JOURNAL OF VIROLOGY, Aug. 1992, p. 4839–4847
0022-538X/92/084839-09$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 66, No. 8

Posttranslational Modification and Subcellular Localization of the p12 Capsid Protein of Herpes Simplex Virus Type 1

DAVID S. McNABB AND RICHARD J. COURTNEY†*

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, 1501 Kings Highway, P.O. Box 33932, Shreveport, Louisiana 71130-3932

Received 1 April 1992/Accepted 13 May 1992

We have previously shown that the 12-kDa capsid protein (p12) of herpes simplex virus type 1 (HSV-1) is a γ_2 (true late) gene product encoded by the UL35 open reading frame (D. S. McNabb and R. J. Courtney, J. Virol. 66:2653–2663, 1992). To extend the characterization of p12, we have investigated the posttranslational modifications and intracellular localization of the 12-kDa polypeptide. These studies have demonstrated that p12 is modified by phosphorylation at serine and threonine residues. In addition, analysis of p12 by acid-urea gel electrophoresis has indicated that the protein can be resolved into three components, designated p12a, p12b, and p12c. Using isotopic-labeling and alkaline phosphatase digestion experiments, we have determined that p12a and p12b are phosphorylated forms of the protein, and p12c is likely to represent the unphosphorylated polypeptide. The kinetics of phosphorylation was examined by pulse-chase radiolabeling, and these studies indicated that p12c can be completely converted into p12a and p12b following a 4-h chase. All three species of p12 were found to be associated with purified HSV-1 virions; however, p12b and p12c represented the most abundant forms of the protein within viral particles. We have also examined the intracellular localization of p12 by cell fractionation and indirect immunofluorescence techniques. These results indicated that p12 is predominantly localized in the nucleus of HSV-1-infected cells and appears to be restricted to specific regions within the nucleus.

Among the 30 or more structural proteins which constitute the herpes simplex virus type 1 (HSV-1) virions (6, 16, 31, 39), only 7 of these polypeptides are components of the nucleocapsids. Gibson and Roizman (13) have identified six proteins, designated VP5, VP19c, VP21, VP22a, VP23, and VP24, as components of capsids isolated from the nuclear fraction of HSV-1-infected cells. Subsequently, Heilman et al. (15) and Cohen et al. (8) identified a seventh capsid protein, designated p12 and NC7, respectively. The three-dimensional structure of the herpesvirus capsids has been partially defined elsewhere (1, 3, 36); however, the precise structural locations and functions of these seven capsid proteins remain unresolved. The major capsid protein (VP5) is thought to be the main component of the hexavalent (36, 41) and possibly the pentavalent capsomers (28, 36). VP19c has been shown to be a DNA-binding protein (4) which appears to be covalently linked to VP5 by disulfide bonding (44). The exact location of VP19c within the capsids remains uncertain, but studies have suggested that it may compose or form the pentavalent capsomers (41) or it may reside within the interior of the capsids (5). VP22a is a component of the core region of the capsids (27, 28). It is a highly processed phosphoprotein (5) which transiently associates with the capsids (13) and is involved in the packaging of viral DNA (32). Braun et al. (4) have suggested that VP21 may represent a higher-molecular-weight form of VP22a. Surface radiolabeling studies have indicated that VP23 is at least partially exposed on the capsid surface (5). The location of VP23 on the capsid surface and the estimated number of molecules per capsid have led to the suggestion that VP23 may form the intercapsomeric fibrils connecting adjacent capsomers (36). The information currently available on VP24 and p12 remains relatively sparse.

Our studies have focused on understanding the structural and functional role of p12 within the viral capsids during the assembly and maturation of HSV. Previous studies have demonstrated that p12 represents a 12-kDa polypeptide which is associated with intranuclear capsids (8, 15). More recent studies by Newcombe and Brown (28) have suggested that p12 (referred to as VP26) may reside in close proximity to the pentavalent capsomers. In an effort to further characterize p12, we have recently identified the UL35 open reading frame as the gene encoding the 12-kDa capsid protein (25). The UL35 open reading frame is predicted to encode a 112-amino-acid protein with a calculated molecular weight of 12,095 (24), which is consistent with the apparent molecular weight of p12 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8, 15, 25). Additionally, we have obtained data which indicate that p12 is more abundant in mature HSV-1 virions than capsids isolated from the nuclear fractions of HSV-infected cells (25). Although we can provide no explanation for these findings, the difference in the amount of DNA associated with each of these particle preparations may be of some relevance. Previous studies have indicated that intranuclear capsids represent a mixed population of DNA-containing and empty viral capsids (22, 37), whereas mature HSV-1 virions represent a relatively homogeneous population of DNA-containing virus particles. Thus, the amount of p12 associated with intranuclear capsids versus mature virions seems to correlate with the amount of DNA present in each of the populations. This has led us to speculate that p12 may somehow interact with the viral DNA present within the nucleocapsids of HSV-1 (25). Analysis of the predicted amino acid sequence of p12 has indicated that the protein is extremely basic, with a calculated pI of 11.6 (26). It is conceivable that a protein with such basic properties could

* Corresponding author.
† Present address: Department of Microbiology and Immunology, Pennsylvania State University College of Medicine, 500 University Drive, P.O. Box 850, Hershey, PA 17033.

interact with the viral DNA and may possibly promote the condensation and/or packaging of the viral genome.

Interestingly, studies by Knopf and Kaerner (20) have identified an acid-extractable 12-kDa basic phosphoprotein (termed BP2) associated with purified HSV-1 virions and chromatin isolated from HSV-infected cells. Analysis of BP2 by acid-urea gel electrophoresis revealed that the polypeptide could be separated into three components, designated BP2a, BP2b, and BP2c, which appeared to differ in phosphate content (20). On the basis of the current data, we cannot definitively conclude that p12 and BP2 are the same polypeptide; however, the similarities in apparent molecular weights and the association of both of these basic proteins with virions strongly imply that this may be true. If these proteins are equivalent, this would provide additional evidence that p12 may interact with the viral DNA in HSV-infected cells and possibly within the virions.

In this article, we report on studies which demonstrate that p12 is posttranslationally modified by phosphorylation at the serine and threonine residues. Further analyses of p12 by acid-urea gel electrophoresis revealed that the 12-kDa polypeptide could be resolved into three components and that these components represent variably phosphorylated species of p12. In addition, studies with purified HSV-1 virions have indicated that all three forms of p12 are contained within the virus. We have also determined the intracellular localization of p12 by cell fractionation and indirect immunofluorescence techniques. These studies demonstrated that p12 is predominantly localized in the nuclei of HSV-infected cells and appears to be sequestered within specific regions of the nucleus.

MATERIALS AND METHODS

Cell culture and virus. HEp-2 cells and human embryonic lung (MRC-5) cells were grown in Eagle's minimal essential medium containing 0.075% sodium bicarbonate and supplemented with 10% newborn and 10% fetal calf serum, respectively. African green monkey kidney (CV-1) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Stocks of the KOS strain of HSV-1 were prepared in MRC-5 cells, and all virus titrations were conducted on African green monkey kidney (Vero) cell monolayers (2).

