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Identification and Characterization of the Herpes Simplex Virus Type 1 Virion Protein Encoded by the UL35 Open Reading Frame

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The UL35 open reading frame (ORF) of herpes simplex virus type 1 (HSV-1) has been predicted from DNA sequence analysis to encode a small polypeptide with a molecular weight of 12,095. We have investigated the protein product of the UL35 ORF by using a *trpE*-UL35 gene fusion to produce a corresponding fusion protein in *Escherichia coli*. The TrpE-UL35 chimeric protein was subsequently isolated and used as a source of immunogen for the production of rabbit polyclonal antiserum directed against the UL35 gene product. The TrpE-UL35 antiserum was found to recognize a 12-kDa protein which was specifically present in HSV-1-infected cells. By utilizing the TrpE-UL35 antiserum, the kinetics of synthesis of the UL35 gene product was examined, and these studies indicate that UL35 is expressed as a γ_2 (true late) gene. The 12-kDa protein recognized by the TrpE-UL35 antiserum was associated with purified HSV-1 virions and type A and B capsids, suggesting that the UL35 ORF may encode the 12-kDa capsid protein variably designated p12, NC7, or VP26. To confirm this assignment, immunoprecipitation and immunoblotting studies were performed to demonstrate that the TrpE-UL35 antiserum reacts with the same polypeptide as an antiserum directed against the purified p12 capsid protein (anti-NC7) (G. H. Cohen, M. Ponce de Leon, H. Diggelmann, W. C. Lawrence, S. K. Vernon, and R. J. Eisenberg, J. Virol. 34:521–531, 1980). Furthermore, the anti-NC7 serum was also found to react with the TrpE-UL35 chimeric protein isolated from *E. coli*, providing additional evidence that the UL35 gene encodes p12. On the basis of these studies, we conclude that UL35 represents a true late gene which encodes the 12-kDa capsid protein of HSV-1.

On the basis of morphological studies, the herpes simplex virus type 1 (HSV-1) virions have four distinct structural regions: an electron-opaque core which contains the viral genome; an icosahedral capsid which encloses the DNA-containing core; an electron-dense material, referred to as the tegument, which surrounds the nucleocapsid; and an outer envelope containing the viral glycoproteins (37, 38, 45, 48). Among the 30 or more structural proteins which comprise the HSV-1 virions (9, 21, 34, 44), only seven polypeptides are structural components of the nucleocapsids. Gibson and Roizman (16) identified six proteins (designated VP5, VP19c, VP21, VP22a, VP23, and VP24) as components of capsids isolated from the nuclei of HSV-infected cells. Subsequently, Heilman et al. (20) and Cohen et al. (11) identified a seventh capsid protein (designated p12 and NC7, respectively) with a molecular weight of 12,000. The genes encoding VP5 (13), VP19c (36, 49), VP22a (35), and VP23 (36) have been identified previously and are designated UL19, UL38, UL26, and UL18, respectively. It has been suggested that VP21 may represent a processed form of VP22a (8) and thus may also be encoded by the UL26 gene. If this is proven to be true, VP24 and p12 are the only remaining capsid proteins which have not been assigned to a specific gene. The identification of the genes encoding each of the capsid proteins is an essential step toward our understanding of herpesvirus assembly. Furthermore, such information is necessary for designing experimental studies of the function(s) of these structural polypeptides using molecular techniques.

HSV-1 has a linear double-stranded DNA genome of approximately 152 kb (28). The knowledge of the complete DNA sequence of the HSV-1 genome (28) and the mapping of the genomic locations of various HSV-specific transcripts (see reference 47 for a summary) have greatly assisted investigators in identifying various HSV-specific gene products. Computer-assisted analyses of the DNA sequence of the HSV genome have suggested the presence of at least 72 separate open reading frames (ORFs) (28). Approximately one-third of the ORFs remain to be characterized with respect to the polypeptides they encode and to determine whether they are structural or nonstructural proteins. In the studies reported herein, we were interested in identifying the gene encoding the p12 capsid protein.

As noted above, previous studies have demonstrated that p12 represents a 12-kDa protein which is associated with the capsids isolated from the nuclei of HSV-infected cells (11, 20). Recent studies by Newcombe and Brown (32) have demonstrated that p12 (termed VP26) and VP22a can be quantitatively removed from capsids by extraction with 2.0 M guanidine hydrochloride. Electron microscopic examination of the extracted capsids revealed the selective removal of the capsid pentons and the internal core proteins. Since VP22a may reside within the capsid (31, 32), these results imply that p12 may be localized in or around the pentons of the HSV capsids. Whether p12 actually forms the pentons or is localized in close proximity to the pentons remains unresolved. Interestingly, previous studies by Knopf and Kaerner (25) suggest that a 12-kDa basic phosphoprotein (termed BP2), which is present in HSV-1 virions and asso-

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ciated with chromatin isolated from HSV-1 infected cells is equivalent to p12 or NC7.

