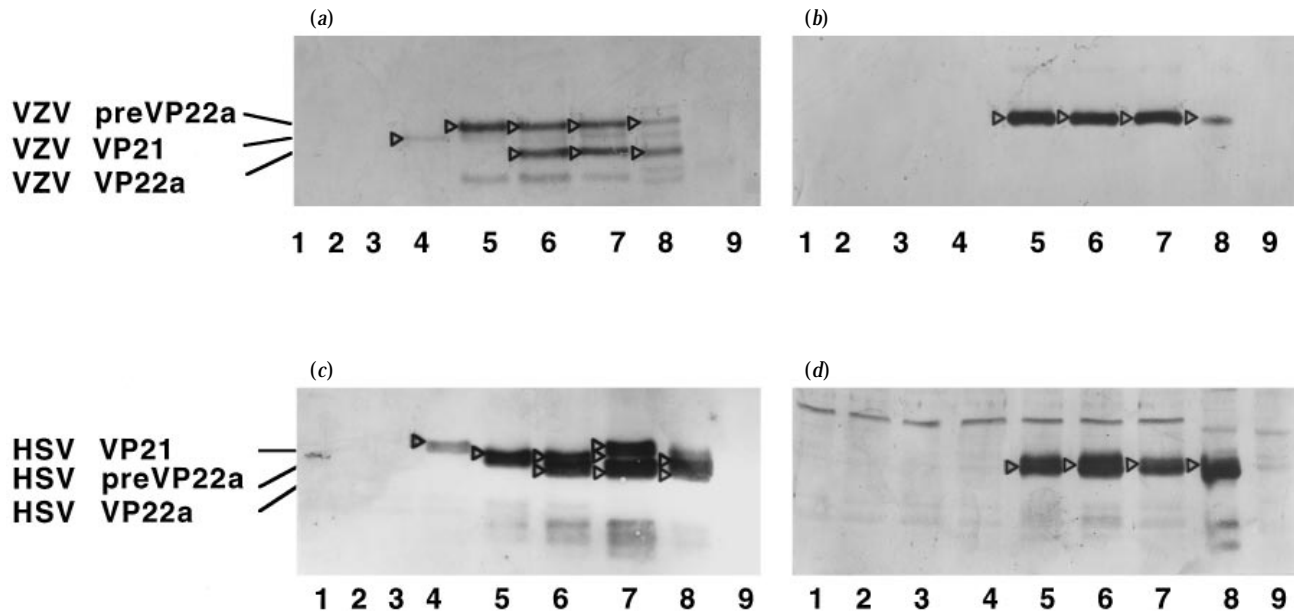


Fig. 2. Cleavage of the VZV and HSV-1 scaffolding proteins by the HSV-1 and VZV proteases. Protein blots of mock- and virus-infected cell polypeptides were separated by SDS-PAGE and probed with (a) antiserum specific for sequences within VZV 33·5 cleaved product, (b) antiserum specific for the carboxy terminus of the full-length 33·5 product, (c) antiserum specific for VP22a and (d) antiserum specific for the carboxy terminus of preVP22a. In (a) and (b) lane 1, mock-infected SF cells; lane 2, PAK6-infected cells; lane 3, AcUL26-infected cells; lane 4, AcVZV33-infected cells; lane 5, AcVZV33·5-infected cells; lane 6, AcVZV33·5 and AcUL26 dually infected cells; lane 7, AcVZV33·5 and AcVZV33 dually infected cells; lane 8, VZV-infected CV-1 cells; lane 9, mock-infected CV-1 cells. In (c) and (d) lane 1, mock-infected SF cells; lane 2, PAK6-infected cells; lane 3, AcVZV33-infected cells; lane 4, AcUL26-infected cells; lane 5, AcUL26·5 infected cells; lane 6, AcUL26·5 and AcVZV33 dually infected cells; lane 7, AcUL26·5 and AcUL26 dually infected cells; lane 8, HSV-1 strain 17 infected BHK cells; lane 9, mock-infected BHK cells.

determine whether similar structures were formed in cells infected with AcVZV33·5 or AcVZV33, thin sections of virus-infected cells were examined under the electron microscope. In AcVZV33-infected cells no distinct structures were observed, probably because the gene 33 product was present in low amounts. In AcVZV33·5-infected cells, however, large numbers of long, hollow, flexible rods, about 50 nm in diameter, were observed, mainly in the nuclei of cells. Some of these rods were arranged in parallel arrays. To confirm that the rods were formed by the 33·5 product, immunoelectron microscopy was carried out using rabbit polyclonal antibody specific for the VZV 33·5 product, tagged with goat anti-rabbit IgG coupled to colloidal gold (Fig. 3). As a control, the immune serum against the VZV protein was replaced with preimmune rabbit serum. Clustering of gold particles over the rods was only observed in thin sections of cells that had been incubated with the immune serum, confirming that these structures were composed of the 33·5 product (compare Fig. 3*a, c*). Scaffold-like particles were also detected in thin-section preparations of AcVZV33·5-infected cells (Fig. 3*a, b*). These particles could be cross-sections of the VZV-specific rods or spherical scaffold-like structures. To investigate whether scaffold-like particles were present in AcVZV33·5-infected cells, an extract of these cells was analysed on a 5–40% (w/w) sucrose gradient. The 33·5 product was present in the pellet fraction and throughout

the gradient, with a greater amount of material being present near the top of the gradient, although a specific light-scattering band was not observed (data not shown). Material in pooled fractions was concentrated by high-speed centrifugation, negatively stained with sodium phosphotungstate and examined under the electron microscope. Large numbers of spherical particles were present in fractions collected near the top of the gradient (Fig. 3*e*), similar in appearance to those formed by the HSV-1 UL26·5 product extracted from capsids (Newcomb & Brown, 1991). These particles were not uniform in size. Low numbers of scaffold-like particles and fragmented rods were observed in fractions from the middle or towards the bottom of the gradient (data not shown).