Antiserum reagents. The immunological reagents used in these studies include hyperimmune monospecific polyclonal rabbit antiserum to ICP8 (11) and TrpE-UL35 antiserum, which is specific for the p12 capsid protein of HSV-1 (25). A mouse monoclonal antibody directed against the 65-kDa DNA-binding protein of HSV-1 has been previously described (23).

Metabolic labeling, immunoprecipitation, and SDS-PAGE. To label viral proteins, HEp-2 cells were mock infected or infected with HSV-1 at a multiplicity of infection of 10 PFU per cell. Following a 1-h adsorption at 37°C, the inoculum was removed and the cells were overlaid with fresh maintenance medium containing 2% calf serum. Prior to radiolabeling, cells were incubated for 1 h in minimal essential medium without methionine and containing 2% dialyzed calf serum. At various times postinfection, cells were radiolabeled with [^{35}S]methionine (100 μCi/ml; specific activity, 700.7 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) and harvested by scraping. The cell pellets were washed in cold phosphate-buffered saline (PBS) and subsequently used in immunoprecipitations. When pulse-chase labeling experiments were performed, mock-infected or HSV-1-infected cells were pulsed for 30 min with 100 μCi of [^{35}S]methionine per ml and chased in the presence of 50-fold excess unlabeled methionine for various periods of time. For $^{32}P_i$ isotopic labeling experiments, cells were incubated for 1 h in phosphate-free medium containing 2% dialyzed calf serum prior to radiolabeling with $^{32}P_i$ (500 μCi/ml; specific activity, 8,500 Ci/mmol; Dupont, NEN Research Products). The [^{3}H]glucosamine (100 μCi/ml; specific activity, 30 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) radiolabeling studies were performed with minimal essential medium containing 2% dialyzed calf serum. Whole-cell pellets or purified HSV-1 virions were resuspended in cold radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 100 μg of tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK] per ml, 2 mM benzamidine), vortexed vigorously, incubated on ice for 5 min, and subjected to sonication. The extracts or purified virions were then boiled for 4 min and clarified by centrifugation for 15 min at 16,000 × *g*. Protein assays were subsequently performed on each sample with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.), with bovine serum albumin as the standard. Equal amounts of total-cell protein were incubated for 3 h at 4°C with the appropriate antibody, and protein A-agarose (50% [vol/vol]; Repligen, Cambridge, Mass.) was subsequently added and the incubation was continued for an additional 2 h at 4°C. The immune complexes were washed four times with cold radioimmunoprecipitation assay buffer, and one final wash was performed with a buffer containing 50 mM Tris-HCl (pH 7.5) and 500 mM NaCl. The bound proteins were eluted by boiling for 3 min in SDS-PAGE sample buffer. Analysis of samples by electrophoresis in SDS-polyacrylamide gels containing 8 or 15% polyacrylamide was performed as described previously (30). Following electrophoresis, the gels were fixed, dried onto 3MM filter paper (Whatman, Inc., Clifton, N.J.), and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.) at −80°C. The [^{35}S]methionine- and [^{3}H]glucosamine-labeled proteins were detected by the use of a fluorographic enhancer (Fluoro-Hance; Research Products International Corp., Mount Prospect, Ill.).

Phosphoamino acid analysis. Following the radiolabeling of HSV-1-infected HEp-2 cells with 500 μCi of $^{32}P_i$ per ml between 12 and 18 h postinfection, cell lysates were prepared and subjected to immunoprecipitation with the TrpE-UL35 antiserum. The immunoprecipitated proteins were separated by SDS-PAGE and electrophoretically transferred to Immobilon P membranes (Millipore Co., Bedford, Mass.), and p12 was localized by autoradiography. The areas of Immobilon P which contained p12 were excised, and the protein was hydrolyzed directly from the membrane by incubation at 110°C for 1 h in 5.7 M HCl (17). The hydrolyzed amino acids were separated by one-dimensional high-voltage electrophoresis on cellulose thin-layer plates (100 μm; EM Science, Gibbstown, N.J.) with pyridine acetate buffer, pH 3.5 (10). The plates were subsequently dried and stained with 0.2% ninhydrin (Sigma Chemicals, St. Louis, Mo.) to visualize the phosphoamino acid markers, and autoradiography was performed with an intensifying screen at −80°C.

Acid-urea gel electrophoresis. For acid-urea gel analysis, isotopically labeled p12 was immunoprecipitated from cell extracts as described above, except that the immune complexes were washed an additional three times in the final wash buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl) to remove the residual detergent. The bound proteins were

eluted with acid-urea sample buffer (0.9 M acetic acid, 6.25 M urea, 0.002% methyl green) by heating the samples at 80°C for 15 min. Electrophoresis was carried out at a constant voltage (10 V/cm) in 15% polyacrylamide gels containing 6.25 M urea and 0.9 M acetic acid (pH 3.2) by the method of Panyim and Chalkley (29) as modified by Knopf and Kaerner (20). Following electrophoresis, the gels were processed for autoradiography as described above.

Alkaline phosphatase treatment of p12. Following immunoprecipitation of p12 from HSV-infected cell extracts as described above, the immune complexes were washed four times with cold radioimmunoprecipitation assay buffer, two times in the final wash buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl) and two times with alkaline phosphatase buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 1 mM $ZnCl_2$). The immune complexes were subsequently resuspended in alkaline phosphatase buffer containing 100 U of calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and incubated at 37°C for 3 h on a rocking platform. Following incubation, the immune complexes were washed three times with radioimmunoprecipitation assay buffer and three times with the final wash buffer before the proteins were eluted in acid-urea sample buffer.

Purification of extracellular virions. Monolayers of HEp-2 cells cultured in roller bottles (850 cm^2) were infected with HSV-1 at a multiplicity of infection of 3 PFU per cell. After a 1-h adsorption at 37°C, maintenance medium supplemented with 2% newborn calf serum was added. At 48 h postinfection, virions were purified from the media by previously described methods (9). Briefly, the extracellular medium was harvested at 48 h postinfection, and the cell debris was removed by low-speed centrifugation. Virions were subsequently pelleted from the supernatant by centrifugation for 1 h at 100,000 × *g*. The virion pellet was gently resuspended in TNE buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA), layered onto a 20 to 60% (wt/vol) continuous sucrose gradient, and centrifuged for 20 h at 50,000 × *g*. After centrifugation, the refractive band containing the virions was harvested by puncturing the side of the tube with a needle and syringe. The recovered virus suspension was diluted, pelleted, and resuspended in water and stored at −80°C.

Immunoblotting. Immunoblotting was performed essentially as reported by Towbin et al. (40), with some modifications (9). The immunoblots were incubated with the appropriate antiserum, and the reactive proteins were visualized by reaction with ^{125}I-labeled protein A (Dupont, NEN Research Products), and then autoradiography was performed at −80°C.