Since the gene encoding p12 had not been identified, we took advantage of these observations concerning the characteristics of BP2 and/or p12 to try to determine whether any of the uncharacterized ORFs within the HSV genome could encode a 12-kDa basic protein. Computer-assisted analyses of the potential ORFs within the HSV-1 genome suggest that the UL35 ORF might encode such a protein. The UL35 ORF is predicted to encode a protein with a molecular weight of 12,095 (28), which is consistent with the apparent molecular weight of p12 (11, 20). Additionally, computer analysis indicates that the predicted amino acid sequence of the UL35 gene product would produce a polypeptide with a calculated pI of 11.6 (29), which is consistent with the basic properties of BP2. The UL35 ORF is located at approximately 0.463 to 0.465 map units on the HSV-1 genome. Analyses of HSV-1 transcripts located within this region of the viral genome suggest that the UL33, UL34, and UL35 ORFs are transcribed late in infection as a nested set of 3′-coterminal mRNAs (47); however, precise mRNA mapping studies need to be performed to confirm this observation. Nevertheless, these results indicate that the UL35 ORF represents a gene which is actively transcribed. Moreover, its appearance late in infection would be consistent with encoding a structural protein of the virion. On the basis of these observations, we reasoned that UL35 represented an attractive candidate for the gene encoding the p12 capsid protein of HSV-1.

In this article, we report the expression of the UL35 ORF in *Escherichia coli* as a TrpE-UL35 chimeric protein and the use of this fusion protein as an antigen for the production of a polyclonal antiserum which specifically recognizes the protein product of the UL35 ORF in HSV-infected cells. The UL35 gene product had an apparent molecular weight of 12,000, which is consistent with the calculated molecular weight of 12,095. The TrpE-UL35 antiserum was also used to examine the kinetics of synthesis of the UL35 gene, and these studies revealed that the UL35 gene product is synthesized as a true late (γ_2) gene product. Immunoblotting experiments also revealed that the 12-kDa protein recognized by the TrpE-UL35 antiserum was present in purified HSV-1 virions as well as type A and B capsids. These results were consistent with the hypothesis that the UL35 gene may encode the 12-kDa capsid protein. This hypothesis was subsequently confirmed by demonstrating the cross-reactivity of anti-NC7 antiserum (prepared against the purified 12-kDa capsid protein) and the TrpE-UL35 antiserum.

MATERIALS AND METHODS

Cell culture and virus. HEp-2 cells and human embryonic lung fibroblasts (MRC-5) cells were grown in Eagle's minimal essential medium containing 0.075% sodium bicarbonate and supplemented with 10% newborn calf serum and 10% fetal calf serum, respectively. 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 as previously described (6).

Enzymes and chemicals. Restriction endonucleases and DNA-modifying enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England Biolabs (Beverly, Mass.), or Promega Corp. (Madison, Wis.) and were used according to the manufacturers' specifications. Radiochemicals were purchased from Dupont, NEN Research Products (Boston, Mass.). Phosphonoacetic acid (PAA), Coomassie brilliant blue R-250, and 3-β-indoleacrylic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Antiserum reagents. Antisera used in these studies include hyperimmune monospecific rabbit antisera to glycoproteins B and C (14), ICP8 (15), and VP5 (29). Antiserum to the p12 capsid protein (anti-NC7) was kindly provided by Gary H. Cohen and Roselyn J. Eisenberg (University of Pennsylvania, Philadelphia) and has been previously described (11).

Construction of a TrpE-UL35 expression plasmid. The pATH21 plasmid, encoding a truncated TrpE protein, was obtained from the American Type Culture Collection (Rockville, Md.) and has been previously described (26). This plasmid produces a 37-kDa fragment of *E. coli* anthranilate synthase encoded by the *trpE* gene. The plasmid pKB135 contains the 9.3-kb *Bam*HI D genomic fragment of HSV-1 (KOS) (Fig. 1A) cloned into the *Bam*HI site of pBR322 (5), and was previously constructed within our laboratory (29). The recombinant expression plasmid pEC135 was constructed by ligation of the UL35-containing 2.2-kb *Sph*I-*Sac*I fragment of pKB135 into the *Sph*I-*Sac*I sites of pATH21 at the 3′ end of the *trpE* gene (Fig. 1B). The plasmid was introduced into *E. coli* TB1 by the $CaCl_2$ transformation procedure (39).