#### Effect of removing the 27 carboxy-terminal amino acids from the gene 33·5 product on its morphology

When the cleaved form of the HSV-1 UL26·5 product was expressed using the baculovirus system, or the full-length protein in the presence of its associated protease, large numbers of scaffold-like particles, many of which were present in regular arrays, were produced. Such structures were rarely observed when the full-length product alone was expressed (Preston *et al.*, 1994). To investigate whether cleavage of the VZV major scaffolding product had an effect on its morphology, cells were

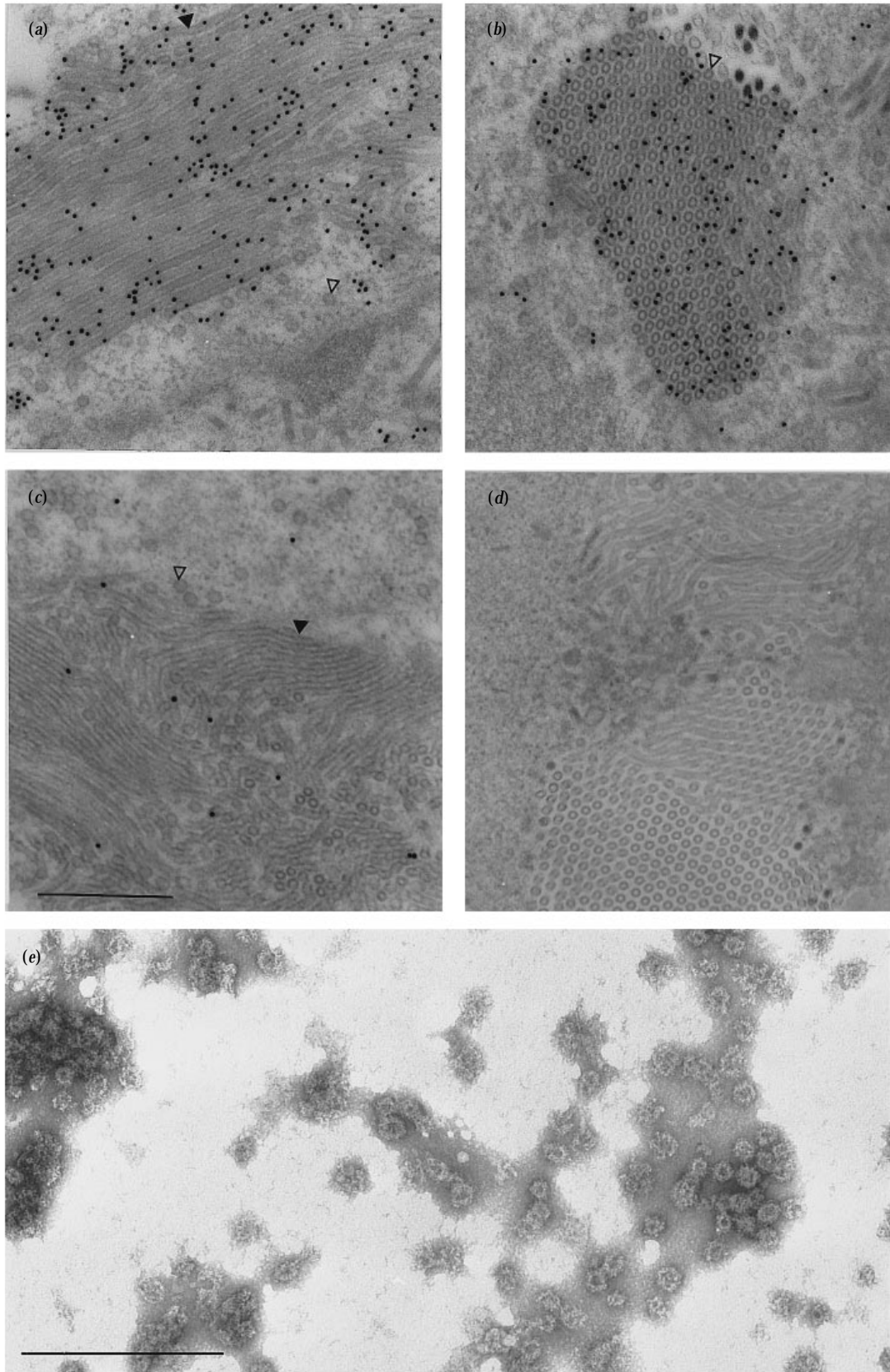


Fig. 3. For legend see facing page.

infected with AcVZV33·5a, a baculovirus expressing the cleaved form of the protein, and samples prepared for electron microscopy. Examination of thin-section preparations of virus-infected cells revealed that, in contrast to the situation with the UL26·5 product, no difference in the morphology of the 33·5 product was observed when its carboxy terminus was removed (Fig. 3*d*). Similarly, no changes in morphology of the scaffolding protein were observed in cells dually infected with AcVZV33 and AcVZV33·5 (data not shown).

