JOURNAL OF VIROLOGY, Jan. 1997, p. 179–190 Vol. 71, No. 1
0022-538X/97/$04.00+0

Human Cytomegalovirus Capsid Assembly Protein Precursor (pUL80.5) Interacts with Itself and with the Major Capsid Protein (pUL86) through Two Different Domains

LISA J. WOOD, MICHAEL K. BAXTER, SCOTT M. PLAFKER, AND WADE GIBSON*

Virology Laboratories, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 1 July 1996/Accepted 11 September 1996

We have used the yeast GAL4 two-hybrid system to examine interactions between the human cytomegalovirus (HCMV) major capsid protein (MCP, encoded by UL86) and the precursor assembly protein (pAP, encoded by UL80.5 and cleaved at its carboxyl end to yield AP) and found that (i) the pAP interacts with the MCP through residues located within the carboxy-terminal 21 amino acids of the pAP, called the carboxyl conserved domain (CCD); (ii) the pAP interacts with itself through a separate region, called the amino conserved domain (ACD), located between amino acids His34 and Arg52 near the amino end of the molecule; (iii) the simian CMV (SCMV) pAP and AP can interact with or replace their HCMV counterparts in these interactions, whereas the herpes simplex virus pAP and AP homologs cannot; and (iv) the HCMV and SCMV maturational proteinase precursors (AC_{pra}, encoded by UL80a and APNG1, respectively) can interact with the pAP and MCP. The ACD and CCD amino acid sequences are highly conserved among members of the betaherpesvirus group and appear to have counterparts in the alpha- and gammaherpesvirus pAP homologs. Deleting the ACD from the HCMV pAP, or substituting Ala for a conserved Leu in the ACD, eliminated detectable pAP self-interaction and also substantially reduced MCP binding in the two-hybrid assay. This finding indicates that the pAP self-interaction influences the pAP-MCP interaction. Immunofluorescence studies corroborated the pAP-MCP interaction detected in the GAL4 two-hybrid experiments and showed that nuclear transport of the MCP was mediated by pAP but not AP. We conclude that the pAP interacts with the MCP, that this interaction is mediated by the CCD and is influenced by pAP self-interaction, and that one function of the pAP-MCP interaction may be to provide a controlled mechanism for transporting the MCP into the nucleus.

Capsid structure and formation in herpes group viruses has been studied most extensively in herpes simplex virus (HSV), where it has been established that the most abundant intranuclear capsid is composed of, and can be constituted in vitro from (52, 76, 80), six protein species (15, 29, 36). Four of these make up the icosahedral outer shell and are also present in the mature virion (29, 53, 54); the other two are internally situated (5) and have a more transient role in capsid formation. The internal proteins are made as precursors that are cleaved during capsid formation. One of them is a proteinase presurser, Pra, whose autoproteolytic cleavage yields two smaller internal proteins, one of which (VP24) remains with the capsid and is present in the mature virion (17, 29, 61). The second internal protein, called ICP35c,d, is cleaved by the proteinase to yield ICP35e,f (also called VP22a, p40, NC-3, or scaffold protein) and is not found in the virion (29, 64).

Counterparts of these six HSV capsid proteins have been identified in cytomegalovirus (CMV). The four outer shell proteins of CMV capsids are called the major capsid protein (MCP, human CMV [HCMV] UL86) (11), the smallest capsid protein (HCMV UL48/49) (6, 28), the minor capsid protein (HCMV UL85) (6), and the minor capsid protein-binding protein (HCMV UL46) (27). The internal proteins A and C_{pra} are derived from the precursor proteinase (AC_{pra}) by autoproteolytic cleavage, and the internal assembly protein (AP) is derived by cleavage from its precursor, pAP (30) (Fig. 1).

Studies done to define the assembly pathway of herpes group viruses have resulted in the identification and characterization of multiple particle forms (e.g., A-, B-, and C-capsids, virions, noninfectious extracellular particles, dense bodies, and light particles [29, 38, 60, 74, 75]). Among these, capsids composed of the four outer shell proteins and the cleaved proteinase and substrate precursors are routinely recovered from nuclei of herpesvirus-infected cells (25, 29) and are typically the most abundant capsid form present. These particles, generally called B-capsids, contain little or no DNA (44, 45, 60, 72), accumulate under conditions of inhibited viral DNA synthesis (22, 45, 58), and, as suggested by pulse-chase radiolabeling experiments (44, 59), may be precursors to DNA-containing particles and functionally analogous to bacteriophage proheads (8, 43). Although immature B-capsids (e.g., pre-B-capsids [26]) containing noncleaved forms of the proteinase and its substrate (i.e., HSV ICP35c,d or CMV pAP) have not been recovered from infected cells, there are three lines of evidence that are consistent with their existence. First, capsids are formed in cells infected at the restrictive temperature with the HSV proteinase mutant *ts*1201, even though cleavage of the proteinase and substrate do not occur at the restrictive temperature (63). Second, the amino and carboxyl halves of the cleaved proteinase precursor are present in approximately equimolar amounts in B-capsids (29, 55), which is compatible with incorporation of the precursor proteinase into the particle first, followed by cleavage. And third, HSV capsids

* Corresponding author. Mailing address: Virology Laboratories, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 955-8680. Fax: (410) 955-3023. E-mail: Wade_Gibson@qmail.bs.jhu.edu.

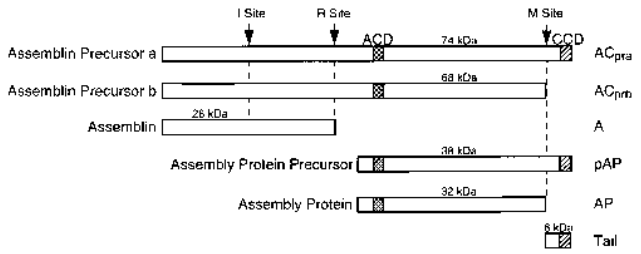

FIG. 1. HCMV proteinase and assembly protein precursors, products, and landmarks. Shown in schematic representation are the HCMV proteinase and assembly protein precursors and their primary cleavage products. The name of each protein is indicated on the left, and its abbreviation is shown on the right. Landmark features of the proteins are indicated, and sequence-derived molecular weights are shown above each. Shaded portions of the top two proteins indicate sequence shared with pAP. The SCMV and HSV homologs of these HCMV proteins that were used in some experiments have similar features (46, 82).

can be isolated that contain a mutant proteinase precursor unable to be cleaved to yield VP21 and VP24, demonstrating that the proteinase precursor can be incorporated into capsids (65).

Although the mechanistic requirement for these internal proteins, and the circumstances and consequences of their cleavage, are only beginning to be understood, there is good evidence that (i) they are required to coordinate association of the outer shell proteins into the capsid structure (18, 76, 80), (ii) they must be cleaved to enable viral DNA to be packaged (23, 63), and (iii) there is a correlation between DNA packaging and their elimination from the particle (39, 64). In addition, there is evidence indicating that the HSV pAP homolog binds to the MCP (18, 37, 79) and enhances its nuclear translocation (57) and that VP22a aggregates with itself when extracted from B-capsids and renatured (53). These observations suggest that the HSV pAP homolog is involved in at least two intermolecular interactions, either or both of which may be critical to capsid formation.

As a means of studying these and other intermolecular interactions among the herpesvirus capsid proteins, we have used the yeast GAL4 two-hybrid system (14, 20). This system is a genetic method for studying protein-protein interactions that takes advantage of the fact that the GAL4 transactivator protein is comprised of two distinct functional domains, a DNA-binding (DB) domain and a transactivation (TA) domain. When the DB and TA domains are cloned into separate expression vectors and coexpressed in yeast cells, they do not interact and there is no expression of the reporter gene, *lacZ*, which encodes β-galactosidase (β-Gal). Likewise, when the DB and TA domains are expressed as fusions with proteins that do not interact, the expression of *lacZ* is not activated (e.g., Fig. 2c to e). However, when the GAL4 domains are expressed as fusions with proteins that do interact, a functional GAL4 transactivator is reconstituted, leading to β-Gal expression that can be detected and measured (e.g., Fig. 2a and b).

We have focused on interactions of the CMV assembly protein precursor in the work reported here and have taken advantage of the two-hybrid system to delineate two domains in this protein that account for its self-interaction and for its interaction with the major capsid protein. An influence of the self-interaction domain on the ability of the assembly protein precursor to interact with MCP was also found, which suggests that pAP multimer formation may be one of the first steps in the capsid assembly pathway.

(Initial reports of this work were presented at the 1995 meetings of the American Society for Virology, 14th Annual Meeting [Austin, Tex., July 8 to 12], Phage and Virus Assembly [Johnstown, Pa., June 16 to 21], Viruses and Cells Gordon Conference [Tilton, N.H., June 11 to 16], and The 20th International Herpesvirus Workshop [Groningen, The Netherlands, July 29 to August 3].)

MATERIALS AND METHODS

Yeast strain and growth media. *Saccharomyces cerevisiae* PCY2 (*MATα Δgal4 Δgal80 URA3::GAL1-lacZ lys2-801^{amber} his- Δ200 trp1- Δ63 leu2 ade2-101^{ochre}*) was used (13). Yeast were grown in yeast extract-peptone-dextrose or in minimal medium containing dextrose (SD) supplemented with an amino acid mixture selective for growth of the transformants (3). SD-leu-trp is SD supplemented with 1× amino acid mixture without leucine and tryptophan.

Yeast transformation. Yeast transformation was by the lithium acetate method performed essentially as described previously (3) except that 50 μl of competent cells was transformed with 5 μg of plasmid DNA without carrier DNA.

β-Gal assays. Yeast colonies were assayed for β-Gal activity by using the yeast colony filter lift assay as described previously (9) except that 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Bethesda Research Laboratories, Gaithersburg, Md.) was used at 1 mg/ml and incubation was at 37°C. The β-Gal activity of liquid yeast cultures was quantitated as described previously (3, 68) except that the units of β-Gal activity were normalized either to the protein concentration of the crude yeast extracts (expressed as nanomoles/minute/milligram of protein) (18, 70) or to cell density (expressed in Miller units) (3, 51). Any value for β-Gal activity presented here is either the mean obtained from assays of at least three dilutions of each crude yeast lysate or the mean calculated from assays performed in triplicate on at least two yeast transformants. The standard deviations of values obtained in each experiment were typically within 10 to 20% of the mean.