Cell fractionation. Monolayers of HEp-2 cells were mock infected or infected with HSV-1 at a multiplicity of infection of 10 PFU per cell. At 18 h postinfection, the cells were harvested by being scraped into cold PBS and pelleted by centrifugation for 5 min at 800 × *g*. The cells were then separated into cytoplasmic and nuclear fractions by using 0.5% Nonidet P-40 in 150 mM NaCl–40 mM phosphate buffer (pH 7.4) essentially as described by Gibson (12). Equivalent amounts of each fraction were resolved by SDS-PAGE and transferred to nitrocellulose, and immunoblotting with the appropriate antiserum was performed.

Indirect immunofluorescence. CV-1 cells were grown on glass coverslips and mock infected or infected with HSV-1 at a multiplicity of infection of 1 PFU per cell. After a 1-h adsorption at 37°C, maintenance medium supplemented with 2% calf serum was added and the incubation was continued until 15 h postinfection. All of the subsequent manipulations were performed at room temperature. The coverslips were washed with PBS and harvested by fixation in 3.7% formaldehyde in PBS for 20 min. Following a 10-min wash with PBS containing 50 mM ammonium chloride, the cells were permeabilized with 0.1% Triton X-100 (vol/vol in PBS) for 4 min at room temperature. After detergent treatment, the coverslips were washed in PBS containing 0.5% bovine serum albumin. To diminish the nonspecific background, the coverslips were incubated for 30 min with a 1:4 dilution of normal goat serum and washed with PBS containing 1% goat serum–0.2% Tween 20. The cells were then incubated with a 1:500 dilution of the TrpE-UL35 antiserum or the same dilution of preimmune serum for 30 min. The monoclonal antibody specific for the 65-kDa DNA-binding protein was used at a 1:50 dilution. Following incubation with the primary antibody, the coverslips were washed three times in PBS containing 1% goat serum–0.2% Tween 20 and stained with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-mouse or goat anti-rabbit; Sigma Chemical Co.) for 30 min. The coverslips were again washed three times in PBS containing 1% goat serum–0.2% Tween 20 and mounted onto glass slides with a drop of mounting medium (PBS containing 50% glycerol–0.1% bovine serum albumin). Microscopy was performed with an Olympus microscope containing a tungsten light source.

RESULTS

Phosphorylation of p12. Our initial hypothesis was that the p12 capsid protein may represent the polypeptide previously designated BP2 by Knopf and Kaerner (20). Since these investigators have shown that BP2 is a phosphoprotein, the first goal of these studies was to determine whether p12 is also phosphorylated within HSV-infected cells. Since our previous studies have demonstrated that p12 is a γ_2 (true late) gene product (25), the isotopic-labeling experiments were performed at late times following HSV-1 infection. Thus, HEp-2 cells were mock infected or infected with HSV-1 and radiolabeled with either 100 μCi of [^{35}S]methionine per ml or 500 μCi of $^{32}P_i$ per ml from 12 to 18 h postinfection. Following radiolabeling, the cells were harvested and the lysates were reacted with TrpE-UL35 antiserum. The immunoprecipitated proteins were resolved by SDS-PAGE and visualized by autoradiography (Fig. 1). These results demonstrated that p12 was isotopically labeled with both [^{35}S]methionine and $^{32}P_i$ (Fig. 1, lanes 3 and 5, respectively), suggesting that the 12-kDa capsid protein is modified by phosphorylation within HSV-infected cells.

Phosphoamino acid analysis of p12. Since p12 is phosphorylated within HSV-infected cells, we wanted to determine which amino acid residues within the protein are modified by phosphate. On the basis of the predicted amino acid sequence of p12, the protein contains 1 tyrosine, 5 serine, and 11 threonine residues which could potentially represent targets for phosphorylation. To determine which amino acid residues are phosphorylated, p12 was immunoprecipitated from HSV-infected cell lysates which had been radiolabeled with 500 μCi of $^{32}P_i$ per ml between 12 and 18 h postinfection. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to Immobilon P membrane, and localized by autoradiography. The pieces of membrane containing p12 were excised and subjected to acid hydrolysis at 110°C for 1 h in 5.7 M HCl. The hydrolysis products were separated by high-voltage electrophoresis on cellulose thin-layer plates, the phosphoamino acid standards were localized by ninhydrin staining, and the radiolabeled phosphoam-

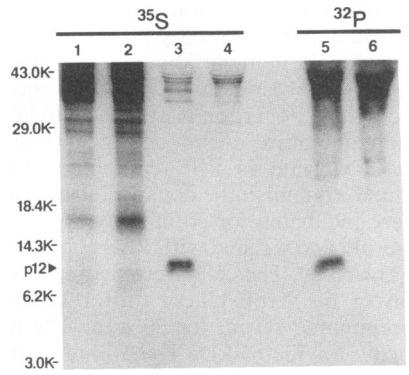

FIG. 1. Phosphorylation of p12. HEp-2 cells were mock infected or infected with HSV-1 and radiolabeled with 100 μCi of [^{35}S]methionine per ml (lanes 1 to 4) or 500 μCi of $^{32}P_i$ per ml (lanes 5 and 6) between 12 and 18 h postinfection. The cell extracts were subjected to immunoprecipitation with the TrpE-UL35 antiserum (lanes 3 to 6), and the proteins were separated by electrophoresis on an SDS-15% polyacrylamide gel. The odd-numbered and even-numbered lanes are HSV- and mock-infected cell extracts, respectively. Lanes 1 and 2 are [^{35}S]methionine-labeled total-cell extracts. The positions of the molecular weight markers and p12 are indicated on the left.

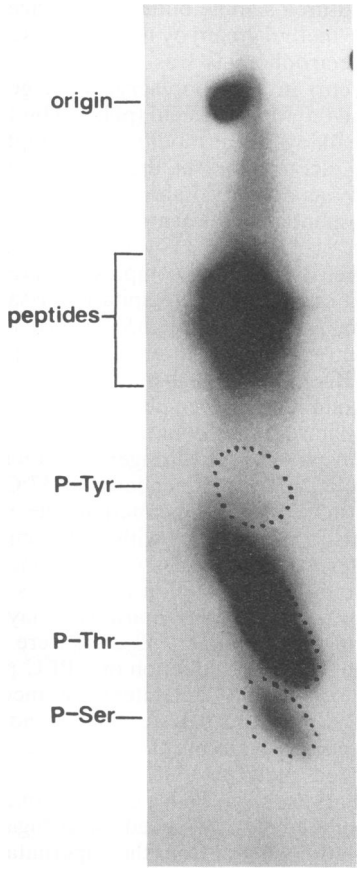

FIG. 2. Phosphoamino acid analysis of p12. The $^{32}P_i$-labeled p12 was isolated, hydrolyzed, and analyzed as described in Materials and Methods. The positions of the phosphotyrosine (P-Tyr), phosphothreonine (P-Thr), and phosphoserine (P-Ser) markers are indicated on the left. The origin and partial hydrolysis products (peptides) are also shown.

ino acids were identified by autoradiography (Fig. 2). These results indicated that p12 is predominantly phosphorylated on threonine residues; however, phosphoserine residues were also detected. There was no evidence to suggest that phosphorylation occurs on tyrosine residues. On the basis of laser densitometric scanning of the autoradiographs, phosphothreonine residues are 80% of the total phosphoamino acids recovered and phosphoserine constitutes the remaining 20%. Since the ratio of threonine to serine residues within the protein is approximately 2:1 and the ratio of phosphothreonine to phosphoserine is 4:1, these data imply that p12 must contain multiple sites for threonine phosphorylation and at least one site for serine phosphorylation.