### The 33·5 product interacts with HSV-1 major capsid protein VP5

The interaction of the HSV-1 UL26·5 product with VP5 is important for localization of VP5 to the nucleus and for capsid formation (Nicholson *et al.*, 1994; Kennard *et al.*, 1995; Matusick-Kumar *et al.*, 1995; Thomsen *et al.*, 1995). Immunofluorescence experiments were performed to determine whether the 33·5 product could interact with HSV-1 VP5 as well as the VZV homologue. BHK cells were either singly transfected or cotransfected with plasmids carrying genes for these proteins under the control of the major IE HCMV promoter and examined at 20 h post-transfection. In cells transfected with gene 33·5 the VZV protein usually localized in discrete regions, normally but not exclusively within the nuclei, whereas in cells expressing HSV-1 VP5 or the VZV homologue alone the major capsid protein was present throughout the cell (Fig. 4*a–c*). In many of the cells containing both HSV-1 VP5 and the 33·5 protein, VP5 colocalized with the VZV protein, suggesting that these two proteins interact. Since a considerable amount of diffuse background staining was observed in both the nucleus and cytoplasm of the cells, the association of HSV-1 VP5 with the 33·5 product appeared to be weak (Fig. 4*d, e*). Similar results were obtained when the VZV major capsid protein (MCP) tagged with pp65 epitope was expressed in place of HSV-1 VP5 although the interaction of the VZV MCP with the 33·5 product appeared to be stronger than that of VP5 with the 33·5 protein, with less background nuclear and cytoplasmic fluorescence detected (Fig. 4*f, g*).

### Construction of a VZV/HSV-1 chimeric scaffolding protein

In HSV-1-infected cells the major scaffolding protein and its associated protease participate in capsid shell formation. The protease, however, is not essential for the formation of capsids but is important in the release of the scaffold from the capsid

(Tatman *et al.*, 1994; Thomsen *et al.*, 1994; Gao *et al.*, 1994). Preliminary experiments in which the scaffold formed by the HSV-1 UL26·5 product was replaced with the VZV 33·5 product in the baculovirus HSV-1 capsid assembly system showed that the VZV protein was a poor substitute for the UL26·5 product. Electron microscopic examination of thin sections of recombinant baculovirus-infected cells containing the VZV scaffolding protein and the three essential outer shell proteins VP5, VP19C and VP23 revealed the presence of large numbers of incomplete and aberrant capsids, with few sealed capsid shells containing an internal scaffold (data not shown).

Previous work has shown that sequences at the carboxy-terminal end of the full-length UL26·5 product interact with VP5 and are crucial for capsid formation (Desai & Person, 1996; Hong *et al.*, 1996). To test whether the carboxy-terminal sequences in the VZV major scaffolding protein were responsible for the inefficient assembly of HSV-1 intact capsid shells, a hybrid gene was designed in which sequences coding for the carboxy-terminal 23 amino acids of the VZV scaffolding protein were replaced with the sequences specifying the carboxy-terminal 22 amino acids from the HSV-1 UL26·5 gene (Fig. 5). The hybrid gene was recombined into the baculovirus expression vector and the resulting recombinant virus, AcVZV33·5C, screened for the ability to make the chimeric scaffolding protein. The hybrid protein was only two amino acids shorter than its VZV counterpart and therefore was expected to migrate on an SDS–polyacrylamide gel in a similar position to the 33·5 product. Analysis of virus-infected cell polypeptides by SDS–PAGE revealed the presence in the AcVZV33·5C-infected cell polypeptide profile of large amounts of a novel protein, corresponding to the hybrid protein, which migrated significantly faster than the VZV product (Fig. 6, lanes 3 and 4). Since amino acids flanking the maturation cleavage site between alanine and serine residues of the UL26·5 product are important for recognition and cleavage of the substrate, the chimeric protein was also examined for the ability to be processed by the HSV-1 or VZV protease (Fig. 6, lanes 1 and 2) (DiIanni *et al.*, 1993*b*; McCann *et al.*, 1994). Both proteases cleaved the chimeric protein and therefore the amino acid sequences flanking the cleavage site in the chimeric protein did not have an adverse effect on cleavage. In addition, the observation that the cleaved form of the chimeric protein comigrated with the cleaved product of the VZV scaffolding protein suggested that the carboxy-terminal sequences and not a mutation elsewhere in the protein were responsible for the aberrant mobility of the protein (Fig. 6, lanes 1, 2, 5 and 6). Similarly, it is possible that the discrepancy in the  $M_r$  of the VZV homologue of VP21 is due to the aberrant mobility of the

Fig. 3. Structure of the VZV major scaffolding protein. (*a–c*) Immunoelectron microscopy of thin sections of cells infected with AcVZV33·5, using VZV antibody 64 (*a, b*) or the preimmune serum (*c*). Solid arrowheads indicate VZV-specific tubular structures; open arrowheads indicate VZV scaffold-like particles. (*d*) Electron micrograph of a thin section of a cell infected with AcVZV33·5a; (*e*) negative stain of material collected near the top of a sucrose gradient of AcVZV33·5-infected cell extract. The bar markers represent 0·5  $\mu$ m.