Plasmids. The GAL4 two-hybrid vectors used in this study were obtained from Daniel Nathans and have been described previously (13). Vector pPC86 encodes

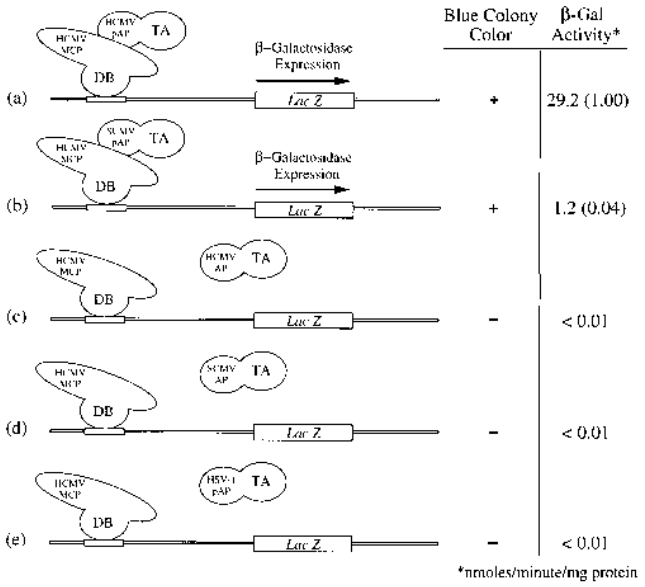

FIG. 2. HCMV MCP interacts with HCMV and SCMV pAPs, but not APs or HSV pAP homolog, in the yeast GAL4 two-hybrid system. Shown are schematic representations of GAL4 two-hybrid coexpression experiments described in the text. The horizontal bar represents DNA, the empty rectangle toward its left end represents the upstream activation sequence (UAS_G) to which the GAL4 complex binds, and the larger rectangle designated *Lac Z* represents the β-Gal-encoding gene. Viral protein fusions with the DB and TA domains of the GAL4 protein are shown as interacting (a and b) or noninteracting (c, d, and e) pairs. MCP denotes HCMV major capsid protein (HCMV MCP); other abbreviations are defined in Fig. 1 or explained in the text. Results of qualitative (filter lift) and quantitative (liquid) β-Gal assays, obtained as described in Materials and Methods, are shown in the right-hand columns as blue colony color (+ denotes positive interaction) and β-Gal activity, respectively. Values in the right-hand column shown in parentheses were normalized to the β-Gal activity obtained for the HCMV pAP-HCMV MCP interaction on line a. The value <0.01 is given for cotransformants with no detected β-Gal activity.

the GAL4 TA domain. Vector pPC97 encodes the GAL4 DB domain and is a derivative of pPC62 (13). pPC97 has been modified so that its polylinker is identical to that of pPC86.

The eukaryotic expression vectors used in this study were pcDNA I/Amp (Invitrogen catalog no. V460-20) and pRSV.5neo (47).

Plasmid construction. Standard techniques were used to construct, clone, and propagate plasmids (69). All constructs were verified by DNA sequence analysis.

(i) Subcloning of viral genes into the two-hybrid vectors. The subcloning of many full-length viral genes into the two-hybrid vectors was carried out according to the following general scheme: (i) PCR amplification of the 5′ end of the gene using a reverse primer that includes a unique internal restriction site and a forward primer that introduces an appropriate polylinker restriction site immediately upstream of the start codon such that upon cloning, the gene is fused in frame to the 3′ end of the GAL4 domain coding sequence; (ii) excision of the rest of the gene from an existing plasmid clone, using the internal restriction site and a polylinker restriction site downstream of the stop codon; and (iii) three-piece ligation of the PCR product, the excised gene fragment, and the vector (cleaved with the appropriate restriction enzymes). For example, the HCMV pAP constructs were cloned with a *Sal*I site upstream, the internal *Xma*I site, and an *Xba*I/*Spe*I fusion downstream (5′*Sal*I-ATG-*Xma*I-*Xba*I/*Spe*I3′). The HCMV MCP constructs were cloned with 5′*Bgl*II-ATG-*Spe*I-*Not*I3′, the simian CMV (SCMV) pAP constructs were cloned with 5′*Sal*I-ATG-*Ava*II-*Bam*HI/*Bgl*II3′, the wild-type SCMV AC_{pra} constructs were cloned with 5′*Sal*I-ATG-*Eco*RV-*Bam*HI/*Bgl*II3′, the wild-type SCMV assemblin constructs were cloned with 5′ *Sal*I-ATG-*Eco*RV-*Bam*HI/*Bgl*II3′, the mutant SCMV assemblin (S118A.S) constructs were cloned with 5′ *Sal*I-ATG-*Eco*RV-*Bam*HI/*Bgl*II3′, and the HSV type 1 (HSV-1) pAP constructs were 5′ *Bgl*II-ATG-*Bsm*I-*Xba*I/*Spe*I3′.

The HCMV AP constructs were produced by cleaving the pAP constructs at the polylinker *Sst*I site, blunting the 3′ overhang with T4 DNA polymerase, cutting at the internal *Xma*I site, and replacing the 3′ end of the gene with the 3′ end from an existing HCMV AP clone. The SCMV AP clones were made by replacing the 3′ end of the SCMV pAP constructs, from an internal *Asp*I site onward, with a PCR product which introduced a stop codon at the maturational (M) site.

The HSV-1 MCP constructs were made by (i) PCR amplification of the gene by using a forward primer which introduced a *Bgl*II site just upstream of the start ATG and a reverse primer which incorporated a *Spe*I site downstream of the stop codon, (ii) ligation into *Bgl*II-*Spe*I-digested pPC97, and (iii) replacement of the internal portion of the gene from the *Bsp*EI site to the *Cel*II site with the corresponding sequence from a genomically derived clone of the gene.

The S118A mutant form of SCMV AC_{pra} (S118A.L) was cloned into pPC86 by cutting the S118A.S construct at an internal *Bsr*GI site and at the *Spe*I site in the polylinker of pPC86 and replacing the 3′ end of the gene with the 3′ end of the wild-type SCMV AC_{pra} construct.

HCMV pAP deletion mutants were generated by PCR amplification using (i) forward primers that introduced a *Sal*I site at the 5′ end such that the gene fragments were fused in frame to the 3′ end of the GAL4 sequence and (ii) reverse primers that introduced either a *Bgl*II (for deletions δc1 to δc5, δn1/δc4, and amino conserved domain ACD†) or an *Eco*RI (δn1 to δn6) site at the 3′ end of the gene fragment. The HCMV carboxyl conserved domain (CCD) was generated by PCR amplification using a forward primer that introduced a *Bam*HI site at the 5′ and a reverse primer that generated a *Spe*I site at the 3′ end of the CCD.

The δACD pAP construct (δACDhcmv) was made in the following way. The HCMV pAP fragment upstream of the ACD (Met1 to Pro33) was generated by PCR amplification using a forward primer that introduced a *Sal*I site at the 5′ end and a reverse primer which introduced a *Spe*I site at the 3′ end of the fragment. The sequence immediately downstream of the ACD (Ser53 onward) was generated by PCR amplification using a forward primer that introduced a *Spe*I site at the 5′ end of the fragment and a reverse primer which included a unique *Xma*I site at the 3′ end. The *Sal*I-*Xma*I fragment from TA-pAPhcmv was replaced with the PCR-generated *Sal*I-*Spe*I and *Spe*I-*Xma*I fragments in a three-piece ligation, such that the ACD sequence in the HCMV pAP was replaced by the *Spe*I site.

The construct encoding the L47A mutant pAP was made by first digesting TA-pAPhcmv with *Sal*I and *Xma*I and then gel isolating the fragment containing the pPC86 backbone and the coding sequence for the carboxyl two-thirds of the pAP gene. Two PCR products were then generated: one using a forward primer that introduced a *Sal*I site at the 5′ end of the fragment and a reverse primer that introduced a unique *Eag*I site at the 3′ end and encoded the alanine substitution for leucine at amino acid 47, and the other using a forward primer that introduced an *Eag*I site at the 5′ end of the fragment and a reverse primer that included the unique *Xma*I site at the 3′ end of the fragment. L47A resulted from a three-piece ligation of the *Sal*I-*Xma*I TA-pAPhcmv fragment with the two PCR products.

A chimera composed of the HCMV CCD fused to the carboxyl end of the HSV pAP homolog (pAPhsv-CCDhcmv) was constructed as follows. First, we replaced the stop codon of the HSV-1 pAP construct with a *Bgl*II site by PCR amplifying the 3′ end of the gene by using a forward primer that included a unique *Afl*III site and a reverse primer that changed the sequence TGATCT to AGATCT. The resulting *Afl*III-*Bgl*II fragment was ligated to an *Eco*RI-*Afl*III fragment containing the remaining portion of the gene in a three-piece ligation with *Eco*RI-*Bgl*II-cleaved vector. The CCD was generated by PCR using a reverse primer to include a *Spe*I site at the 3′ end of the sequence and a forward primer that introduced a *Bam*HI restriction site. The CCD fragment was inserted downstream of an in frame with the HSV-1 pAP gene *Bgl*II/*Bam*HI-*Spe*I.

A second chimera encoding the HSV pAP tail fused to the carboxyl end of the HCMV pAP (pAPhcmv-Tailhsv) was produced by a three-piece ligation of (i) a PCR product encoding the carboxy-terminal 25 amino acids of the HSV-1 pAP with a flanking *Xho*I site at the 5′ end and an *Sst*I site after the stop codon, (ii) an *Xma*I-*Xho*I fragment from TA-pAPhcmv which encodes the carboxyl two-thirds of the HCMV pAP, and (iii) the *Xma*I-*Sst*I fragment from TA-pAPhcmv which contains the vector portion of the construct along with the coding sequence for the amino-terminal third of the HCMV pAP.

A third chimera composed of the ACD-containing fragment Ala31-Tyr59 fused to the amino terminus of the HSV pAP homolog was constructed in the following way. The HCMV fragment Ala31-Tyr59 (referred to as ACD†) was generated by PCR amplification using a forward primer that included a *Sal*I restriction site at the 5′ end of the sequence and a reverse primer that introduced a *Bgl*II site at the 3′ end. The ACD† fragment was ligated to the HSV-1 pAP construct, which had been digested with *Sal*I and *Bgl*II, so that it was upstream of and in frame with the 5′ end of the HSV-1 pAP gene.

(ii) Subcloning of viral genes into eukaryotic expression vectors. The CMV MCP gene was cloned into the eukaryotic expression vector pcDNA I/Amp by excision from the GAL4 two-hybrid DB-MCPhcmv construct with *Eco*RI-*Not*I digestion and ligation into *Eco*RI-*Not*I-digested vector. The SCMV pAP and AP were cloned into the eukaryotic expression vector pRSV.5neo. The SCMV pAP construct, AW1, has been described previously (84). The SCMV AP was excised from the two-hybrid TA-APscmv construct by *Sal*I-*Spe*I digestion and then ligated into *Sal*I-*Xba*I-digested pRSV.5neo.

Mammalian cells. COS-7 African green monkey cells (a kind gift from Carolyn Machamer, Johns Hopkins University School of Medicine) were grown in Dulbecco's modified Eagle's medium (catalog no. 12100-061; GIBCO, Grand Island, N.Y.) containing 10% (vol/vol) fetal calf serum (HyClone, Logan, Utah), penicillin (100 U/ml), streptomycin (100 U/ml), and nystatin (10 U/ml) (GIBCO).

Transfection. COS-7 African green monkey kidney cells were seeded (≈10,000 cells/well) onto eight-well chamber slides (Supercell, Erie, Pa.) and allowed to attach and grow overnight at 37°C in maintenance medium.

Cells were transfected by a modification of the DEAE-dextran technique, described previously (1). Briefly, the maintenance medium was aspirated, and the plasmid DNAs (1.5 mg of the MCP plasmid and 1 mg of the pAP and AP plasmids were transfected per well), in 250 μl of phosphate-buffered saline (PBS) containing 25 μg of DEAE-dextran (Pharmacia LKB, Uppsala, Sweden) per ml, were added to the cells and incubated at 37°C for 30 min. The DNA-containing transfection solution was then aspirated and replaced with maintenance medium containing chloroquine (10 μg/ml; Sigma, St. Louis, Mo.). Three to four hours later, the drug-containing medium was removed and replaced with maintenance medium, and the cultures were incubated at 37°C for 3 days.