Fusion protein isolation. The TrpE-UL35 fusion protein synthesized by *E. coli* TB1 formed insoluble aggregates which were isolated from cell lysates as previously described (26). Briefly, *E. coli* TB1-containing pEC135 was streaked onto Luria-Bertani agar plates containing 50 μg of ampicillin per ml and 20 μg of tryptophan per ml and grown overnight at 37°C. A loop of cells from the overnight culture was used to inoculate modified M9 medium containing tryptophan (26) and grown with vigorous shaking at 37°C to mid-logarithmic phase. To induce the expression of *trpE*-UL35, the entire culture was diluted 10-fold in modified M9 medium without tryptophan and grown for an additional 2 h. The culture was subsequently treated with 10 μg of 3-β-indoleacrylic acid per ml, and incubation was continued for 4 h.

The induced cells were harvested by centrifugation for 5 min at 3,300 × *g*, washed once in 10 mM Tris-HCl (pH 7.5), and pelleted again. The cell pellet was resuspended in 50 mM Tris-HCl (pH 7.5)–5 mM EDTA–1 mM phenylmethylsulfonyl fluoride–3 mg of lysozyme per ml and kept on ice for 2 h. Next, NaCl was added to a final concentration of 0.3 M and mixed by gently inverting the tubes. Subsequently, Nonidet P-40 was added to a final concentration of 0.7% and the lysate was gently mixed. To shear the DNA, the samples were sonicated with 30-s bursts until the viscosity was reduced. To separate the soluble and insoluble fractions, the samples were centrifuged for 10 min at 9,000 × *g*, and the insoluble pellet was subsequently washed once in 10 mM Tris-HCl (pH 7.5)–1 M NaCl and once in 10 mM Tris-HCl (pH 7.5). The insoluble pellet was resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 3 min, and then centrifuged for 15 min at 16,000 × *g* to remove any insoluble material. The fusion protein was then separated by preparative SDS-PAGE, and the gel was stained with 0.05% (wt/vol) Coomassie brilliant blue R-250 dissolved in dionized water (19). The gel was briefly destained with deionized water, the fusion protein was excised, and the gel fragments were stored at −80°C.

The TrpE and TrpE-UL35 proteins to be used for subsequent immunoblot analyses were eluted from the gel fragments essentially as described by Beemon and Hunter (3). Briefly, the gel fragments containing the proteins were