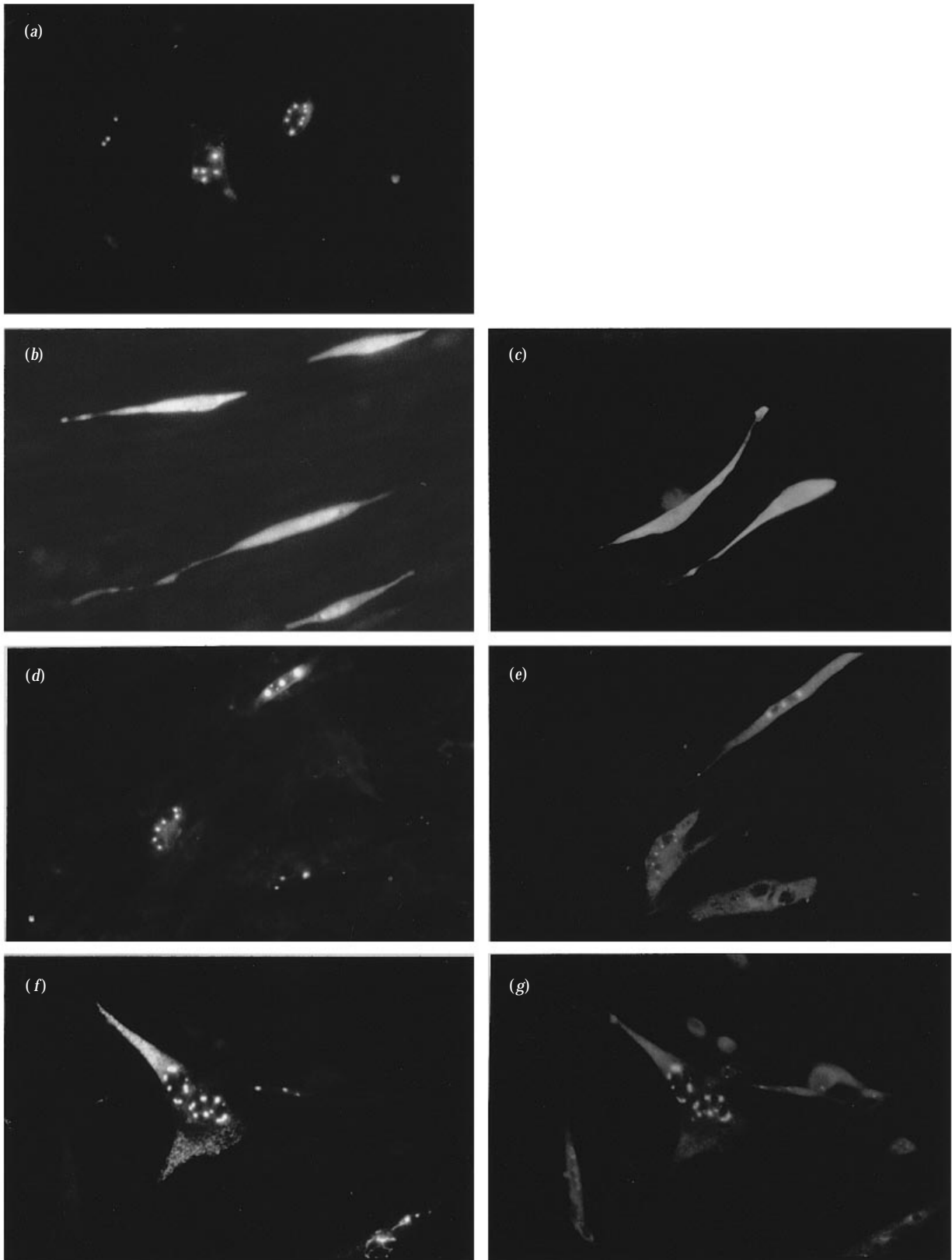


Fig. 4. Interaction of the VZV 33.5 product with the VZV and HSV-1 major capsid proteins. Cells were transfected with plasmids containing the genes (a) VZV 33.5; (b) HSV-1 UL19 (VP5); (c) VZV 40 (HSV-1 VP5 homologue); (d, e) VZV 33.5 and HSV-1UL19; (f, g) VZV 33.5 and VZV 40. The distribution of VZV 33.5 was examined using rabbit antiserum 64 (a, d-g);

### C-terminal end of the chimeric scaffolding protein

	↓	
VZV	VEASSKAPLIQGSTADDADMFQNMVGRE	27aa
Chimera	VEASSKAHVVDVTARAADLFVQSMMGAR	25aa
HSV-1	VNASSAAHVVDVTARAADLFVQSMMGAR	25aa

Fig. 5. Carboxy-terminal end of the VZV/HSV-1 chimeric scaffolding protein. VZV amino acid sequences present in the chimeric protein are shaded. The arrow indicates the predicted cleavage site of the scaffolding protease and the numbers on the right-hand side refer to the number of amino acids removed by the protease.

full-length 33·5 product rather than the VZV homologue of VP2I, which lacks the carboxy-terminal 27 amino acids. The sequence of the 3' end of the chimeric gene was confirmed by DNA sequence analysis of the recombinant gene inserted into the virus and the protein sequence was verified by protein blot analysis of the baculovirus-expressed protein using VZV and HSV-1 antibodies specific for the carboxy termini of the homologous proteins only (data not shown).

### Comparison of HSV-1 capsid shell formation, using HSV-1, VZV and the chimeric scaffolding proteins

SF cells were dually infected with AcUL18/19/38, together with a baculovirus expressing either the HSV-1, VZV or chimeric scaffolding protein. At 50 h post-infection, the cells were harvested and samples prepared for electron microscopy. Numerous capsids containing large internal scaffolds were observed in thin-section preparations of cells coinfecting with AcUL26·5 and the baculovirus expressing the three essential HSV-1 capsid shell proteins (Fig. 7*a*). In cells mixedly infected with AcVZV33·5 and AcUL18/19/38, however, there were fewer scaffold-containing capsids and many of these contained small cores. Incomplete capsid shells were also observed (Fig. 7*b*) as was found previously. By contrast, the population of capsids detected in cells containing the chimeric scaffolding protein, VP5, VP19C and VP23 was more uniform than those produced using the VZV scaffolding protein (Fig. 7*b, c*) and similar in appearance to those formed using the UL26·5 product. To compare the efficiency of HSV-1 capsid assembly using the different scaffolding proteins, cells were multiply infected with baculoviruses AcUL18/19/38, AcUL35 and AcUL26·5 or AcVZV33·5 or AcVZV33·5C. The baculovirus AcUL35, expressing VP26, was included in the infection because higher yields of purified capsids were achieved when this capsid protein was present (F. Rixon, unpublished data). At 72 h post-infection, cells were harvested and extracts were analysed on sucrose gradients. A strong opaque band was present in the gradient of the extract containing the chimeric scaffolding protein, in a similar position to the band of

intermediate capsids in the gradient of the extract containing the UL26·5 protein (Fig. 7*d*). The light-scattering material higher up in the gradient was probably due to the presence of tubular structures formed by the chimeric scaffolding protein since these structures were observed in the corresponding thin-section preparations of cells. A faint band in the position of intermediate capsids was just detectable in the gradient of the sample containing the VZV 33·5 product. In addition, light-scattering material was also observed throughout the gradient and was probably a result of large numbers of incomplete and aberrant capsid structures in addition to the tubular structures. The bands were collected, diluted and the material concentrated by high-speed centrifugation. Intact capsids were detected in negatively stained preparations of the material from all the gradients (data not shown).