Immunofluorescence. Three days after transfection, the maintenance medium was aspirated from the cultures and the cell layers were washed once with PBS, fixed for 25 min at room temperature in PBS containing 3% paraformaldehyde (electron microscopy grade; Polysciences, Inc., Warrington, Pa.), and then washed again three times with TN buffer (10 mM Tris HCl [pH 7.4], 0.9% NaCl) containing 10 mM glycine (TNgly). The fixed cells were permeabilized by incubating them for 4 min in TNgly buffer containing 0.5% Triton X-100 and then washed three times with TNgly buffer. The permeabilized cells were incubated at room temperature for 35 min with a rabbit antipeptide antiserum to either MCP (anti-MCP [unpublished data]) or the CMV AP (anti-N1 [71]). Anti-MCP and anti-N1 were used at dilutions of 1:500 and 1:80, respectively, in 5% bovine serum albumin (BSA) (Calbiochem-Novabiochem Corp., La Jolla, Calif.) made in TN containing 0.02% NaN_3). After removal of the primary antibodies, the cells were washed three times with TNgly, incubated at room temperature for 35 min with whole goat serum (1:20 in 5% BSA; catalog no. 005-000-121; Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) to block nonspecific binding of the secondary antibodies, washed three times with TNgly, incubated at room temperature for 35 min with fluorescein isothiocyanate-labeled goat anti-rabbit antibodies (1:150 in 5% BSA; catalog no. 111-095-144; Jackson Immunoresearch Laboratories), and washed three times in TNgly. Coverslips were attached to the processed slides with a drop of Mowiol mounting medium, which contained Mowiol 4-88 (catalog no. 475904; Calbiochem, San Diego, Calif.) and the antifade agent DABCO (Sigma), prepared as described previously (35). Cells were examined and photographed with an Olympus BH-2 microscope equipped with a 40× SPlan objective lens, using Kodak Ektachrome Elite 400 ASA film.

RESULTS

All two-hybrid constructs used in the work reported here encoded a fusion between the amino end of a viral protein and the carboxyl end of the GAL4 DB or TA domain. These fusion constructs have been named by their GAL4 domain (i.e., TA or DB), followed by the viral sequence present (e.g., pAP,

TABLE 1. Requirement of the CCD of HCMV and HSV pAP homolog for interaction with MCP[a]

Assay	DB domain fusion	TA domain fusion	Blue colony color[b]	β-Gal activity[c] (nmol/min/mg of protein [relative activity])	
				Expt 1	Expt 2
a	MCPhcmv	pAPhcmv	+	5.8 (1.0)	11.1 (1.0)
b	MCPhcmv	pAPhsv-CCDhcmv	+	25.9 (4.5)	57.4 (5.2)
c	MCPhsv	pAPhsv-CCDhcmv	+	65.0 (11.2)	138.9 (12.5)
d	MCPhsv	pAPhsv	+	271.5 (46.7)	521.6 (47.0)
e	MCPhsv	pAPhcmv	−	<0.01	<0.01
f	MCPhsv	pAPhcmv-Tailhsv	+	63.9 (10.9)	56.3 (5.1)
g	MCPhcmv	pAPhcmv-Tailhsv	+	26.5 (4.6)	103.9 (9.4)

[a] Cotransformations were done as described in the text with plasmids expressing the DB and TA fusion proteins indicated. Abbreviations are explained in the text, Fig. 1, and the legend to Fig. 2.
[b] Results of qualitative filter lift assays.
[c] Results of quantitative liquid assays from two independent experiments. Values were calculated and normalized as described in the legend to Fig. 2 and Materials and Methods.

MCP, or deletion name), followed by the viral origin of the sequence in lowercase letters (e.g., hcmv, scmv, or hsv).

All fusion proteins that gave positive results in cotransformations with other fusion proteins were tested to verify that they did not yield false-positive reactions. This was done by cotransforming the fusion protein construct with the complementary GAL4 vector and then assaying the transformed cells for β-Gal expression by the filter lift assay. Only the HSV pAP homolog in the GAL4 DB vector (i.e., DB-pAPhsv), cotransformed with the GAL4 TA vector, gave a false-positive reaction and was not used in further experiments.

We found that although the relative levels of β-Gal activity were generally consistent from experiment to experiment, the absolute values varied significantly (Table 1). To control for this fluctuation, so that the β-Gal activities of different transformants could be compared, each experiment included either an HCMV pAP/pAP or an HCMV pAP/MCP cotransformation for normalizing the measurements in that set.

HCMV pAP and MCP interact. We first tested for an interaction between the HCMV pAP and MCP by cotransforming yeast with the DB-MCPhcmv and TA-pAPhcmv plasmids. The transformants were plated on SD-leu-trp, and a yeast colony filter lift assay was done 2 days later. Permeabilized colonies on the nitrocellulose replica turned blue after incubation with X-Gal, indicating that the two viral proteins interact (Fig. 2a and Table 1, assay a; also see Fig. 4, pAP, and Table 4, assay a). The relative strength of the interaction was determined by calculating units of β-Gal activity for each yeast transformant in a liquid assay (Fig. 2a; also see Tables 1 and 4, assays a).

When the experiment was repeated using the reciprocal constructs (i.e., TA-MCPhcmv plus DB-pAPhcmv), no interaction was detected by either assay. We attribute this negative result to nonreactivity of the TA-MCPhcmv fusion protein (e.g., conformation or low abundance), because the DB-pAPhcmv construct reacted well in other assays (e.g., Table 2, assays a, c, and e).

pAP-MCP interaction shows species specificity. Given the probable functional similarity between the assembly protein homologs of different herpesviruses (38), we next tested whether the pAP of one virus could substitute for that of another in MCP binding. We did this by determining whether the pAP from another betaherpesvirus (i.e., SCMV) or from an alphaherpesvirus (i.e., HSV-1) (for nomenclature, see reference 67) could substitute for the HCMV pAP in interacting with the HCMV MCP. Yeast colony filter lift assays showed that the SCMV pAP interacted with the HCMV MCP but that the HSV pAP homolog did not (Fig. 2b and e, respectively). Failure of the HSV pAP homolog to interact with the HCMV MCP was not due to lack of expression or stability of the HSV-1 pAP fusion protein because it showed a strong interaction with the HSV MCP (Table 1, assay d).

The HCMV pAP appeared to interact more strongly than the SCMV pAP with the HCMV MCP (compare Fig. 2a and b); however, additional experiments revealed that all yeast cotransformants expressing the SCMV pAP as a TA fusion showed much lower β-Gal activities than those expressing the same protein as a DB fusion (e.g., 29-fold [Table 2, assays c and d] 6-fold [Table 4, assays f and g]). The experiment could not be repeated for comparison with the pAP genes in the GAL4 DB vector because the TA-MCPhcmv construct needed as the partner was not active (see above). Such vector-dependent or polarity effects on β-Gal activity have been observed by others (19, 21, 31, 40) and indicate that factors other than the strength of the interaction between fusion proteins can influence these results (e.g., amount and stability of transcript or protein or conformational or other changes in the fusion protein).

CCD of CMV pAP is required for interaction with MCP. The next series of experiments was done to identify the CMV pAP domain involved in MCP binding. We began by comparing the amino acid sequences of the HCMV and SCMV pAPs (interactive with HCMV MCP) with that of the HSV pAP homolog (not interactive with HCMV MCP) and identified two highly conserved domains (Fig. 3). One has been noted

TABLE 2. Interaction of CMV pAPs with each other and with the HSV pAP homolog[a]

Assay	DB domain fusion	TA domain fusion	Blue colony color[b]	β-Gal activity[c] (Miller units [relative activity])
a	pAPhcmv	pAPhcmv	+	39.4 (1.0)
b	APhcmv	APhcmv	+	135.7 (3.4)
c	pAPhcmv	pAPscmv	+	3.9 (0.1)
d	pAPscmv	pAPhcmv	+	113.6 (2.9)
e	pAPhcmv	pAPhsv	−	<0.01
f	pAPscmv	pAPhsv	−	<0.01

[a] Cotransformations were done as described in the text with plasmids expressing the DB and TA fusion proteins indicated. Abbreviations are explained in the text and Fig. 1.
[b] Results of qualitative filter lift assays.
[c] Results of quantitative liquid assays. Values were claculated and normalized as described in the legend to Fig. 2 and Materials and Methods.

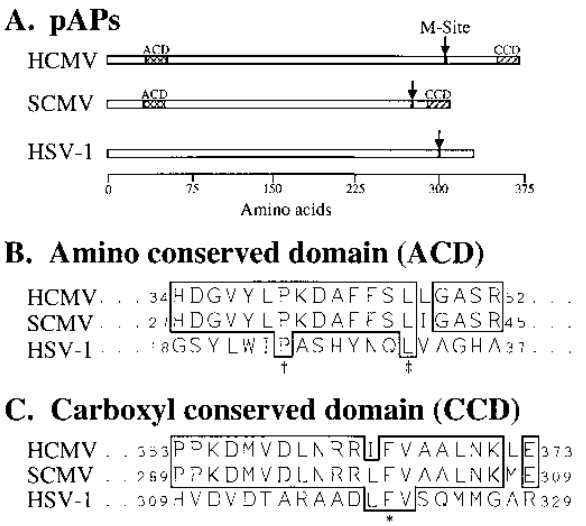

FIG. 3. Amino and carboxyl conserved domains of CMV pAP. (A) Schematic representation of the HCMV, SCMV, and HSV-1 pAPs. Relative amino acid length is indicated on the scale at bottom. Hatched boxes labeled ACD and CCD indicate conserved amino acid sequences discussed in text and specified in panels B and C. Arrows at right end of bars indicate M cleavage sites (84). (B) Alignment of the 19-amino-acid conserved sequence near the amino ends of the HCMV and SCMV pAPs, and the corresponding sequence of the HSV pAP homolog. The program PILEUP from the Genetics Computer Group sequence analysis software package was used for the alignment. Numbers at beginning and end of sequences indicate amino acid positions in sequences; regions of amino acid identity between sequences are boxed; the symbols ‡ and † indicate the conserved Leu and Pro discussed in the text. (C) Alignment of the 21-amino-acid conserved sequence at the carboxyl ends of the HCMV and SCMV pAPs, and the corresponding sequence of the HSV pAP homolog. PILEUP was used for the alignment; numbers and boxing are as explained in panel B; the symbol * indicates the conserved Phe discussed in the text. Sequences used for the alignments were from GenBank: HCMV strain AD169 (12), SCMV strain Colburn (82), and HSV-1 (50).

before (66) and comprises the carboxy-terminal 21 amino acids of the two CMV proteins (Fig. 3A and C, CCD; also see Fig. 6). Nineteen of these 21 residues are identical. The second domain is located toward the amino end of both proteins and is comprised of a 19-amino-acid sequence, 18 residues of which are identical (Fig. 3A and B, ACD; also see Fig. 6). Neither of these sequences show strong homology to corresponding regions of the HSV pAP homolog (Fig. 3B and C; also see Fig. 6).