Analysis of p12 by acid-urea gel electrophoresis. Knopf and Kaerner (20) have previously demonstrated that BP2 could be resolved into multiple components by acid-urea gel electrophoresis. If p12 and BP2 are the same polypeptide, one would predict that p12 could also be resolved into multiple species by acid-urea gel electrophoresis. To directly address this hypothesis, HEp-2 cells were mock infected or infected with HSV-1 and radiolabeled with either 100 μCi of [^{35}S]methionine per ml or 500 μCi of $^{32}P_i$ per ml from 12 to 18 h postinfection. Following harvesting, the cell lysates were subjected to immunoprecipitation with preimmune serum or TrpE-UL35 antiserum and the immunoprecipitated proteins were incubated for 3 h at 37°C in the absence or presence of 100 U of calf intestinal alkaline phosphatase. The proteins were subsequently separated by acid-urea gel electrophoresis and visualized by autoradiography (Fig. 3). The preimmune serum demonstrated no reactivity to proteins present in HSV- or mock-infected cell extracts (Fig. 3, lanes 1 and 2). In contrast, the TrpE-UL35 antiserum reacted with two proteins, designated p12a and p12b, which were present in lysates of HSV-infected cells radiolabeled with either [^{35}S]methionine or $^{32}P_i$ (Fig. 3, lanes 3 and 7, respectively). Similar polypeptides were not identified in immunoprecipitates from mock-infected cell lysates (Fig. 3, lanes 4 and 8), suggesting that these proteins may represent variably phosphorylated products of p12. The phosphorylation of p12 at multiple threonine and serine residues (Fig. 2) would certainly support such a possibility. To directly test this hypothesis, aliquots of the [^{35}S]methionine-labeled HSV- and mock-infected cell lysates were subjected to immunoprecipitation with the TrpE-UL35 antiserum, incubated in the presence of calf intestinal alkaline phosphatase to remove the phosphate moieties, and the samples were analyzed in parallel by acid-urea gel electrophoresis (Fig. 3, lanes 5 and 6). These data demonstrate that removal of the phosphate from p12a and p12b converts these proteins into a single polypeptide, designated p12c. These results suggest that p12a and p12b are variably phosphorylated forms of the 12-kDa capsid protein, and p12c is likely to be the unphosphorylated polypeptide. On the basis of these results, we cannot definitively conclude that the alkaline phosphatase removed all of the phosphate moieties from p12a and p12b to generate p12c; however, these data demonstrate that p12a, p12b, and p12c are related forms of the same 12-kDa capsid protein. Considering the fact that a higher degree of phosphorylation would render p12 less basic, one would presume that p12a has the highest phosphate content. This supposition is supported by the relative migration of the dephosphorylated protein (p12c) in acid-urea gels.

Kinetics of p12 phosphorylation. Since p12a, p12b, and

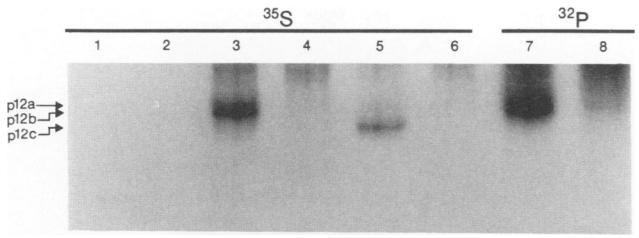

FIG. 3. Analysis of p12 by acid-urea gel electrophoresis. HEp-2 cells were mock infected or infected with HSV-1 and radiolabeled with 100 μCi of [^{35}S]methionine per ml (lanes 1 to 6) or 500 μCi of $^{32}P_i$ per ml (lanes 7 and 8) between 12 and 18 h postinfection. Following harvesting, the cell lysates were reacted with either preimmune serum (lanes 1 and 2) or TrpE-UL35 antiserum (lanes 3 to 8) and the immune complexes were incubated in the presence (lanes 5 and 6) or absence (lanes 1 to 4 and 7 and 8) of calf intestinal alkaline phosphatase as described in Materials and Methods. The proteins were subsequently separated by acid-urea gel electrophoresis and visualized by autoradiography. The odd-numbered lanes are HSV-infected extracts, and even-numbered lanes are mock-infected cell extracts. The positions of p12a, p12b, and p12c are indicated on the left.

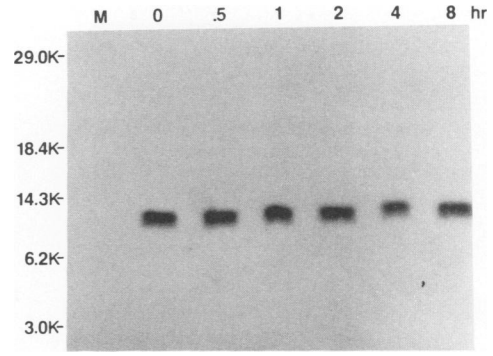

FIG. 4. Pulse-chase analysis of p12 by SDS-PAGE. HEp-2 cells were mock infected or infected with HSV-1 and at 12 h postinfection the cells were pulsed with 100 μCi of [^{35}S]methionine per ml for 30 min, followed by a chase in the presence of excess unlabeled methionine. At various times after pulse-labeling, the cells were harvested and the lysates were reacted with the TrpE-UL35 antiserum. The immunoprecipitated proteins were separated by SDS–15% polyacrylamide gels and visualized by fluorography. The pulse (0) and the various times of chase (hr) are indicated above the lanes. The mock-infected cells (M) were harvested immediately after the pulse. The positions of the molecular weight markers are indicated on the left.

p12c are related forms of the 12-kDa capsid protein, we wanted to determine the kinetics of processing of p12 into its phosphorylated components. To provide markers for the three species of p12, HSV- and mock-infected HEp-2 cells were pulse-labeled with 100 μCi of [^{35}S]methionine per ml between 8 and 12 h postinfection. The cell lysates were reacted with the TrpE-UL35 antiserum, and the immune complexes were incubated in the presence or absence of calf intestinal alkaline phosphatase and resolved by acid-urea gel electrophoresis in parallel with the pulse-chase studies (see Fig. 5A).