### Predicted secondary structure of herpesvirus scaffolding proteins

The predicted secondary structure for herpesvirus scaffolding proteins was obtained using the program Protein-Predict (Rost, 1996). Fig. 8 shows the prediction based on the alignment of seven alphaherpesvirus proteins. The amino acids conserved in the viruses used in the analysis are also indicated in Fig. 8.

### Discussion

In AcVZV33·5-infected cells, the 33·5 protein formed long, flexible hollow rods which were not observed in cells containing large amounts of the UL26·5 product. This structure was not dependent on the presence of the 27 carboxy-terminal amino acids since it was also formed by the cleaved form of the 33·5 product. The finding that the VZV protein can adopt several different structures is in keeping with the predicted secondary structure of herpesvirus scaffolding proteins. The results of this analysis suggest that the scaffolding protein is unstructured with a flexible morphology, consistent with its biological role in which it not only has to interact with the major capsid protein and assemble into a spherical structure but also has to disassemble for DNA packaging to occur. Six regions of conserved amino acids, including the protease cleavage site, were identified. Three of these regions were present in predicted helical regions of the protein, one of which contains the VP5 binding domain located near the carboxy terminus (Hong *et al.*, 1995). The two internal helical regions are strong candidates for sites involved in the interaction of the scaffolding protein with itself.

HSV-1 VP5 distribution was examined using mouse monoclonal antibody LP12 (*b, d, e*) and the distribution of the VZV homologue of HSV-1 VP5 using mouse monoclonal antibody 9220 (*c, f, g*). The secondary antibody used in (*a*), (*d*) and (*f*) was TRITC-GAR whereas in (*b*), (*c*), (*e*) and (*g*) FITC-GAM was used. Panels (*d*) and (*e*), (*f*) and (*g*) represent duplicate exposures of single fields.