To test the influence of these domains on the pAP-MCP interaction, we first deleted the entire tail sequence (i.e., M site to carboxy terminus, including the CCD [Fig. 1]) from the HCMV and SCMV pAP to generate the mature form of each (i.e., AP) as GAL4 fusion proteins (i.e., TA-APhcmv and TA-APscmv). Neither of the mature APs interacted with the HCMV MCP (Fig. 2c and d; Fig. 4, AP). Similarly, when just the carboxy-terminal half of the HCMV pAP tail was deleted (i.e., 37 amino acids), no interaction with the MCP was detected (Fig. 4, δc1). These results indicate that sequences within the carboxyl half of the tail of the HCMV and SCMV pAPs are required for MCP binding.

Given that the 21-amino-acid CCD is the most highly conserved sequence present in the SCMV and HCMV pAP tails, we tested its ability to interact with the HCMV MCP. Three constructs were made for this purpose. The first was a fusion of the HCMV CCD with the GAL4 TA domain (TA-CCDhcmv). No interaction between this construct and the MCP was detected (Fig. 4, δn7). The second construct, in which the entire 65-amino-acid HCMV pAP tail was fused to the GAL4 TA domain (Fig. 4, δn6), also showed no interaction with the MCP. The third construct was made to test the possibility that the CCD sequence was not presented correctly in the first two constructs. Considering that the HSV pAP homolog may have general structural similarities with the HCMV pAP, even though it is not interactive with the HCMV MCP (Fig. 2e), we fused the HCMV CCD to the carboxyl end of the HSV-1 pAP to create the HSV/HCMV chimera, TA-pAPhsv-CCDhcmv. This chimera did interact with HCMV MCP (Table 1, assay b), demonstrating that when the 21-amino-acid CCD of the HCMV pAP is added to the carboxyl end of the HSV pAP homolog, the HSV protein is converted from noninteractive to interactive with the HCMV MCP. Interestingly, this HSV/CMV chimera interacted fivefold better with the HCMV MCP than did the HCMV pAP itself (Table 1; compare assays a and b). Evidence described below suggests that this effect is due to a stronger self-interaction of the HSV pAP than the HCMV pAP.

An analogous chimera, composed of the 25-amino-acid HSV-1 pAP tail fused to the carboxyl end of the HCMV pAP, was made to determine whether the HSV pAP-MCP interaction, like that of the counterpart CMV proteins, is mediated by residues located within the HSV pAP homolog tail. This chimera interacted with the HSV MCP (Table 1, assay f), indicating that residues in the tail of the HSV pAP homolog mediate its binding to the HSV MCP. In contrast to the results described above for the pAPhsv-CCDhcmv chimera, interaction of the pAPhcmv-Tailhsv chimera with the HSV MCP was decreased by four- to ninefold compared with the HSVpAP-MCP interaction (Table 1; compare assays f and d). The apparent influence of the pAP domain of these chimeras on their MCP interactions is noteworthy and is further discussed below.

To determine whether these chimeric pAPs were still able to interact with their homologous MCPs, we did two additional cotransformations: TA-pAPhsv-CCDhcmv plus DB-MCPhsv and TA-pAPhcmv-Tailhsv plus DB-MCPhcmv. Both homologous interactions occurred (Table 1, assays c and g). The HCMV pAP with an HSV tail interacted somewhat better with the HCMV MCP than did the native HCMV pAP (Table 1; compare assays g and a), whereas the HSV chimera pAP with the HCMV CCD on its carboxyl end interacted somewhat more weakly with the HSV MCP than did the native HSV pAP homolog (Table 1; compare assays c and d). Because the HCMV pAP does not interact with the HSV MCP (Table 1, assay e) and the HSV pAP homolog does not interact with the HCMV MCP (Fig. 2e), these results indicate that the homologous pAP-MCP interaction of both chimeras is mediated by its internally situated MCP-binding domain and demonstrate that the MCP-interactive domain of the pAP does not have to be at its extreme carboxy terminus to be functional.

The tail of pAP participates in translocation of MCP into nucleus. Results of our two-hybrid experiments indicated that the MCP-interactive domain of the CMV pAP is located in its tail. To corroborate this in a more functionally representative assay, we compared the abilities of the two forms of the CMV assembly protein, the pAP (with tail) and the AP (without tail), to change the subcellular localization of the HCMV MCP in transfected cells. The HSV MCP distributes throughout the cytoplasm and nucleus of transfected cells in the absence of other viral proteins, but when coexpressed with the HSV pAP homolog, the HSV MCP localizes predominantly to the nucleus (57). If interaction between the CMV pAP and MCP is mediated only by the CCD, and if interaction between the MCP and pAP is required for MCP nuclear translocation, we expected that cotransfections of the CMV MCP and pAP (but

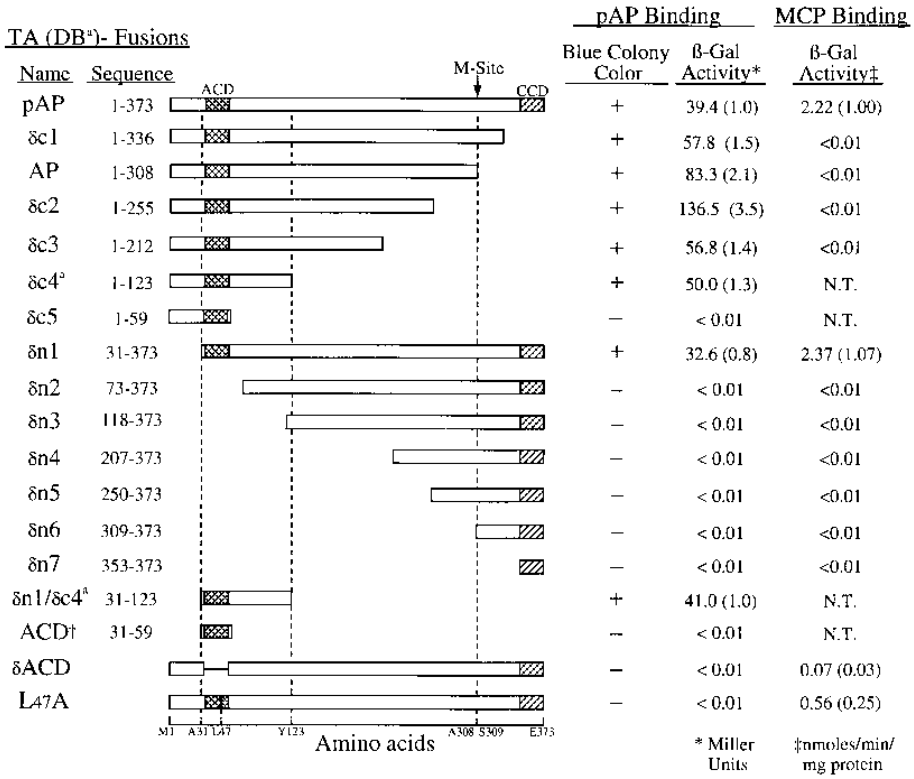

FIG. 4. Deletional mapping of pAP self-interaction domain. HCMV pAP deletion mutants, represented by the horizontal open boxes, were constructed and tested for the ability to interact with the HCMV pAP and MCP in the two-hybrid system, all as described in the text. The names of the deletion mutants and the amino acid sequences that they contain are indicated in the left two columns. The ACD, CCD, M site, and amino acids referred to in the text are indicated above and below the protein representations. Interaction between the mutants and HCMV pAP (pAP Binding) was determined qualitatively by the filter lift assay (Blue Colony Color) and quantitatively by the liquid assay (β-Gal Activity*). Interaction between the mutants and HCMV MCP (MCP Binding) was determined by liquid assay (β-Gal Activity‡). Assays were done and data are presented as described in the legend to Fig. 2 and Materials and Methods. The results shown for names footnoted with the letter a are for deletions expressed as DB fusions; all other deletion mutants were expressed as TA fusions. Only deletions expressed as TA fusions could be tested for interaction with the MCP. ACD† is the ACD with several amino and carboxyl flanking residues, as explained in the text. In δACD, the 19-amino-acid ACD is replaced by Thr and Ser which correspond to a *Spe*I site used in constructing this mutant (see Materials and Methods). N.T. indicates that the pair was not tested.

not AP) would result in nuclear translocation of the HCMV MCP.

This was tested by transfecting COS-7 cells with a plasmid encoding the HCMV MCP, alone or together with a second plasmid encoding either the SCMV pAP or AP, as described in Materials and Methods. Three days after transfection, the cells were fixed, permeabilized, incubated with either anti-MCP (specific for the carboxyl end of the MCP) or anti-N1 (specific for the amino end of both the pAP and AP), and examined by indirect immunofluorescence. We found that the MCP expressed alone was confined to the cytoplasm (Fig. 5A) and that both the pAP (Fig. 5B) and AP (Fig. 5C) were localized entirely in the nucleus when expressed alone. When MCP and pAP were coexpressed, we observed nuclear localization of both the MCP (Fig. 5D1 to D3) and the pAP (Fig. 5E) and a more coalesced pattern of MCP fluorescence than was seen in cells expressing either of the proteins alone. Parenthetically, some cells in the pAP/MCP coexpression experiments exhibited cytoplasmic MCP localization, consistent with a less than complete efficiency of cotransfection using the DEAE-dextran protocol (reference 41 and data not shown). In contrast to the results obtained when pAP and MCP were coexpressed, no nuclear localization of the MCP was seen in cells coexpressing the MCP and AP (Fig. 5F), even though the transfection efficiency for the plasmid encoding AP appeared to be equivalent to that encoding pAP in these coexpressions (e.g., compare Fig. 5G and E). Thus, the tail of the pAP, which is required for the pAP-MCP interaction in the two-hybrid system (Fig. 2a to d; Fig. 4, pAP and AP), is also needed to promote nuclear localization of the HCMV MCP in transfected cells.

HCMV pAP interacts with itself. During the course of the experiments described above, we observed that the HCMV pAP interacted with itself when coexpressed as fusion proteins with the GAL4 DB and TA domains (i.e., DB-pAPhcmv plus TA-pAPhcmv) (Table 2, assay a; Fig. 4, pAP; Table 3, assay a; Table 4, assay d). The HCMV AP also interacted with itself, and the interaction was stronger than the pAP-pAP interaction (Table 2; compare assays a and b), suggesting that the CMV pAP tail interferes with self-interaction in the two-hybrid system. The HCMV pAP self-interaction, like the pAP-MCP interaction, showed virus species specificity in that the HCMV pAP interacted with the SCMV pAP but neither of the CMV pAPs interacted with the HSV pAP homolog (Table 2, assays c to f).