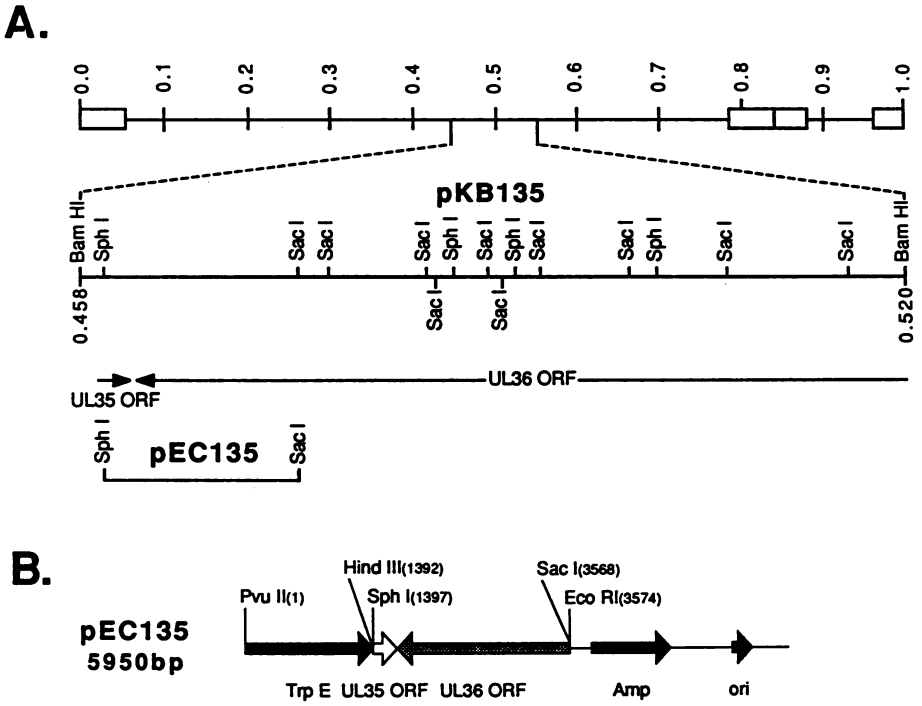

FIG. 1. Location in the HSV-1 genome of the UL35 gene and viral DNA fragments used in this study. (A) Beneath the diagram of the HSV-1 genome (top line), the *Bam*HI D genomic fragment of HSV-1 (KOS) present in plasmid pKB135 (map coordinates 0.458 through 0.520) has been expanded to show the locations of the relevant restriction sites used in this study. The location and direction of the UL35 and the 3′ portion of the UL36 ORFs are shown beneath pKB135. Next is shown the location of the genomic fragment in plasmid pEC135 used to express the UL35 ORF in *E. coli*. (B) Linear diagram of the plasmid pEC135 used to express the UL35 ORF in *E. coli* TB1 as a TrpE-UL35 fusion protein. The translational termination codon used by TrpE-UL35 is that of the UL35 gene.

resuspended in 1 ml of 0.05 M NH_4HCO_3 containing 0.1% SDS and homogenized by multiple passages through a fine wire mesh. The samples were diluted to a final volume of approximately 1 ml/100 cm^2 of gel with the same buffer. The homogenate (containing a final concentration of 5% 2-mercaptoethanol) was boiled for 5 min and placed on a rotary shaker for 48 h at 37°C to allow the proteins to diffuse from the gel fragments. The gel fragments were subsequently pelleted by centrifugation at 12,000 × *g* for 10 min, and the supernatant was removed. The gel fragments were washed in 0.2 volume of the same buffer and pelleted, and the second supernatant was removed and combined with the first. The supernatants were lyophylized to dryness, and the samples were resuspended in SDS-PAGE sample buffer and stored at −20°C.

Generation of TrpE-UL35 antiserum. In preparation for immunization, gel fragments containing the TrpE-UL35 chimeric protein were combined with equal volumes of phosphate-buffered saline (PBS) and Freund's complete adjuvant (GIBCO Laboratories, Grand Island, N.Y.). This material was injected intramuscularly at multiple sites into a female New Zealand White rabbit. Booster injections of antigen in Freund's incomplete adjuvant were administered biweekly. Approximately 75 μg of antigen was used for the primary injection; subsequent injections contained 30 to 50 μg of the chimeric protein. Blood was obtained from the marginal ear vein 10 to 14 days after the third and subsequent booster injections and was allowed to clot at 37°C for 15 min, and the clot contracted overnight at 4°C. The resulting serum was collected by low-speed centrifugation (1,500 × *g* for 10 min at 4°C) followed by high-speed centrifugation (12,000 × *g* for 10 min at 4°C) and stored at −20°C.

Metabolic labeling, immunoprecipitation, and SDS-PAGE. To label viral proteins, HEp-2 cells were mock infected or infected with HSV-1 (KOS) at a multiplicity of 10 PFU per cell. After 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 (50 to 100 μCi/ml; specific activity, 700.7 Ci/mmol; Dupont, NEN Research Products) and harvested by scraping; they were washed in cold PBS and subsequently used in immunoprecipitations. Whole cell pellets were solubilized in cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl [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], 2 mM benzamidine), vortexed vigorously, incubated on ice for 5 min, and disrupted by sonication. Extracts were then boiled for 4 min and clarified by centrifugation for 15 min at 16,000 × *g*. Protein assays were subsequently performed for each sample by using the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.) with bovine serum albumin as the standard. Equal amounts of total cell protein were then incubated for 3 h at 4°C with the appropriate antiserum, and subsequently, protein A-agarose beads (50% [vol/vol]; Repligen, Cambridge, Mass.) were added and the mixture was allowed to incubate for 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 then eluted by boiling in SDS-PAGE sample buffer. Analysis of samples by SDS-PAGE was performed as described previously (33). Samples were electrophoresed in 7, 10, 12, or 15% polyacrylamide gels cross-linked with *N*, *N*-methylene-bisacrylamide (28.2:0.8 ratio of acrylamide to methylene-bisacrylamide). After electrophoresis, the slab gels either were directly visualized by staining with 0.25% (wt/vol) Coomassie blue R-250 or silver (30) or were fixed and dried onto Whatman 3MM filter paper (Whatman, Inc., Clifton, N.J.); the dried gels were then exposed to Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.) at −80°C. The [^{35}S]methionine-labeled proteins were detected by the use of a fluorographic enhancer (Fluoro-Hance; Research Products International Corp., Mount Prospect, Ill.).