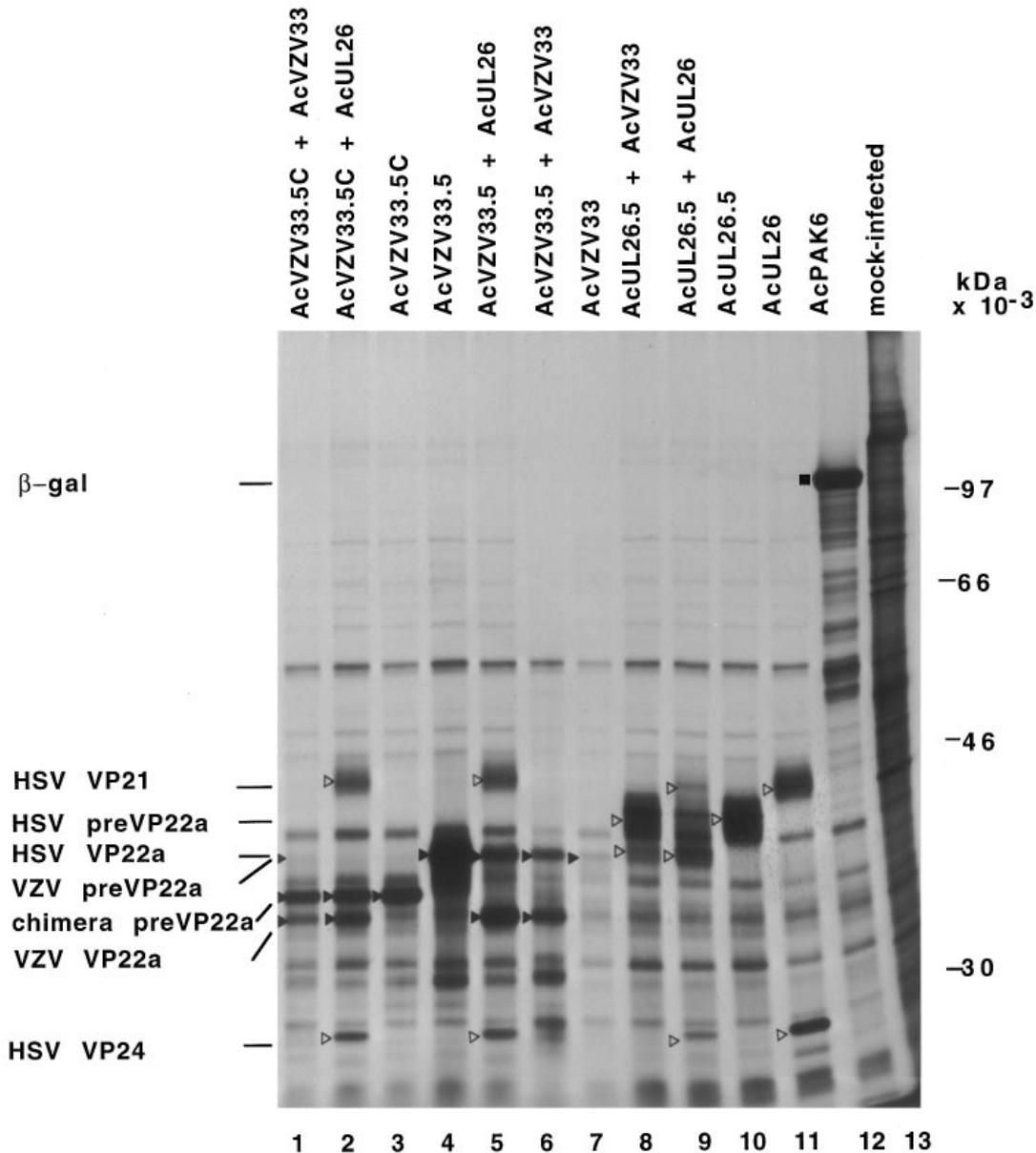
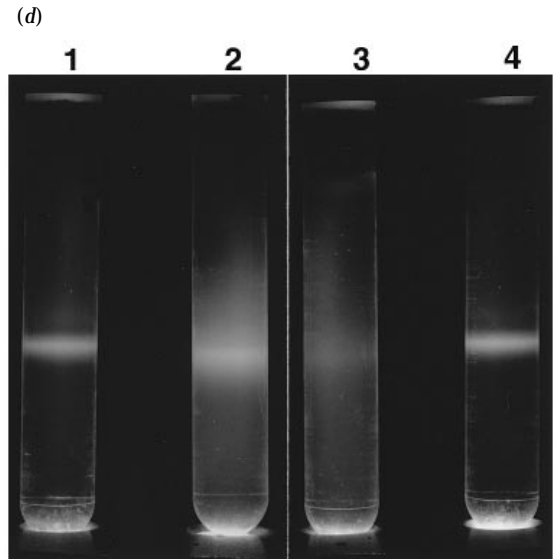
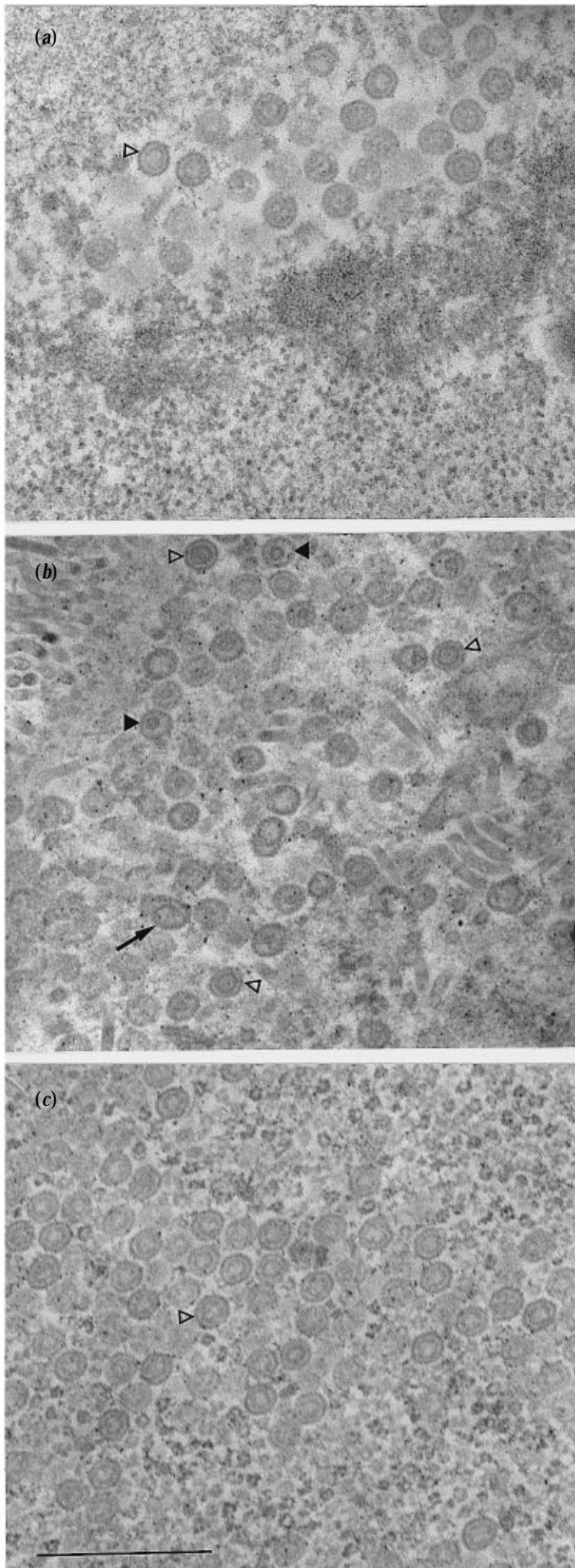


Fig. 6. Characterization of the VZV/HSV-1 chimeric scaffolding protein. Mock- and virus-infected cells were labelled with [<sup>35</sup>S]methionine and the polypeptides analysed on an SDS-10% polyacrylamide gel. The positions of the herpesvirus gene products are indicated on the left-hand side and the positions of molecular mass markers are shown on the right. ►, VZV or chimeric products; ▷, HSV-1 products; ■, β-galactosidase.

Although the VZV 33·5 product has only 29% amino acid identity with its HSV-1 counterpart, it can substitute for the HSV-1 homologue in HSV-1 capsid assembly, albeit less efficiently, using the baculovirus system. Our finding that the hybrid VZV/HSV-1 scaffolding protein was a much more effective replacement for the 26·5 product than the VZV homologue in the assembly of the HSV-1 capsid indicates that a strong interaction between VP5 and the carboxy-terminal end of the scaffolding protein is important for capsid formation and that considerable variation in the rest of the scaffolding protein is tolerated. Not only were a greater number of intact

capsids produced when the chimeric protein was used instead of the VZV product as a scaffold but the population of capsids was more uniform in morphology, consisting mainly of large cored capsids. The presence of a significant number of small cored capsids in the population of intact capsids formed in the presence of the VZV 33·5 product but in the absence of the protease suggests that small cored capsids result from the loss of strong contact between the scaffold and the major capsid protein rather than a conformation change induced by cleavage near the carboxy-terminal end of the scaffolding protein. Bovine herpesvirus type 1 (BHV-1) scaffolding protein, which



**Fig. 7.** Comparison of capsid shell formation, using the HSV-1, VZV and chimeric scaffolding proteins. Electron micrographs of thin sections of a cell dually infected with AcUL26·5 and AcUL18/19/38 (a), AcVZV33·5 and AcUL18/19/38 (b) and AcVZV33·5C and AcUL18/19/38 (c). The bar marker represents 0·5  $\mu\text{m}$ . Open arrowheads indicate large cored capsids, solid arrowheads denote small cored capsids and the arrow indicates an incomplete capsid. Material from the virus-infected cells was sedimented through 5–40% (w/w) sucrose gradients and the gradients are shown in panel (d). The scaffolding protein present in (1) and (4) is the UL26·5 product; that in (2) is the chimeric protein and in (3) the VZV 33·5 product. (1) and (4) are the same sample.