The ACD of CMV pAP is required for pAP self-interaction. Our preliminary mapping of the domain responsible for HCMV pAP self-interaction ruled out the CCD but not the ACD, because the AP was able to interact with itself (Table 2, assay b). We used a series of deletion mutants to identify the pAP self-interactive domain and focused on the ACD as a candidate. The constructs that were tested are shown in Fig. 4, and the assay results that were obtained can be summarized as

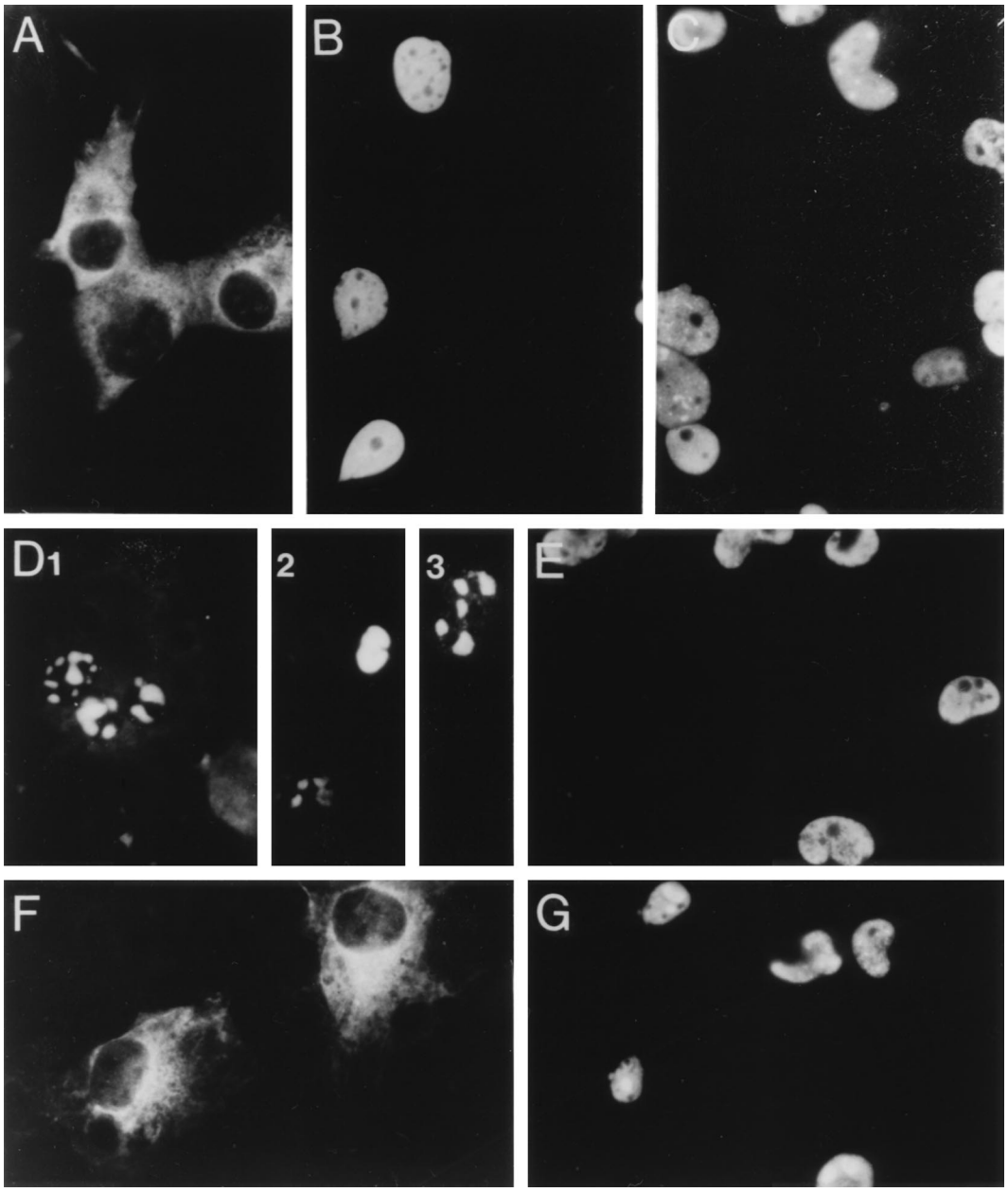

FIG. 5. The HCMV pAP tail promotes translocation of MCP into the nucleus. COS-7 cells were transfected, fixed and permeabilized, reacted with antisera to the CMV MCP or pAP, and examined by indirect immunofluorescence, all as described in the text. Shown are photomicrographs of representative cells in each preparation. The top three panels show cells expressing only the HCMV MCP and stained with anti-MCP (A), only the SCMV pAP and stained with anti-N1 (against the amino end of pAP) (B), and only SCMV AP and stained with anti-N1 (C). The middle panels show cells coexpressing HCMV MCP and SCMV pAP and stained with either anti-MCP (D1 to D3) or anti-N1 (E). The bottom panels show cells coexpressing HCMV MCP and SCMV AP and stained with either anti-MCP (F) or anti-N1 (G). Panels D1 to D3 are three different fields of the same preparation.

follows. (i) Deletion of the tail, and up to 250 amino acids from its carboxyl end, did not eliminate the ability of pAP to interact with full-length pAP (Fig. 4, δc1, AP, δc2, δc3, and δc4). All of these carboxy-terminal deletions interacted better with pAP than did full-length pAP (Fig. 4, β-Gal Activity). Deletion of the carboxyl 314 residues abolished self-interaction (Fig. 4, δc5). (ii) Deletion of the amino-terminal 30 residues did not noticeably affect interaction with pAP, compared with the pAP-pAP interaction (Fig. 4, δn1), but all deletions that removed the 19-amino-acid ACD (i.e., Fig. 4, δn2 to δn7) abolished interaction with pAP. (iii) All constructs containing the sequence Ala31-Tyr123, including a double deletion mutant lacking both the amino-terminal 30 residues and the carboxy-terminal 250 residues (Fig. 4, δn1/δc4), interacted with the pAP. Thus, deletional analysis mapped the sequence responsible for pAP self-interaction to the region between Ala31 and Tyr123, which contains the ACD.

Considering that nonreactivity of the shortest ACD-containing constructs (i.e., Fig. 4, δc5 and ACD†) may be due to some indirect effect (e.g., size or presentation), as was observed for the shortest CCD constructs (Fig. 4, δn6 and δn7), we made three additional constructs. The first was a deletion mutant in

TABLE 3. Requirement of the ACD for HCMV pAP self-interaction[a]

Assay	DB domain fusion	TA domain fusion	Blue colony color[b]	β-Gal activity[c] (nmol/min/mg of protein [relative activity])
a	pAPhcmv	pAPhcmv	+	8.0 (1.0)
b	pAPhcmv	ACD†hcmv-pAPhsv	+	10.3 (1.3)
c	pAPscmv	ACD†hcmv-pAPhsv	+	10.5 (1.3)
d	pAPhcmv	δACDhcmv	−	<0.01
e	δn1/δc4hcmv	ACD†hcmv-pAPhsv	+	47.1 (5.9)

[a] Cotransformations were done as described in the text with plasmids expressing the DB and TA fusion proteins indicated. Abbreviations are explained in the text, Fig. 1, and the legend to Fig. 4.
[b] Results of qualitative filter lift assays.
[c] Results of quantitative liquid assays. Values were calculated and normalized as described in the legend to Fig. 2 and Materials and Methods.

which the 19-amino-acid ACD was removed from the pAP (Fig. 4, δACD). This mutation eliminated detectable interaction with the HCMV pAP (Fig. 4, δACD; also see Table 3, assay d). The second construct had the point mutation Leu47 to Ala. This change altered one of the three amino acids that are highly conserved among all herpesvirus ACDs (marked with ‡ in Fig. 6). This mutant also failed to give detectable interaction with the pAP (Fig. 4, L47A).

Because the lack of interaction between pAP and δACD, or between pAP and L47A, could be attributed to conformational changes that affected a different site, our third construct was a chimera composed of the HCMV ACD† (HCMV sequence Ala31-Tyr59, which contains the HCMV ACD) fused to the amino terminus of the HSV pAP homolog (TA-ACD†hcmv-pAPhsv), which in its native form does not interact with HCMV or SCMV pAP (Table 2, assays e and f). ACD† differs from ACD by a three-residue amino extension and a seven-residue carboxyl extension that were added to provide short spacer sequences between it and the GAL4 TA domain at one end and the HSV pAP homolog at the other. This chimera interacted with both the HCMV and SCMV pAPs (Table 3, assays b and c), consistent with the ACD being the sequence involved in the pAP self-interaction. The ACD†hcmv-pAPhsv chimera interacted with the 93-amino-acid sequence of the double deletion mutant DB-δn1/δc4hcmv (Table 3, assay e), providing direct evidence that pAP self-interaction is between two ACD-containing domains.

The amino-end-deleted pAPs, the δACD pAP, and the L47A pAP were also tested for their interaction with the HCMV MCP. Surprisingly (i.e., because the pAP-MCP interaction mapped to the CCD at the carboxyl end of the pAP), only one of these mutants interacted well with the MCP. Removing the amino-terminal 30 residues from the pAP had no observed effect on its interaction with the MCP in a liquid assay (Fig. 4, δn1). However, when the amino-terminal 72 amino acids or the ACD was removed, or when Leu47 was replaced with Ala, interaction with the MCP was either eliminated or reduced by 32- or 4-fold, respectively (Fig. 4, δACD, δn2, and L47A). Removal of the amino-terminal 117 residues, or more, abolished MCP interaction (Fig. 4, δn3 to δn7). Thus, the 19-amino-acid ACD and possibly additional residues between Ser53 and Pro117 appear to contribute to the pAP-MCP interaction, as well as mediate the pAP-pAP interaction, in the GAL4 two-hybrid system.

The CMV proteinase precursor also interacts with CMV MCP and pAP. Because of their in-frame, 3′-coterminal genetic organization, the entire amino acid sequence of the pAP is present in the carboxyl half of the herpesvirus proteinase precursor (Fig. 1) (46, 82). As a result, the proteinase precursor also contains the ACD and CCD (Fig. 1, AC_{pra}) and, consequently, the potential to interact with the MCP, the pAP, and itself. We tested for these interactions by making a GAL4 TA fusion with the SCMV proteinase precursor, AC_{pra} (TA-AC_{pra}scmv), and coexpressing it with GAL4 DB fusions of either the HCMV MCP (DB-MCPhcmv), the SCMV pAP (DB-pAPscmv), or itself (DB-AC_{pra}scmv). Results of filter lift assays indicate that this active proteinase fusion protein interacted with the pAP and itself (Table 4, assays f and h) but not with the MCP (Table 4, assay b). However, a weak interaction was detected between AC_{pra} and the MCP in a liquid assay (Table 4, assay b, β-Gal activity).

To test the possibility that the poor reactivity between the MCP and the wild-type proteinase construct was due to autoproteolytic elimination of its CCD-containing tail (i.e., M-site cleavage), we made a new fusion construct, TA-S118A.Lscmv, with a proteolytically inactive mutant of AC_{pra} (i.e., S118A.L, where "L" stands for "long" or full-length proteinase precursor [83]). Coexpression of the MCP with this inactive proteinase gave a positive reaction in the filter lift assay and an approximately fourfold-greater reaction strength than the AC_{pra}/MCP pair in the liquid assays (Table 4; compare assays b and c). These results are consistent with M-site cleavage contributing to the observed lack of interaction between AC_{pra} and the MCP in the filter lift assay. Nevertheless, the relative strength of the interaction between the inactive proteinase and the MCP was slightly weaker than between the pAP and the MCP (≈2-fold; compare Fig. 2b with Table 4, assay c), suggesting that the amino half of the proteinase precursor may hinder its MCP interaction in the two-hybrid system.