For the pulse-chase studies, monolayers of HEp-2 cells were mock infected or infected with HSV-1, and at 12 h postinfection the cells were radiolabeled with 100 μCi of [^{35}S]methionine per ml for 30 min and harvested immediately or chased in the presence of 50-fold-excess-unlabeled methionine for various periods of time. Following harvesting, the cell lysates were reacted with the TrpE-UL35 antiserum and the immunoprecipitates were resolved by either SDS-PAGE or acid-urea gel electrophoresis. When the proteins were separated by SDS-PAGE, a single 12-kDa polypeptide was observed, and the protein appeared to be stable for at least 8 h after synthesis (Fig. 4). To evaluate the processing of p12 into its multiple components, acid-urea gel electrophoresis was performed with parallel samples (Fig. 5B). Following a 30-min pulse, p12b and p12c were found to be present in equal amounts, with little p12a detectable. After 30 min of chase, p12a increased slightly, and this increase became more evident following 1 h of chase. Consistent with a precursor-product relationship, p12c was found to simultaneously decrease in abundance. Interestingly, the amount of p12b appeared to remain relatively constant throughout the chase period, whereas p12c was completely processed within 4 h to the more slowly migrating products. Concomitantly, the processing of p12a and p12b appeared to reach an equilibrium after 4 h, with the relative abundance of the two proteins remaining constant through the 8-h chase period. Surprisingly, we have never detected p12c following pulse-labeling for extended periods of time (Fig. 5A). It is conceivable that the steady-state levels of p12c may be relatively low within HSV-infected cells. Nevertheless, the phosphorylation of p12 within HSV-infected cells results in three forms of the polypeptide as evaluated by acid-urea gel electrophoresis. Moreover, the presence of these three components of p12 is analogous to the results previously reported for BP2 (20) and provides strong evidence that BP2 and p12 are the same polypeptide.

Virion-associated forms of p12. Since three variants of p12 were identified within HSV-infected cells, we wanted to determine whether any specific form of the polypeptide was preferentially associated with purified HSV-1 virions. Thus, HSV-1 virions were purified from the medium of HSV-1-infected HEp-2 cells as described in Materials and Methods. The HSV-1 virions were resuspended in cold radioimmunoprecipitation assay buffer, sonicated, and boiled for 4 min to

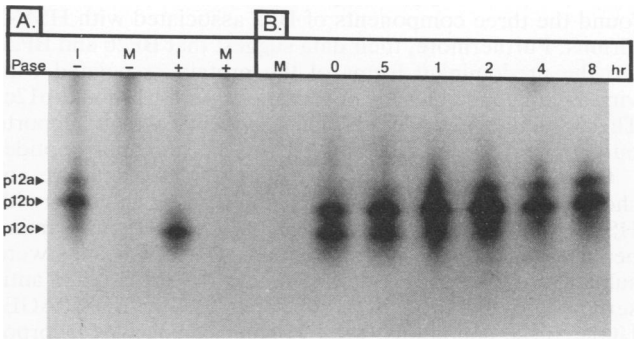

FIG. 5. Pulse-chase analysis of p12 by acid-urea gel electrophoresis. (A) HSV-1-infected (I) and mock-infected (M) HEp-2 cells were pulse-labeled with 100 μCi of [^{35}S]methionine per ml from 8 to 12 h postinfection and the cell lysates were reacted with TrpE-UL35 antiserum. The immune complexes were incubated in the absence (−) or presence (+) of calf intestinal alkaline phosphatase (Pase) and resolved by acid-urea gel electrophoresis. (B) Samples identical to those described in the legend to Fig. 4 were separated by acid-urea gel electrophoresis and visualized by fluorography. The pulse (0) and various times of chase (hr) are indicated above the lanes. The mock-infected cells (M) were harvested immediately after the pulse. The positions of p12a, p12b, and p12c are indicated at the left.

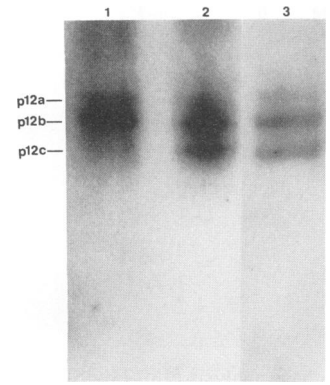

FIG. 6. Virion-associated forms of p12. HSV-infected HEp-2 cells harvested at 18 h postinfection (lane 1) or purified HSV-1 virions (lanes 2 and 3) were solubilized and immunoprecipitations were performed with anti-TrpE-UL35 serum. The immunoprecipitated proteins were separated by acid-urea gel electrophoresis, transferred to nitrocellulose, and immunoblotted with the TrpE-UL35 antiserum. Lane 3 represents the resolved components of the virion-associated p12 from a separate experiment which demonstrated better resolution of p12a. The positions of p12a, p12b, and p12c are indicated on the left.

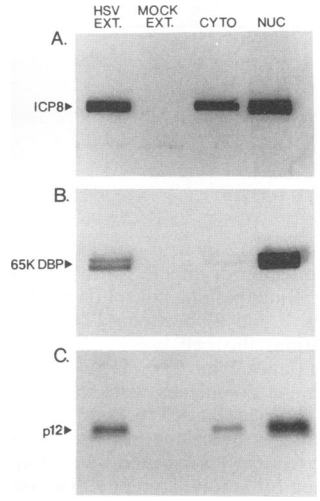

FIG. 7. Analysis of the intracellular distribution of p12 by cell fractionation. HEp-2 cells were mock infected or infected with HSV-1 and harvested at 18 h postinfection. The cells were separated into cytoplasmic (cyto) and nuclear (nuc) fractions by using 0.5% Nonidet P-40 in phosphate-buffered saline as described by Gibson (12). The proteins present in equivalent volumes of each fraction were separated by electrophoresis through SDS-8% polyacrylamide gels (A and B) or an SDS–15% polyacrylamide gel (C) and transferred to nitrocellulose. Following transfer, immunoblotting was performed with antiserum specific for ICP8 (A), 65-kDa DNA-binding protein (65K DBP) (B) or p12 (C). The reactive proteins were visualized by incubation with ^{125}I-labeled protein A and autoradiography. HSV-infected (HSV ext.) and mock-infected (mock ext.) total-cell extracts were analyzed in parallel.

solubilize the virion polypeptides, and the samples were subsequently reacted with the TrpE-UL35 antiserum. The immunoprecipitated proteins were separated by acid-urea gel electrophoresis, transferred to nitrocellulose, and immunoblotted with the TrpE-UL35 antiserum (Fig. 6). As a reference for identifying the various components of p12, the 12-kDa polypeptide was immunoprecipitated from HSV-infected cell extracts harvested at 18 h postinfection and analyzed in parallel (Fig. 6, lane 1). These data indicate that all three forms of p12 are associated with purified HSV-1 virions (Fig. 6, lane 2 and 3); however, p12b and p12c were the predominant forms of the polypeptide within the virus. This was somewhat surprising, considering the relatively low abundance of p12c within HSV-infected cells (Fig. 6, lane 1). Interestingly, Knopf and Kaerner (20) have also found the three components of BP2 associated with HSV-1 virions. Furthermore, their data suggest that BP2b and BP2c are the predominant forms of the protein associated with virions, analogous to our observations with p12b and p12c. These findings provide additional evidence which supports our supposition that BP2 and p12 are the same polypeptide.