- Camacho, A. & Tabares, E. (1996).** Characterization of the genes, including that encoding the viral proteinase, contained in *Bam*HI restriction fragment 9 of the pseudorabies virus genome. *Journal of General Virology* **77**, 1865–1874.
- Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A., III, Kouzarides, T., Martignetti, J. A., Preddie, E., Satchwell, S. C., Tomlinson, P., Weston, K. M. & Barrell, B. G. (1990).** Analysis of the protein coding content of the sequences of human cytomegalovirus strain AD169. *Current Topics in Microbiology and Immunology* **154**, 129–173.
- Davison, A. J. & Scott, J. E. (1983).** Molecular cloning of the varicella-zoster virus genome and derivation of six restriction endonuclease maps. *Journal of General Virology* **64**, 1811–1814.
- Davison, A. J. & Scott, J. E. (1986).** The complete DNA sequence of varicella-zoster virus. *Journal of General Virology* **67**, 1759–1816.
- Davison, M. D., Rixon, F. J. & Davison, A. J. (1992).** Identification of genes encoding two capsid proteins (VP24 and VP26) of herpes simplex virus type 1. *Journal of General Virology* **73**, 2709–2713.
- Desai, P. & Person, S. (1996).** Molecular interactions between the HSV-1 capsid proteins as measured by the yeast two-hybrid system. *Virology* **220**, 516–521.
- Devereux, J., Haeblerli, P. & Smithies, O. (1984).** A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387–395.
- Dilanni, C. L., Drier, D. A., Deckman, I. C., McCann, P. J., III, Liu, F., Roizman, B., Colonna, R. J. & Cordingley, M. G. (1993a).** Identification of the herpes simplex virus-1 protease cleavage sites by direct sequence analysis of autoproteolytic cleavage products. *Journal of Biological Chemistry* **268**, 2048–2051.
- Dilanni, C. L., Mapelli, C., Drier, D. A., Tsao, J., Natarajan, S., Riexinger, D., Festin, S. M., Bolgar, M., Yamanaka, G., Weinheimer, S. P., Meyers, C. A., Colonna, R. J. & Cordingley, M. G. (1993b).** In vitro activity of the herpes simplex virus type 1 protease with peptide substrates. *Journal of Biological Chemistry* **268**, 25449–25454.
- Dilanni, C. L., Stevens, J. T., Bolgar, M., O'Boyle, D. R., II, Weinheimer, S. P. & Colonna, R. J. (1994).** Identification of the serine residue at the active site of the herpes simplex virus type-1 protease. *Journal of Biological Chemistry* **269**, 12672–12676.
- Friedrichs, W. E. & Grose, C. (1986).** Varicella-zoster virus p32/p36 complex is present in both the viral capsid and the nuclear matrix of the infected cell. *Journal of Virology* **57**, 155–164.
- Gao, M., Matusick-Kumar, L., Hurlburt, W., Feuer Ditus, S., Newcomb, W. W., Brown, J. C., McCann, P. J., III, Deckman, I. & Colonna, R. J. (1994).** The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. *Journal of Virology* **68**, 3702–3712.
- Gompels, U. A., Nicholas, J., Lawrence, G., Jones, M., Thomson, B. J., Martin, M. E. D., Efstathiou, S., Craxton, M. & Macaulay, H. A. (1995).** The DNA sequence of human herpesvirus-6: structure, coding content and genome evolution. *Virology* **209**, 29–51.
- Griffin, A. M. (1990).** The complete nucleotide sequence of the capsid p40 gene from infectious laryngotracheitis virus. *Nucleic Acids Research* **18**, 3664.
- Haanes, E. J., Thomsen, D. R., Martin, S., Homa, F. L. & Lowery, D. E. (1995).** The bovine herpesvirus 1 maturational proteinase and scaffold proteins can substitute for the homologous herpes simplex virus type 1 proteins in the formation of hybrid type B capsids. *Journal of Virology* **69**, 7375–7379.
- Hong, Z., Beaudet-Miller, M., Durkin, J., Zhang, R. & Kwong, A. D. (1996).** Identification of a minimal hydrophobic domain in the herpes simplex virus type 1 scaffolding protein which is required for interaction with the major capsid protein. *Journal of Virology* **70**, 533–540.
- Kennard, J., Rixon, F. J., McDougall, I. M., Tatman, J. D. & Preston, V. G. (1995).** The 25 amino acid residues at the carboxy terminus of the herpes simplex virus type 1 UL26 protein are required for the formation of the capsid shell around the scaffold. *Journal of General Virology* **76**, 1611–1621.
- Liu, F. & Roizman, B. (1991a).** The promoter, transcriptional unit, and coding sequence of herpes simplex virus 1 family 35 proteins are contained within and in frame with the UL26 open reading frame. *Journal of Virology* **65**, 206–212.
- Liu, F. & Roizman, B. (1991b).** The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. *Journal of Virology* **65**, 5149–5156.
- Liu, F. & Roizman, B. (1993).** Characterization of the protease and other products of the amino-terminus-proximal cleavage of the herpes simplex virus 1 UL26 protein. *Journal of Virology* **67**, 1300–1309.
- Livingstone, C. & Jones, I. (1989).** Baculovirus expression vectors with single strand capability. *Nucleic Acids Research* **17**, 2366.
- McCann, P. J., III, O'Boyle, D. R., II & Deckman, I. C. (1994).** Investigation of the specificity of the herpes simplex virus type 1 protease by point mutagenesis of the autoproteolysis sites. *Journal of Virology* **68**, 526–529.
- McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E. & Taylor, P. (1988).** The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *Journal of General Virology* **69**, 1531–1574.
- McLauchlan, J., Leifkens, K. & Stow, N. D. (1994).** The herpes simplex virus type 1 UL37 gene product is a component of virus particles. *Journal of General Virology* **75**, 2047–2052.
- Matusick-Kumar, L., Hurlburt, W., Weinheimer, S. P., Newcomb, W. W., Brown, J. C. & Gao, M. (1994).** Phenotype of the herpes simplex virus type 1 protease substrate ICP35 mutant virus. *Journal of Virology* **68**, 5384–5394.
- Matusick-Kumar, L., Newcomb, W. W., Brown, J. C., McCann, P. J., III, Hurlburt, W., Weinheimer, S. P. & Gao, M. (1995).** The C-terminal 25 amino acids of the protease and its substrate ICP35 of herpes simplex virus type 1 are involved in the formation of sealed capsids. *Journal of Virology* **69**, 4347–4356.
- Newcomb, W. W. & Brown, J. C. (1991).** Structure of the herpes simplex virus capsid: effects of extraction with guanidine hydrochloride and partial reconstitution of extracted capsids. *Journal of Virology* **65**, 613–620.
- Newcomb, W. W., Trus, B. L., Booy, F. P., Steven, A. C., Wall, J. S. & Brown, J. C. (1993).** Structure of the herpes simplex virus capsid: molecular composition of the pentons and triplexes. *Journal of Molecular Biology* **232**, 499–511.
- Nicholas, J. (1996).** Determination and analysis of the complete nucleotide sequence of human herpesvirus 7. *Journal of Virology* **70**, 5975–5989.
- Nicholson, P., Addison, C., Cross, A. M., Kennard, J. C., Preston, V. G. & Rixon, F. J. (1994).** Localization of the herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires the abundant scaffolding protein VP22a. *Journal of General Virology* **75**, 1091–1099.
- Preston, V. G., Coates, J. A. V. & Rixon, F. J. (1983).** Identification and characterization of a herpes simplex virus gene product required for encapsidation of viral DNA. *Journal of Virology* **45**, 1056–1064.