When assemblin, the proteolytic domain of AC_{pra}, was tested as a TA fusion protein (TA-Ascmv), no self-interaction was detected (DB-Ascmv), nor were interactions detected between it and the pAP (DB-pAPscmv) or the HCMV MCP (DB-MCPhcmv) (data not shown). The inactive assemblin mutant, S118A.S (i.e., TA-S118A.S, where "S" stands for "short" or assemblin form of the proteinase), was also nonreactive with itself, the pAP, and the MCP when tested as a TA fusion (data not shown). Thus, these experiments did not detect the enzyme-substrate interactions that potentially occurred (e.g., assemblin with its own I site or with pAP M site), even when the S118A mutant enzyme or a noncleavable M-site mutant (i.e.,

TABLE 4. Interaction of the CMV proteinase precursor with CMV MCP, pAP, and itself[a]

Assay	DB domain fusion	TA domain fusion	Blue colony color[b]	β-Gal activity[c] (nmol/min/mg of protein [relative activity])
a	MCPhcmv	pAPhcmv	+	23.8 (1.000)
b	MCPhcmv	AC_{pra}scmv	−	0.1 (0.004)
c	MCPhcmv	S118A.Lscmv	+	0.4 (0.017)
d	pAPhcmv	pAPhcmv	+	32.5 (1.000)
e	pAPscmv	pAPscmv	+	14.8 (0.455)
f	pAPscmv	AC_{pra}scmv	+	10.7 (0.329)
g	AC_{pra}scmv	pAPscmv	+	1.7 (0.052)
h	AC_{pra}scmv	AC_{pra}scmv	+	0.2 (0.006)
i	pAPscmv	S118A.Lscmv	+	16.7 (0.514)

[a] Cotransformations were done as described in the text with plasmids expressing the DB and TA fusion proteins indicated. AC_{pra} is the SCMV proteinase precursor, and S118A.Lscmv is an inactive form of the SCMV proteinase having Ala substituted for the Ser nucleophile (83). Other abbreviations are explained in the text, Fig. 1, and the legend to Fig. 2.
[b] Results of qualitative filter lift assays.
[c] Results of quantitative liquid assays. Values were calculated and normalized as described in the legend to Fig. 2 and Materials and Methods.

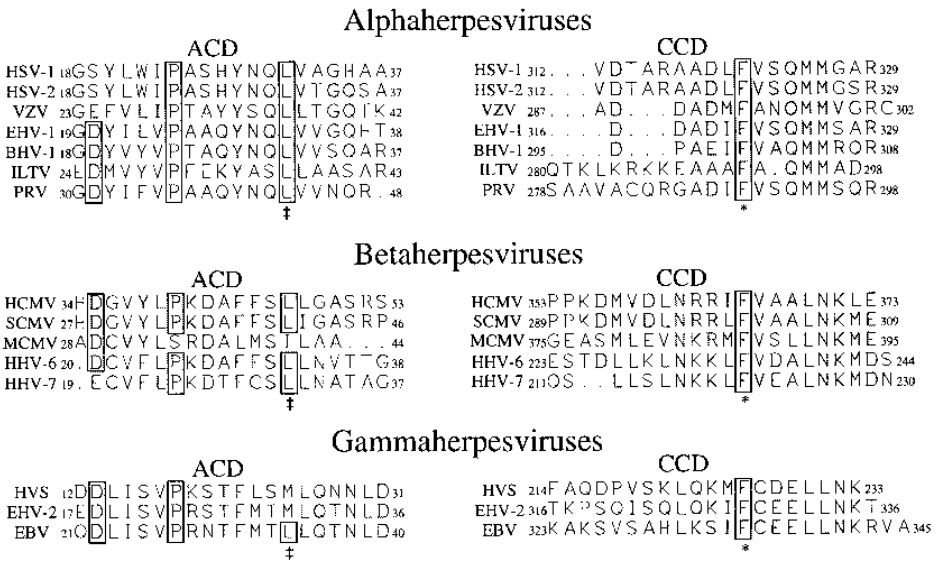

FIG. 6. Amino acid sequence alignments of the HCMV ACD and CCD and their apparent homologs in other herpesviruses. Alignment of the pAP sequences was done by using the program PILEUP. Sequences were obtained from GenBank: HCMV strain AD169 (12), SCMV strain Colburn (82), murine CMV (MCMV) (48), human herpesvirus 6 (HHV-6) (32), human herpesvirus 7 (HHV-7) (56), HSV-1 (50), HSV-2 (73), varicella-zoster virus (VZV) (16), equine herpesvirus 1 (EHV-1) (78), bovine herpesvirus 1 (BHV-1) (34), infectious laryngotracheitis virus (ILTV) (33), pseudorabies virus (PRV) (10), herpesvirus saimiri (HVS) (2), equine herpesvirus 2 (EHV-2) (77), Epstein-Barr virus (EBV) (4). Classification as alpha-, beta-, or gammaherpesviruses is based on the nomenclature of Roizman et al. (67). Amino acids that are identical or chemically similar among >50% of the viruses within a subfamily (e.g., alphaherpesviruses) are indicated by shading. Residues within the HCMV ACD and CCD that are perfectly conserved among >70% of all herpesviruses are boxed. The first and last amino acids of each domain are numbered. * indicates the Phe residue conserved throughout all herpesvirus CCD homologs; ‡ indicates the well-conserved Leu residue whose substitution abolished the pAP self-interaction.

A556$^-$) (83) was used in an attempt to slow or stabilize the interaction (data not shown).

DISCUSSION

We have used the GAL4 two-hybrid system to demonstrate that the CMV pAP interacts with itself and with the MCP through two different domains. Interaction with the MCP is through a 21-amino-acid sequence, called the CCD, at the carboxy terminus of the molecule; the self-interaction is through a 19-amino-acid sequence, called the ACD, near the amino end of the molecule (Fig. 3). All herpesvirus pAP homologs have ACD and CCD counterparts, and these domains are conserved and distinct within each of the three herpesvirus groups (i.e., alpha, beta, and gamma) (Fig. 6). Correspondingly, the pAP of another betaherpesvirus (i.e., SCMV) could substitute for the HCMV pAP in both the MCP interactions and self-interactions, but the pAP of an alphaherpesvirus (i.e., HSV) could not (Fig. 2 and Table 2). An influence of the ACD on the pAP-MCP interaction was identified and suggests that pAP multimerization may be an early step in organizing the MCP into precapsomeric elements. Evidence supporting these conclusions is discussed below as it relates to previous findings and provides insight into the capsid assembly pathway of CMV and, by extension, other herpesgroup viruses.

pAP-MCP interaction. Our evidence that the pAP-MCP interaction is mediated by the 21-amino-acid CCD is twofold. First, HCMV pAP deletion mutants lacking the CCD (i.e., deletion of the 65-amino-acid tail or its carboxy-terminal 37 amino acids) failed to interact with the HCMV MCP (Fig. 4, AP and δc1). A corresponding deletion of the entire 25-amino-acid tail of the HSV pAP homolog likewise eliminated its interaction with the HSV MCP in GAL4 two-hybrid (18) and coimmunoprecipitation (79) experiments. Second, fusion of the CMV CCD to a protein not interactive with the CMV MCP (e.g., HSV pAP homolog) converted it to MCP-interactive (Table 1, assay b). A comparable result was obtained when the counterpart 25-amino-acid tail domain of the HSV pAP homolog was fused to the HCMV pAP and expressed with the HSV MCP (Table 1, assay f). The capacity of these HCMV/HSV pAP chimeras to interact with the MCPs of both HCMV and HSV (Table 1, assays c and g) indicates that their resident MCP-binding domains (e.g., CCD of HCMV pAP) can function when situated internally and suggests that interaction of these sequences with the MCP is relatively unhindered by steric constraints.

It has recently been shown that an even smaller sequence (i.e., 12 amino acids) from near the carboxyl end of the tail of the HSV pAP homolog can convert glutathione *S*-transferase (GST) from noninteractive to interactive with the HSV MCP (37) and that this interaction is hydrophobic and critically dependent upon the presence of Phe321, which is conserved among all herpesvirus pAP homologs (Fig. 6) (37). A similar study done with the HCMV pAP has identified a 16-amino-acid sequence within the CCD that promotes interaction of its GST fusion protein with the HCMV MCP (7). The biological importance of these carboxy-terminal domains was demonstrated by the finding that capsid formation was perturbed, both in the recombinant baculovirus HSV capsid assembly system (42, 79) and in mutant HSV-infected cells (49), when the mature form of the protein (without tail) was used in place of the precursor (with tail).

The CMV pAP-MCP interaction detected by the two-hybrid experiments was corroborated by immunofluorescence localization assays, which also enabled us to begin investigating the biological significance of this interaction. Results of the study indicated that the CMV MCP did not enter the nucleus when expressed alone but did when expressed together with the CMV pAP (Fig. 5). The mature CMV AP, which retains both of the pAP nuclear localization signals (NLS) (62), did not

promote MCP nuclear translocation. We interpret these results as indicating that the CMV MCP interacts with the tail domain of the pAP in the cytoplasm, that this interaction provides the MCP with an NLS-bearing escort that enables it to be translocated into the nucleus, and that the resulting pAP/MCP complexes appear to coalesce or partition within the nucleus (Fig. 5D1 to D3).

Different results were obtained in a similar study of the counterpart HSV proteins. Unlike the CMV MCP, which remained in the cytoplasm when expressed alone, the HSV MCP was distributed throughout the cell (57). Because this protein is too large to diffuse into the nucleus (i.e., ≈40- to 60-kDa size cutoff [24]), this finding indicates that the HSV MCP may have a weak NLS, be partially degraded into fragments small enough to enter by diffusion and yet retain antigenic reactivity, or utilize a cellular protein as a nuclear translocation escort. Also in contrast to its CMV counterpart, the HSV AP homolog (i.e., without tail) was reported to promote nuclear translocation of the HSV MCP (42). Whether this effect indicates the presence of a second MCP-interactive domain within the AP portion of the pAP that was not detected by GAL4 two-hybrid (18) or coimmunoprecipitation (79) experiments, or is due to a secondary effect, is not resolved and remains an important question.

pAP self-interaction. The following evidence supports our conclusion that pAP self-interaction is mediated by the ACD (i.e., HCMV pAP, His34-Arg52). (i) A set of deletion mutants defined the interactive domain as being within the sequence Ala31 to Tyr123 (Fig. 4). (ii) A small ACD-containing fragment (i.e., ACD†) converted a protein (i.e., HSV pAP homolog) from noninteractive to interactive with the HCMV pAP when fused to its amino terminus (Table 3, assay b). (iii) Selective deletion of the ACD eliminated detectable pAP self-interaction (Fig. 4, δACD). (iv) Replacement of the ACD Leu47 with Ala, which would not be expected to dramatically affect overall conformation, also eliminated pAP self-interaction (Fig. 4, L47A). And finally, (v) ACD† (Ala31-Tyr59) interacted with deletion mutant δn1/δc4 (Ala31-Tyr123) (Table 3, assay e) indicating that the sequence with which ACD interacts is present between Ala31 and Tyr123.

Because the ACD is nearly perfectly conserved between the HCMV and SCMV pAPs (Fig. 3B), it seemed likely that the sequence in the δn1/δc4 mutant with which it interacts would also be highly conserved. The fact that the ACD is the only sequence within this region of the two proteins that is well conserved in both HCMV and SCMV suggests that pAP self-interaction occurs by the ACD of one pAP interacting with the ACD of another. If so, it is plausible that each ACD has two interactive surfaces (e.g., "lock and key" model [Fig. 7A]) that could enable multiple pAP monomers to associate into higher-order structures (e.g., Fig. 7B).