Glycosylation of p12. In experiments whose data are not shown, HEp-2 cells were mock infected or infected with HSV-1 and radiolabeled with 100 μCi of [^{3}H]glucosamine per ml from 2 to 18 h postinfection. The cell lysates were subjected to immunoprecipitation with the TrpE-UL35 antiserum, and the proteins were separated by SDS-PAGE. Under these conditions, the [^{3}H]glucosamine was incorporated into infected-cell proteins, but p12 was not radiolabeled, suggesting that the protein is not glycosylated.

Intracellular localization of p12. Previous studies by Cohen et al. (8) have demonstrated that NC7 (p12) was detectable only within the nuclei of HSV-1-infected cells by indirect immunofluorescence; however, the intensity of the fluorescence signal was relatively weak. Since p12 is associated with HSV capsids (8, 15, 25, 28), we expected that the 12-kDa polypeptide must be localized within the nucleus; however, we wanted to determine the relative abundance of p12 within the nuclei and cytoplasm of HSV-infected cells using subcellular fractionation. Thus, HEp-2 cells were mock infected or infected with HSV-1 and harvested at 18 h postinfection. The cells were subsequently separated into cytoplasmic and nuclear fractions by the detergent fractionation method previously described by Gibson (12). The proteins present in equivalent volumes of each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the TrpE-UL35 antiserum (Fig. 7C). As controls, we also examined the intracellular distribution of ICP8 (Fig. 7A) and the 65-kDa DNA-binding protein (Fig. 7B), using antisera specific for these proteins. These results indicated that p12 is found predominantly within the nuclear fractions of HSV-infected cells. The segregation of ICP8 and the 65-kDa DNA-binding protein predominantly within the nuclear fractions of HSV-infected cells is consistent with previously published reports (19, 43) and demonstrates the validity of the fractionation procedure. Laser densitometric scanning of the autoradiographs indicates that approximately 80% of the total 12-kDa capsid protein is present within the nuclear fraction. The nuclear fraction contained approximately 65 and 97% of the total ICP8 and 65-kDa DNA-binding protein present within the HSV-infected cells, respectively.

To confirm the results of the subcellular fractionation studies and to further examine the intracellular localization of p12, indirect immunofluorescence studies were performed with the TrpE-UL35 antiserum as described in Materials and Methods. For comparison and as a control, the 65-kDa DNA-binding protein was examined in parallel. In experiments whose data are not shown, no specific staining was observed in mock-infected cells reacted with either the preimmune serum or the TrpE-UL35 antiserum. The preim-

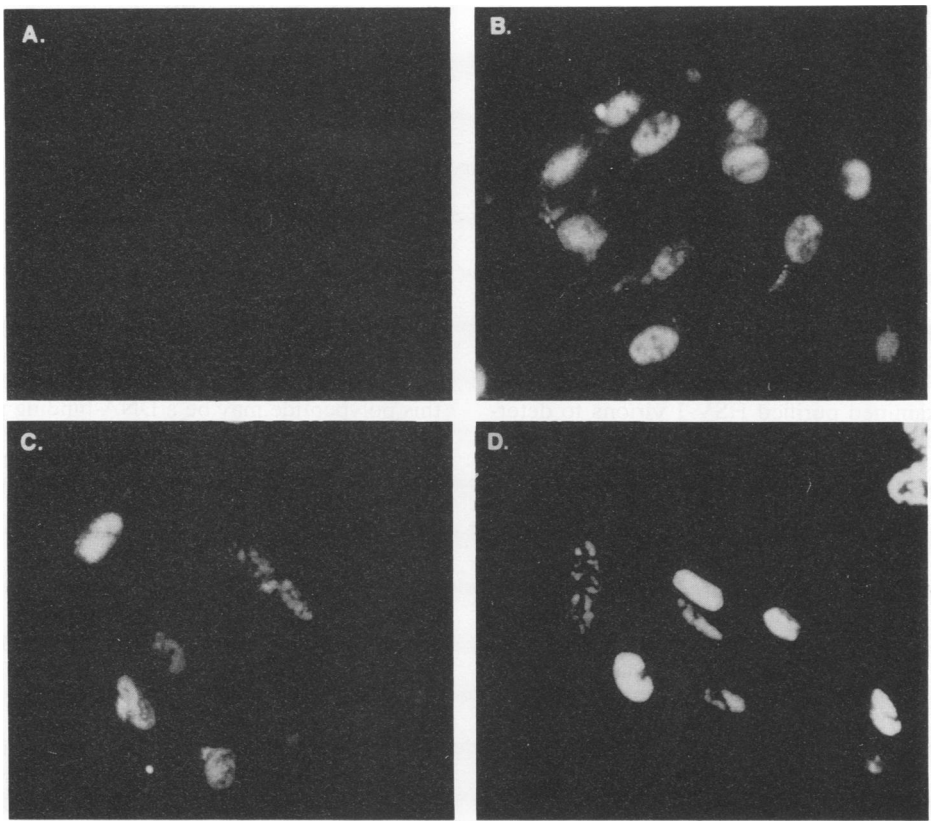

FIG. 8. Intracellular localization of p12 by indirect immunofluorescence. HSV-1-infected CV-1 cells were harvested at 15 h postinfection and incubated in the presence of rabbit preimmune serum (A), TrpE-UL35 antiserum (B and C), or a monoclonal antibody specific for 65-kDa DNA-binding protein (D). The cells were subsequently stained with fluorescein isothiocyanate-conjugated goat anti-rabbit (A to C) or goat anti-mouse (D) secondary antibody.

mune serum showed no reactivity to HSV-1-infected cells harvested at 15 h postinfection (Fig. 8A). In contrast, HSV-infected cells reacted with the TrpE-UL35 antiserum showed bright fluorescence within the nucleus (Fig. 8B and C). Interestingly, the p12 staining pattern within the nucleus was not diffuse but seemed to be localized within specific regions of the nucleus. The 65-kDa DNA-binding protein was also found to be sequestered within specific regions of the nucleus (Fig. 8D), and the staining pattern was similar to that observed for p12 (compare Fig. 8C and D). Previous studies have demonstrated that the 65-kDa DNA-binding protein is localized within distinct regions of the nucleus (14) and appears to colocalize with other DNA-associated proteins (14, 18). At present, we have not used double-fluorochrome-labeling immunofluorescence to directly demonstrate that p12 and the 65-kDa DNA-binding protein colocalize, but we believe such studies are warranted in the future since p12 may also interact with the viral DNA (20, 25).

DISCUSSION

In the studies described in this article, we have examined the posttranslational modifications and subcellular localization of the 12-kDa capsid protein. Pulse-labeling studies with $^{32}P_i$ demonstrated that p12 is modified by phosphorylation within HSV-infected cells. In addition, phosphoamino acid analysis of p12 indicated that the phosphorylation occurs on both serine and threonine residues. Quantitative examination of these results indicates a 4:1 ratio of threonine-to-serine phosphorylation, suggesting that p12 contains several sites for phosphate addition. Consistent with this hypothesis, three electrophoretically distinct species of p12, designated p12a, p12b, and p12c, were identified by acid-urea gel electrophoresis. Isotopic labeling studies performed with $^{32}P_i$ indicate that p12a and p12b represent phosphorylated forms of the capsid protein. These results were further supported by the experiments in which p12 was incubated in the presence or absence of calf intestinal alkaline phosphatase to remove the phosphate moieties. Analysis of these samples by acid-urea gel electrophoresis revealed that both p12a and p12b were converted to the faster-migrating p12c, implying that p12c represents the unphosphorylated form of the polypeptide.