- Preston, V. G., Rixon, F. J., McDougall, I. M., McGregor, M. & Al Kobaisi, M. F. (1992). Processing of the herpes simplex virus assembly protein ICP35 near its carboxy terminal end requires the product of the whole of the UL26 reading frame. *Virology* **186**, 87–98.
- Preston, V. G., Al-Kobaisi, M. F., McDougall, I. M. & Rixon, F. J. (1994). The herpes simplex virus gene UL26 proteinase in the presence of the UL26.5 gene product promotes the formation of scaffold-like structures. *Journal of General Virology* **75**, 2355–2366.
- Rixon, F. J., Cross, A. M., Addison, C. & Preston, V. G. (1988). The products of herpes simplex virus type 1 gene UL26 are strongly associated with empty but not full capsids. *Journal of General Virology* **69**, 2879–2891.
- Rost, B. (1996). PHD: predicting one-dimensional protein structure by profile based neural networks. *Methods in Enzymology* **266**, 525–539.
- Schrag, J. D., Prasad, B. V. V., Rixon, F. J. & Chiu, W. (1989). Three-dimensional structure of the HSV-1 nucleocapsid. *Cell* **56**, 651–660.
- Steffy, K. R., Schoen, S. & Chen, C.-M. (1995). Nucleotide sequence of the herpes simplex virus type 2 gene encoding the protease and capsid protein ICP35. *Journal of General Virology* **76**, 1069–1072.
- Tatman, J. D., Preston, V. G., Nicholson, P., Elliott, R. M. & Rixon, F. J. (1994). Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *Journal of General Virology* **75**, 1101–1113.
- Telford, E. A. R., Watson, M. S., McBride, K. & Davison, A. J. (1992). The DNA sequence of equine herpesvirus-1. *Virology* **189**, 304–316.
- Telford, E. A. R., Watson, M. S., Aird, H. C., Perry, J. & Davison, A. J. (1995). The DNA sequence of equine herpesvirus 2. *Journal of Molecular Biology* **249**, 520–528.
- Thomsen, D. R., Roof, L. L. & Homa, F. L. (1994). Assembly of herpes simplex virus (HSV) intermediate capsids in insect cells infected with recombinant baculoviruses expressing HSV capsid proteins. *Journal of Virology* **68**, 2442–2457.
- Thomsen, D. R., Newcomb, W. W., Brown, J. C. & Homa, F. L. (1995). Assembly of the herpes simplex virus capsid: requirement for the carboxy-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes. *Journal of Virology* **69**, 3690–3703.
- Welch, A. R., Woods, A. S., McNally, L. M., Cotter, J. R. & Gibson, W. (1991). A herpesvirus maturational proteinase, assemblin: identification of its gene, putative active site domain, and cleavage site. *Proceedings for the National Academy of Sciences, USA* **88**, 10792–10796.
- Zhou, Z. H., Prasad, B. V. V., Jakana, J., Rixon, F. J. & Chiu, W. (1994). Protein subunit structures in the herpes simplex virus capsid determined from 400-kV spot-scan electron cryomicroscopy. *Journal of Molecular Biology* **242**, 456–469.

---

Received 18 November 1996; Accepted 19 March 1997