A self-interaction for the HSV pAP homolog, initially suggested by in vitro reconstitution experiments with the HSV AP counterpart, VP22a (53), was demonstrated by using the two-hybrid system (18), and the major interactive domain was mapped to the region of Gly151 to Gly198, well away from the predicted HSV counterpart of the CMV ACD (i.e., HSV Gly18 to Ala37 [Fig. 6]). More recent work has confirmed these results for the primary self-interactive region of the HSV pAP homolog and has defined it as being between His164 and His219 (reference 15a). Interestingly, these later studies have detected several additional domains of the HSV pAP homolog that are involved in its self-interaction, but none of them appears to be a direct homolog of the CMV ACD.

Effect of ACD on pAP-MCP interaction. One of the most intriguing observations to come out of this work was the apparent influence of the ACD on the pAP-MCP interaction. This influence was first suggested by the results of experiments designed to test the interaction of the CMV/HSV chimera pAPs with CMV and HSV MCPs. Data from those experiments showed that interaction of the CMV CCD with the CMV MCP was influenced by the AP sequence to which it was attached. Specifically, fusion of the CMV CCD to the carboxy terminus of the HSV pAP homolog, which interacts comparatively strongly with its MCP (Table 1, assay d), resulted in a chimera (DB-pAPhsv-CCDhcmv) that interacted more strongly with the HCMV MCP than did the native HCMV pAP (compare Fig. 5a and b). Conversely, when the HSV tail was fused to the terminus of the HCMV pAP, which interacts comparatively weakly with its MCP (Table 1, assay a), the resulting chimera (DB-pAPhcmv-Tailhsv) interacted more weakly with the HSV MCP than did the native HSV pAP homolog (Table 1; compare assays d and f).

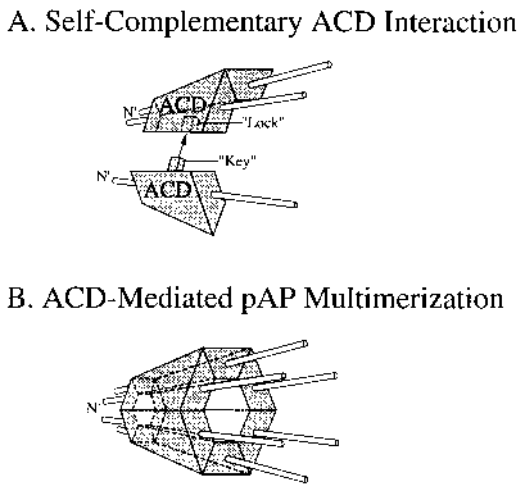

FIG. 7. Hypothetical "lock and key" model of CMV pAP self-complementarity and ACD-mediated multimerization. (A) A schematic representation of the self-complementary interaction between the ACDs of two CMV pAPs. The "key" face of one domain is suggested to oligomerize with the complementary "lock" face of another. Rods represent the amino and carboxyl ends of the pAP molecule. (B) Diagram illustrating a hypothetical hexameric structure resulting from the ACD-mediated interaction of six pAP molecules.

We also found that all deletions that eliminated the ACD, and consequently pAP self-interaction, also dramatically reduced the pAP-MCP interaction (Fig. 4). The most notable of these was δACD, which lacked only the specific ACD sequence and gave severely reduced MCP binding (Fig. 4 and data not shown). Because a substantial reduction in MCP binding was also seen with a point mutation in one of the highly conserved ACD residues (Fig. 4, L47A), it seems unlikely that the result was simply due to an unexpected, deletion-induced conformational change in the pAP. A similar effect of the self-interactive domain on MCP binding has been observed for the HSV pAP homolog (reference 15a).

Taken together, these findings indicate that the pAP self-interaction markedly enhances the pAP-MCP interaction and that the self-interacting and MCP-interacting portions of the molecule need not be from the same pAP species (e.g., HSV tail works on CMV pAP). We have not tested whether an unrelated, self-interacting protein can replace the viral pAP in potentiating MCP interaction. However, this possibility is consistent with the finding that short carboxy-terminal peptides of either the HSV or CMV pAP fused to GST, a protein that forms dimers (81), interact with their respective MCPs in vitro

(7, 37). It would be instructive, in this connection, to know whether the strength of these interactions would be altered by using a nondimerizing fusion partner, such as the maltose-binding protein.

Implications for assembly. Because the CMV MCP appears unable to enter the nucleus unescorted (Fig. 5A), pAP-MCP interaction is expected to occur in the cytoplasm of CMV-infected cells. Our results indicate that the pAP-MCP interaction is enhanced when the pAP is able to multimerize, suggesting that pAP self-association is important for the efficient formation or stabilization of pAP/MCP complexes and that it may represent an initiating or accelerating step in the capsid assembly pathway. Self-associated pAP monomers, with or without bound MCP, could recruit into the complex both additional pAPs (through ACD interactions) and MCPs (through CCD interactions). Growth of the nascent complex could proceed continuously (e.g., sequential addition of pAP and MCP monomers) or discontinuously (e.g., stepwise association of preformed pAP_2/MCP_2 complexes or of pAP multimers with MCP monomers). Because there is no evidence that capsids can form in the cytoplasm of herpesvirus-infected cells, the putative pAP/MCP assemblages presumably would be of limited size and complexity (e.g., hexameric or pentameric protocapsomer).

The role of the pAP in this cytoplasmic complex is suggested to be at least twofold. First, it could provide a mechanism to organize the MCP into the structural elements required for subsequent steps in capsid assembly (e.g., association with the triplex proteins). For example, pAP conformation may dictate that its most stable multimer is a hexamer, as suggested in Fig. 7, thereby enabling its self-interaction to provide the directive force to organize six MCP monomers into hexon precursors. By extension, pentons could be formed by replacing some or all of the pAP molecules with the proteinase precursor (e.g., AC_{pra}), which contains the entire pAP sequence as its carboxyl end (Fig. 1), but may have a conformation more compatible with fivefold symmetry. A second role for the pAP in this putative precapsomeric cytoplasmic complex would be to supply the NLS needed for translocating it into the nucleus, where the capsid assembly process would continue.

ACKNOWLEDGMENTS

We thank Daniel Nathans and Pierre Chevray for providing the GAL4 vectors pPC86 and pPC97, *S. cerevisiae* PCY2, and advice on using the GAL4 two-hybrid system. We thank Stan Person and Prashant Desai for advice about β-Gal assays and for helpful discussions.

M.K.B. is a student in the Biochemistry, Cellular, and Molecular Biology training program and was supported in part by U.S. Public Health Service grant GM07445. S.M.P. is a student in the Pharmacology and Molecular Sciences training program and was supported by USPHS grant GM07626. This work was aided by USPHS research grants AI13718 and AI32957.

REFERENCES

1. **Adams, G. A., and J. K. Rose.** 1985. Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. Cell **41:**1007–1015.
2. **Albrecht, J.-C., J. Nicholas, D. Biller, K. R. Cameron, B. Biesinger, C. Newman, S. Wettmann, M. A. Craxton, H. Coleman, B. Fleckenstein, and R. W. Honess.** 1992. Primary structure of the herpesvirus saimiri genome. J. Virol. **66:**5047–5058.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Wiley, New York, N.Y.
4. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Sequin, P. S. Tufnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310:**207–211.
5. **Baker, T. S., W. W. Newcomb, F. P. Booy, J. C. Brown, and A. C. Steven.** 1990. Three-dimensional structures of maturable and abortive capsid of equine herpesvirus 1 from cryoelectron microscopy. J. Virol. **64:**563–573.
6. **Baldick, C. J., and T. E. Shenk.** 1996. Proteins associated with purified human cytomegalovirus particles. J. Virol. **70:**6097–6105.
7. **Beaudet-Miller, M., R. Zhang, J. Durkin, W. Gibson, A. D. Kwong, and Z. Hong.** 1996. Virus-specific interaction between the human cytomegalovirus major capsid protein and the C terminus of the assembly protein precurser. J. Virol. **70:**8081–8088.
8. **Black, L. W., and M. K. Showe.** 1984. Morphogenesis of the T4 head. American Society for Microbiology, Washington, D.C.
9. **Breeden, L., and K. Nasmyth.** 1985. Regulation of the yeast HO gene. Cold Spring Harbor Symp. Quant. Biol. **50:**643–650.
10. **Camacho, A., and E. Tabares.** 1994. Submitted to GenBank. Geninfo accession number 609218.
11. **Chee, M., S. A. Rudolph, B. Plachter, B. Barrell, and G. Jahn.** 1989. Identification of the major capsid protein gene of human cytomegalovirus. J. Virol. **63:**1345–1353.
12. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immuno. **154:**125–169.
13. **Chevray, P. M., and D. Nathans.** 1992. Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. Proc. Natl. Acad. Sci. USA **89:**5789–5793.
14. **Chien, C., P. L. Bartel, R. Sternglanz, and S. Fields.** 1991. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA **88:**9578–9582.
15. **Cohen, G. H., M. Ponce de Leon, H. Diggelmann, W. C. Lawrence, S. K. Vernon, and R. J. Eisenberg.** 1980. Structural analysis of the capsid polypeptides of herpes simplex virus types 1 and 2. J. Virol. **34:**521–531.

15a. **Cordingley, M. G.** Personal communication.

16. **Davison, A. J., and J. E. Scott.** 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67:**1759–1816.
17. **Davison, M. D., F. J. Rixon, and A. J. Davison.** 1992. Identification of genes encoding two capsid proteins (VP24 and VP26) of herpes simplex virus type 1. J. Gen. Virol. **73:**2709–2713.
18. **Desai, P., and S. Person.** 1996. Molecular interactions between the HSV-1 capsid proteins as measured by the yeast two-hybrid system. Virology **220:** 516–521.
19. **Estojak, J., R. Brent, and E. A. Golemis.** 1995. Correlation of two-hybrid affinity data with in vitro measurements. Mol. Cell. Biol. **15:**5820–5829.
20. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
21. **Finley, R., and R. Brent.** 1994. Interaction mating reveals binary and ternary connections between Drosophila cell cycle regulators. Proc. Natl. Acad. Sci. USA **91:**12980–12984.
22. **Friedmann, A., J. E. Coward, H. S. Rosenkranz, and C. Morgan.** 1975. Electron microscopic studies on assembly of herpes simplex virus upon removal of hydroxyurea block. J. Gen. Virol. **26:**171–181.
23. **Gao, M., L. Matusick-Kumar, W. Hurlburt, S. F. DiTusa, W. W. Newcomb, J. C. Brown, P. J. McCann, I. Deckman, and R. J. Colonno.** 1994. The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. J. Virol. **68:**3702–3712.
24. **Gerace, L., and B. Burke.** 1988. Functional organization of the nuclear envelope. Annu. Rev. Cell Biol. **4:**335–374.
25. **Gibson, W.** 1981. Structural and nonstructural proteins of strain Colburn cytomegalovirus. Virology **111:**516–537.
26. **Gibson, W.** 1991. Cytomegalovirus protein structure and function. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.
27. **Gibson, W., M. K. Baxter, and K. S. Clopper.** 1996. Cytomegalovirus "missing" capsid protein identified as heat-aggregatable product of human cytomegalovirus UL46. J. Virol. **70:**7454–7461.
28. **Gibson, W., K. S. Clopper, W. J. Britt, and M. K. Baxter.** 1996. Human cytomegalovirus (HCMV) "smallest capsid protein" identified as product of small open reading frame located between HCMV UL48 and UL49. J. Virol. **70:**5680–5683.
29. **Gibson, W., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10:**1044–1052.
30. **Gibson, W., A. R. Welch, and M. R. T. Hall.** 1995. Assemblin, a herpes virus serine maturational proteinase and new molecular target for antivirals. Perspect. Drug Discov. Des. **2:**413–426.
31. **Gilks, C. B., S. E. Bear, H. L. Grimes, and P. N. Tsichlis.** 1993. Progression of interleukin-2 (IL-2)-dependent rat T cell lymphoma lines in IL-2-independent growth following activation of a gene (*Gfi-1*) encoding a novel zinc finger protein. Mol. Cell. Biol. **13:**1759–1768.
32. **Gompels, U. A., J. Nicholas, G. Lawrence, M. Jones, B. J. Thomson, M. E. D. Martin, S. Efstathiou, M. Craxton, and H. A. Macaulay.** 1995. The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. Virology **209:**29–51.
33. **Griffin, A. M.** 1990. The complete sequence of the capsid p40 gene from