To determine the kinetics of phosphorylation and to directly demonstrate the relationship between the three species of p12, pulse-chase radiolabeling studies were performed. These studies demonstrated that p12c could be completely converted into p12a and p12b following a 4-h chase period. Furthermore, after a 4-h chase, the relative abundance of p12a and p12b reached an equilibrium, which remained constant throughout the 8-h chase period. These data imply that the phosphorylation of p12 is quite stable and that the cycling between the phosphorylated and nonphosphorylated species of the protein seems improbable, albeit such a phenomenon has been observed with other HSV-

specific polypeptides (42). Recently, studies by Purves et al. (34) have demonstrated that the HSV-1 serine/threonine protein kinase encoded by the US3 gene is responsible for the phosphorylation of the UL34 gene product. The phosphorylation site recognized by the US3 protein kinase has been determined previously (21, 33), and the consensus sequence is $(R)_n$-X-(S/T)-Y-Y, where *n* is 3 or more, X prefers Arg, Ala, Val, Pro, or Ser, and Y has a similar bias except that acidic residues and proline are not acceptable. Computer-assisted analysis of the predicted amino acid sequence of p12 (UL35) indicated that no consensus phosphorylation sites for the US3 protein kinase are present within the 12-kDa protein; however, we cannot rule out the possibility that p12 is phosphorylated by the protein kinase encoded by the UL13 gene of HSV-1 (7, 38) or a cellular protein kinase.

We have also examined purified HSV-1 virions to determine whether specific forms of p12 are preferentially associated with the virions during assembly. These results indicated that the virions contain all three species of the polypeptide; however, the predominant forms were p12b and p12c. These results were surprising, since p12c is difficult to detect within HSV-infected cells by pulse-labeling or immunoblotting. As mentioned in Results, we have inferred that the difficulties in detecting p12c are probably related to the transient nature of this polypeptide; however, such a conclusion does not explain the apparent abundance of this protein within virions. Such a disparity could be explained if p12 were to somehow interact with the viral DNA contained within the capsid. Since the phosphorylation of p12 would render the protein less basic, then the component containing the fewest phosphate moieties (p12c) would represent the most basic form of the polypeptide, with p12a representing the least basic. This conclusion is supported by the relative mobilities of p12a, p12b, and p12c toward the cathode when resolved by acid-urea gel electrophoresis (Fig. 5). Considering this information, it is conceivable that p12c could have a higher affinity for the viral genome and be packaged along with the viral DNA into the capsids during assembly, thereby resulting in a disproportionately high concentration of p12c within the virions. In support of this hypothesis, studies by Wilcox et al. (42) have demonstrated that phosphorylation of certain HSV-specific proteins can modulate their affinity for DNA. It is conceivable that the high concentration of p12c found within HSV-1 virions could result from the dephosphorylation of p12a and p12b following disruption of the virions, but such a dephosphorylation event would require the presence of a phosphatase activity within the HSV-1 virions or a contaminating cellular phosphatase present in the virion preparations. To our knowledge, there are no data which indicate that HSV-1 virions contain a protein having phosphatase activity. Furthermore, the pulse-chase radiolabeling studies (Fig. 5) suggest that the phosphate moieties on the cell-associated p12a and p12b are relatively stable following cell lysis, extract preparation, and immunoprecipitation. Thus, the generation of the virion-associated p12c as a result of nonspecific or specific phosphatase activity following the disruption of HSV-1 virions seems unlikely. In addition, the relative amounts of p12a, p12b, and p12c that we observed in HSV-1 virions are consistent with that previously reported by Knopf and Kaerner for BP2 (20). We are currently determining the affinity of p12 for DNA by DNA-cellulose chromatography. If p12 does demonstrate DNA-binding activity, then quantitating the relative amounts of the p12a, p12b, and p12c which bind to the DNA should allow us to directly determine whether phosphorylation modulates the affinity of p12 for the viral DNA.

One of our goals in this study was to assess the relationship between p12 and BP2 and to determine whether these 12-kDa virion proteins are the same polypeptide. By comparing the properties of p12 described in this report with the properties of BP2 (20), a compelling argument that these proteins are in fact identical can be made. First, p12 and BP2 are basic 12-kDa phosphoproteins which are associated with HSV-1 virions (20, 25) (Fig. 1). Second, both proteins can be resolved into three components when analyzed by acid-urea gel electrophoresis (20) (Fig. 3). Finally, the relative abundance of each species of p12 within HSV-1 virions is similar to that previously reported for BP2 (20) (Fig. 6). On the basis of these findings, we conclude that BP2 and p12 are the same polypeptide. As mentioned in the introduction, Knopf and Kaerner (20) have shown that BP2 is associated with the chromatin isolated from HSV-infected cells, suggesting that this polypeptide may be a DNA-binding protein encoded by HSV-1. Thus, it is conceivable that p12 interacts with the viral DNA in HSV-infected cells and within the virions.

Analysis of the intracellular localization of p12 by subcellular fractionation and indirect immunofluorescence has demonstrated that the protein is predominantly localized within the nucleus. In addition, the immunofluorescence studies suggest that p12 is restricted to specific regions within the infected cell nucleus. This pattern of fluorescence was similar to that identified for the 65-kDa DNA-binding protein (Fig. 8D) (14); however, double-fluorochrome-labeling immunofluorescence studies will need to be performed to directly evaluate the colocalization of these proteins. Several studies have indicated that HSV-encoded proteins which associate with the viral DNA, such as ICP4, ICP8, and the 65-kDa DNA-binding protein, colocalize in discrete regions of the infected cell nucleus referred to as "replication compartments" (14, 18, 35). The localization of p12 and the 65-kDa DNA-binding protein within the same regions of the nucleus would prove interesting since p12 may also interact with the viral DNA within HSV-infected cells (20, 25).

We are currently focusing our studies on addressing the association of p12 with the viral DNA within HSV-infected cells and virions using DNA-cellulose chromatography and protein-DNA cross-linking. Such studies should provide interesting information concerning the interactions of p12 with the viral genome. Since no mutations have been mapped to the UL35 gene, we do not know whether p12 represents an essential gene product. The construction of an HSV-1 mutant containing a null allele of the UL35 gene will allow us to address this point and provide a useful tool for determining the functional role of this small basic protein in virus assembly and maturation.

ACKNOWLEDGMENTS

We thank William Newcombe and Jay Brown for interesting and helpful discussions concerning p12.

This work was supported in part by Public Health Service grant AI29026 from the National Institutes of Health.

REFERENCES

1. **Baker, T. S., W. W. Newcombe, F. P. Booy, J. C. Brown, and A. C. Steven.** 1990. Three-dimensional structures of maturable and abortive capsids of equine herpesvirus 1 from cryoelectron microscopy. J. Virol. **64**:563–573.
2. **Bone, D. R., and R. J. Courtney.** 1974. A temperature-sensitive mutant of herpes simplex virus type 1 defective in the synthesis of the major capsid protein. J. Gen. Virol. **24**:17–27.
3. **Booy, F. P., W. W. Newcombe, B. L. Trus, J. C. Brown, T. S. Baker, and A. C. Steven.** 1991. Liquid-crystalline, phage-like

packing of encapsidated DNA in herpes simplex virus. Cell **64**:1007–1015.
4. **Braun, D. K., W. Batterson, and B. Roizman.** 1984. Identification and genetic mapping of a herpes simplex virus capsid protein that binds DNA. J. Virol. **50**:645–648.
5. **Braun, D. K., B. Roizman, and L. Pereira.** 1984. Characterization of posttranslational products of herpes simplex virus gene 35 proteins binding to the surfaces of full capsids but not empty capsids. J. Virol. **49**:142–153.
6. **Cassai, E. N., M. Sarmiento, and P. G. Spear.** 1975. Comparison of the virion proteins specified by herpes simplex virus types 1 and 2. J. Virol. **16**:1327–1331.
7. **Chee, M. S., G. L. Lawrence, and B. G. Barrell.** 1989. Alpha-, beta-, and gammaherpesviruses encode a putative phosphotransferase. J. Gen. Virol. **70**:1151–1160.
8. **Cohen, G. H., M. Ponce de Leon, H. Diggelmann, W. C. Lawrence, S. 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.
9. **Compton, T., and R. J. Courtney.** 1984. Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus type 1-infected cells. J. Virol. **49**:594–597.
10. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–402.
11. **Flannery, V. L., R. J. Courtney, and P. A. Schaffer.** 1977. Expression of an early, nonstructural antigen of herpes simplex virus in cells transformed in vitro by herpes simplex virus. J. Virol. **21**:284–291.
12. **Gibson, W.** 1981. Structural and nonstructural proteins of strain Colburn cytomegalovirus. Virology **111**:516–537.
13. **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.
14. **Goodrich, L. D., P. A. Schaffer, D. I. Dorsky, C. S. Crumpacker, and D. S. Parris.** 1990. Localization of the herpes simplex virus type 1 65-kilodalton DNA-binding protein and DNA polymerase in the presence and absence of viral DNA synthesis. J. Virol. **64**:5738–5749.
15. **Heilman, C. J., Jr., M. Zweig, 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.
16. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XIII. The virion polypeptides of type 1 strains. J. Virol. **14**:640–651.
17. **Kamps, M. P., and B. M. Sefton.** 1989. Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. Anal. Biochem. **176**:22–27.
18. **Knipe, D. M., D. Senechek, S. A. Rice, and J. L. Smith.** 1987. Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. J. Virol. **61**:276–284.
19. **Knipe, D. M., and A. E. Spang.** 1982. Definition of a series of stages in the association of two herpesviral proteins with the cell nucleus. J. Virol. **43**:314–324.
20. **Knopf, K. W., and H. C. Kaerner.** 1980. Virus-specific basic phosphoproteins associated with herpes simplex virus type 1 (HSV-1) particles and the chromatin of HSV-1-infected cells. J. Gen. Virol. **46**:405–414.
21. **Leader, D. P., A. Donella-Deana, F. Marchiori, F. C. Purves, and L. A. Pinna.** 1991. Further definition of the substrate specificity of the alphaherpesvirus protein kinase and comparison with protein kinases A and C. Biochim. Biophys. Acta **1091**:426–431.
22. **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.
23. **Martin, S., R. J. Courtney, G. Fowler, and B. T. Rouse.** 1988. Herpes simplex virus type 1-specific cytotoxic T lymphocytes recognize virus nonstructural proteins. J. Virol. **62**:2265–2273.
24. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
25. **McNabb, D. S., and R. J. Courtney.** 1992. Identification and characterization of the herpes simplex virus type 1 virion protein encoded by the UL35 open reading frame. J. Virol. **66**:2653–2663.
26. **McNabb, D. S., and R. J. Courtney.** Unpublished data.
27. **Newcombe, W. W., and J. C. Brown.** 1989. Use of Ar^+ plasma etching to localize structural proteins in the capsid of herpes simplex virus type 1. J. Virol. **63**:4697–4702.
28. **Newcombe, 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.
29. **Panyim, S., and R. Chalkley.** 1969. High resolution acrylamide gel electrophoresis of histones. Arch. Biochem. Biophys. **130**: 337–346.
30. **Powell, K. L., and R. J. Courtney.** 1975. Polypeptides synthesized in herpes simplex virus type 2-infected HEp-2 cells. Virology **66**:217–228.
31. **Powell, K. L., and D. H. Watson.** 1975. Some structural antigens of herpes simplex virus type 1. J. Gen. Virol. **29**:167–177.
32. **Preston, V. G., J. A. V. 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.
33. **Purves, F. C., A. Donella-Deana, F. Marchiori, D. P. Leader, and L. A. Pinna.** 1986. The substrate specificity of the protein kinase induced in cells infected with herpesviruses: studies with synthetic substrates indicate structural requirements distinct from other protein kinases. Biochim. Biophys. Acta **889**:208–215.
34. **Purves, F. C., D. Spector, and B. Roizman.** 1991. The herpes simplex virus 1 protein kinase encoded by the US3 gene mediates posttranslational modification of the phosphoprotein encoded by the UL34 gene. J. Virol. **65**:5757–5764.
35. **Quinlan, M. P., L. B. Chen, and D. M. Knipe.** 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell **36**:857–868.
36. **Schrag, J. D., B. V. Venkataram Prasad, F. J. Rixon, and W. Chiu.** 1989. Three-dimensional structure of the HSV-1 nucleocapsid. Cell **56**:651–660.
37. **Sherman, G., and S. L. Bachenheimer.** 1988. Characterization of intranuclear capsids made by ts morphogenic mutants of HSV-1. Virology **163**:471–480.
38. **Smith, R. F., and T. F. Smith.** 1989. Identification of new protein kinase-related genes in three herpesviruses, herpes simplex virus, varicella-zoster virus, and Epstein-Barr virus. J. Virol. **63**:450–455.
39. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9**:143–159.
40. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
41. **Vernon, S., M. Ponce de Leon, G. Cohen, R. Eisenberg, and B. Rubin.** 1981. Morphological components of herpesvirus. III. Localization of herpes simplex virus type 1 nucleocapsid polypeptides by immune electron microscopy. J. Gen. Virol. **54**:39–46.
42. **Wilcox, K. W., A. Kohn, E. Sklyanskaya, and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33**:167–182.
43. **Yao, F., and R. J. Courtney.** 1989. A major transcriptional regulatory protein (ICP4) of herpes simplex virus type 1 is associated with purified virions. J. Virol. **63**:3338–3344.
44. **Zweig, M., C. J. Heilman, Jr., and B. Hampar.** 1979. Identification of disulfide-linked protein complexes in the nucleocapsids of herpes simplex virus type 2. Virology **94**:442–450.