infectious laryngotracheitis virus. Nucleic Acids Res. **18:**3664.
34. **Haanes, E. J., D. R. Thomsen, S. Martin, F. L. Homa, and D. E. Lowery.** 1995. 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. J. Virol. **69:**7375–7379.
35. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Heilman, C. J., M. Zwieg, J. R. Stephenson, and B. Hampar.** 1979. Isolation of a nucleocapsid polypeptide of herpes simplex virus types 1 and 2 possessing immunologically type-specific and cross-reactive determinants. J. Virol. **29:**34–42.
37. **Hong, Z., M. Beaudet-Miller, J. Burkin, R. Zhang, and A. D. Kwong.** 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. J. Virol. **70:**533–540.
38. **Irmiere, A., and W. Gibson.** 1983. Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. Virology **130:**118–133.
39. **Irmiere, A., and W. Gibson.** 1985. Isolation of human cytomegalovirus intranuclear capsids, characterization of their protein constituents, and demonstration that the B-capsid assembly protein is also abundant in noninfectious enveloped particles. J. Virol. **56:**277–283.
40. **Kamens, J., and R. Brent.** 1991. A yeast transcription assay defines distinct *rel* and *dorsal* DNA recognition sequences. New Biol. **3:**1005–1013.
41. **Kang, K. I., J. Devin, F. Cadepond, N. Jibard, A. Guiochon-Mantel, E.-E. Baulieu, and M.-G. Catelli.** 1994. In vivo functional protein-protein interaction: nuclear targeted hsp90 shifts cytoplasmic steroid receptor mutants into the nucleus. Proc. Natl. Acad. Sci. USA **91:**340–344.
42. **Kennard, J., F. J. Rixon, I. S. McDougall, J. D. Tatman, and V. G. Preston.** 1995. The 25 amino acid residues at the carboxy terminus of the herpes simplex virus type 1 UL26.5 protein are required for the formation of the capsid shell around the scaffold. J. Gen. Virol. **76:**1611–1621.
43. **King, J., and S. Casjens.** 1974. Catalytic head assembling protein in virus morphogenesis. Nature (London) **251:**112–119.
44. **Ladin, B. F., M. L. Blankenship, and T. Ben-Porat.** 1980. Replication of herpesvirus DNA. V. Maturation of concatemeric DNA of pseudorabies virus to genome length is related to capsid formation. J. Virol. **33:**1151–1164.
45. **Lee, J. Y., A. Irmiere, and W. Gibson.** 1988. Primate cytomegalovirus assembly: evidence that DNA packaging occurs subsequent to B capsid assembly. Virology **167:**87–96.
46. **Liu, F., and B. Roizman.** 1991. The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. J. Virol. **65:**5149–5156.
47. **Long, E. O., S. Rosen-Bronson, D. R. Karp, M. Malnati, R. P. Sekaly, and D. Jaraquemada.** 1991. Efficient cDNA expression vectors for stable and transient expression of HLA-DR in transfected fibroblast and lymphoid cells. Hum. Immunol. **31:**229–235.
48. **Loutsch, J. M., N. J. Galvin, M. L. Bryant, and B. C. Holwerda.** 1994. Cloning and sequence analysis of murine cytomegalovirus protease and capsid assembly protein genes. Biochem. Biophys. Res. Commun. **203:**472–478.
49. **Matusick-Kumar, L., W. W. Newcomb, J. C. Brown, P. J. McCann, W. Hurlburt, S. P. Weinheimer, and M. Gao.** 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. J. Virol. **69:**4347–4356.
50. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
51. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Newcomb, W. B., F. L. Homa, D. R. Thomsen, Z. Ye, and J. C. Brown.** 1994. Cell-free assembly of the herpes simplex virus capsid. J. Virol. **68:**6059–6063.
53. **Newcomb, W. W., and J. C. Brown.** 1991. Structure of the herpes simplex virus capsid: effects of extraction with guanidine hydrochloride and partial reconstitution of extracted capsids. J. Virol. **65:**613–620.
54. **Newcomb, W. W., J. C. Brown, F. P. Booy, and A. C. Steven.** 1989. Nucleocapsid mass and capsomer protein stoichiometry in equine herpesvirus 1: scanning transmission electron microscopic study. J. Virol. **63:**3777–3783.
55. **Newcomb, W. W., B. L. Trus, F. P. Booy, A. C. Steven, J. S. Wall, and J. C. Brown.** 1993. Structure of the herpes simplex virus capsid: molecular composition of the pentons and the triplexes. J. Mol. Biol. **232:**499–511.
56. **Nicholas, J.** 1996. Determination and analysis of the complete nucleotide sequence of human herpesvirus 7. J. Virol. **70:**5975–5989.
57. **Nicholson, P., C. Addison, A. M. Cross, J. Kennard, V. G. Preston, and F. J. Rixon.** 1994. Localization of the herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires the abundant scaffolding protein VP22a. J. Gen. Virol. **75:**1091–1099.
58. **O'Callaghan, D. J., J. M. Hyde, G. A. Gentry, and C. C. Randall.** 1968. Kinetics of viral deoxyribonucleic acid, protein, and infectious particle production and alterations in host macromolecular syntheses in equine abortion (herpes) virus-infected cells. J. Virol. **2:**793–804.
59. **O'Callaghan, D. J., and C. C. Randall.** 1976. Molecular anatomy of herpesviruses: recent studies. Prog. Med. Virol. **22:**152–210.
60. **Perdue, M. L., J. C. Cohen, M. C. Kemp, C. C. Randall, and D. J. O'Callaghan.** 1975. Characterization of three species of nucleocapsids of equine herpesvirus type-1 (EHV-1). Virology **64:**187–204.
61. **Person, S., S. Laquerre, P. Desai, and J. Hempel.** 1993. Herpes simplex virus type 1 capsid protein VP21 originates within the UL26 open reading frame. J. Gen. Virol. **74:**2269–2273.
62. **Plafker, S. M., and W. Gibson.** Unpublished data.
63. **Preston, V. G., J. A. Coates, and F. J. Rixon.** 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. J. Virol. **45:**1056–1064.
64. **Rixon, F. J., A. M. Cross, C. Addison, and V. G. Preston.** 1988. The products of the herpes simplex virus type 1 gene UL26 which are involved in DNA packaging are strongly associated with empty but not full capsids. J. Gen. Virol. **69:**2879–2891.
65. **Robertson, B. J., P. J. McCann III, L. Matusick-Kumar, W. W. Newcomb, J. C. Brown, R. J. Colonno, and M. Gao.** 1996. Separate functional domains of the herpes simplex virus type 1 protease: evidence for cleavage inside capsids. J. Virol. **70:**4317–4328.
66. **Robson, L., and W. Gibson.** 1989. Primate cytomegalovirus assembly protein: genome location and nucleotide sequence. J. Virol. **63:**669–676.
67. **Roizman, R. B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, K. Wolf.** 1981. Herpesviridae. Definition, provisional nomenclature and taxonomy. Intervirology **16:**201–217.
68. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
69. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
70. **Schacterle, G. R., and R. L. Pollack.** 1973. A simplified method for the quantitative assay of small amounts of protein in biological material. Anal. Biochem. **51:**654–655.
71. **Schenk, P., A. S. Woods, and W. Gibson.** 1991. The 45-kDa protein of cytomegalovirus (Colburn) B-capsids is an amino-terminal extension form of the assembly protein. J. Virol. **65:**1525–1529.
72. **Sherman, G., and S. L. Bachenheimer.** 1988. Characterization of intranuclear capsids made by ts morphogenic mutants of HSV-1. Virology **163:**471–480.
73. **Steffy, K. R., S. Schoen, and C. M. Chen.** 1995. Nucleotide sequence of the herpes simplex virus type 2 gene encoding the protease and capsid protein ICP35. J. Gen. Virol. **76:**1069–1072.
74. **Szilagyi, J. F., and C. Cunningham.** 1991. Identification and characterization of a novel non-infectious herpes simplex virus-related particle. J. Gen. Virol. **72:**661–668.
75. **Talbot, P., and J. D. Almeida.** 1977. Human cytomegalovirus: purification of enveloped virions and dense bodies. J. Gen. Virol. **36:**345–349.
76. **Tatman, J. D., V. G. Preston, P. Nicholson, R. M. Elliott, and F. J. Rixon.** 1994. Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. J. Gen. Virol. **75:**1101–1113.
77. **Telford, E. A., M. S. Watson, H. C. Aird, J. Perry, and A. J. Davison.** 1995. The DNA sequence of equine herpesvirus type 2. J. Mol. Biol. **249:**520–528.
78. **Telford, E. A. R., M. S. Watson, K. McBride, and A. J. Davison.** 1992. The DNA sequence of equine herpes 1. Virology **189:**304–316.
79. **Thomsen, D. R., W. W. Newcomb, J. C. Brown, and F. L. Homa.** 1995. Assembly of the herpes simplex virus capsid: requirement for the carboxyl-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes. J. Virol. **69:**3690–3703.
80. **Thomsen, D. R., L. L. Roof, and F. L. Homa.** 1994. Assembly of herpes simplex virus (HSV) intermediate capsids in insect cells infected with recombinant baculoviruses expressing HSV capsid proteins. J. Virol. **68:**2442–2457.
81. **Walker, J., P. Crowley, A. D. Moreman, and J. Barrett.** 1993. Biochemical properties of cloned glutathione *S*-transferases from *Schistosoma mansoni* and *Schistosoma japonicum*. Mol. Biochem. Parasitol. **61:**255–264.
82. **Welch, A. R., L. M. McNally, and W. Gibson.** 1991. Cytomegalovirus assembly protein nested gene family: four 3′-coterminal transcripts encode four in-frame, overlapping proteins. J. Virol. **65:**4091–4100.
83. **Welch, A. R., L. M. McNally, M. R. T. Hall, and W. Gibson.** 1993. Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine. J. Virol. **67:**7360–7372.
84. **Welch, A. R., A. S. Woods, L. M. McNally, R. J. Cotter, and W. Gibson.** 1991. A herpesvirus maturational protease, *assemblin*: identification of its gene, putative active site domain, and cleavage site. Proc. Natl. Acad. Sci. USA **88:**10792–10796.