Immunoblotting. Immunoblotting was performed essentially as reported by Towbin et al. (46), with some modifications (12). The immunoblots were incubated with the appropriate rabbit antiserum, and the reactive proteins were visualized by reaction with ^{125}I-labeled protein A (Dupont, NEN Research Products) and then autoradiographed at −80°C.

PAA inhibition of viral DNA synthesis. HEp-2 cells were incubated in minimal essential medium containing 2% calf serum and 300 μg of PAA for 2 h prior to HSV-1 (KOS) infection, and this level was maintained throughout infection.

Purification of extracellular virions. Monolayers of HEp-2 cells cultured in roller bottles (850 cm^2) were infected at a multiplicity of 3 PFU per cell. After 1 h of adsorption at 37°C, maintenance medium supplemented with 2% newborn calf serum was added. All incubations were carried out at 37°C. At 48 h postinfection, virions were purified by previously described methods (12). Briefly, extracellular virions were harvested from the media at 48 h postinfection and cell debris was removed by low-speed centrifugation. Virions were 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). The virus suspension was then 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 virus 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.

Isolation of intranuclear virus capsids. HEp-2 cells were infected with HSV-1 (KOS) at a multiplicity of 3 PFU per cell, and type A and B viral capsids were purified from the nuclear fraction essentially as described by Gibson and Roizman (16). Briefly, infected cells were harvested at 22 h postinfection and collected by centrifugation at 1,000 × *g* for 10 min. The pellet was resuspended in 10 mM Tris-HCl (pH 7.2)–150 mM NaCl–2 mM $MgCl_2$–1% Nonidet P-40. After incubation for 30 min at 0°C, the nuclei were pelleted from the suspension by centrifugation at 1,000 × *g* for 10 min and lysed by the addition of 0.5% deoxycholate in the presence of 50 μg of DNase I per ml for 15 min at 37°C. This was followed by a further 5-min incubation at 0°C in the presence of 0.5% Brij 58 and 0.5 M urea. The extract was clarified by centrifugation for 10 min at 7,000 × *g*. The supernatant was layered onto a 10 to 40% (wt/wt) linear sucrose gradient and centrifuged at 70,000 × *g* for 1 h at 4°C. After centrifugation, two refractile bands located near the middle of the tube were collected and stored at −80°C.

RESULTS

Expression of the UL35 gene in *E. coli*. On the basis of the predicted molecular weight and pI of the UL35 gene product, we reasoned that this ORF was a likely candidate to encode the 12-kDa basic phosphoprotein (BP2) previously described by Knopf and Kaerner (25). It also seemed plausible that BP2 was identical to the 12-kDa capsid protein previously described by Cohen et al. (11) and Heilman et al. (20). Thus, the objectives of this study were to identify the protein product of the UL35 ORF, to examine the kinetics of synthesis of the protein, and to determine whether the polypeptide encoded by the UL35 ORF was indeed the p12 capsid protein. Our approach was to express the UL35 ORF as a fusion protein in *E. coli* and to subsequently isolate the chimeric protein to use as an immunogen for the production of rabbit polyclonal antiserum which would specifically recognize a polypeptide present in HSV-infected cells.

The expression of the UL35 ORF in *E. coli* was accomplished by constructing a *trpE*-UL35 gene fusion by using *E. coli* expression plasmid pATH21 (26). The TrpE-UL35 expression plasmid (Fig. 1B), designated pEC135, was constructed by subcloning the 2.2-kb *Sph*I-*Sac*I fragment of pKB135 (Fig. 1A) into pATH21, as described in Materials and Methods. The resulting gene fusion contained 323 codons of the *trpE* gene fused in frame with the 3′ 91 codons of the predicted 112-codon UL35 ORF.

To identify the *trpE*-UL35 gene product in *E. coli*, the following cultures were grown and induced as described in Materials and Methods: (i) *E. coli* TB1 (induced), (ii) *E. coli* TB1 containing pATH21 (induced), (iii) *E. coli* TB1 containing pEC135 (uninduced), and (iv) *E. coli* TB1 containing pEC135 (induced). After induction, total lysates were prepared from each culture and subsequently separated into soluble and insoluble fractions. The proteins present in each fraction were separated by SDS-PAGE and visualized by Coomassie blue staining (Fig. 2). *E. coli* TB1 containing pATH21 produced a 37-kDa protein under inducing conditions (Fig. 2, lanes 2, 6, and 10) which was absent in *E. coli* TB1 cells alone (Fig. 2, lanes 1, 5, and 9). The appearance of the 37-kDa protein under inducing conditions is consistent with the apparent molecular weight of the truncated TrpE protein (26) and suggested that the *trpE* gene promoter was functioning under inducing conditions. Samples from cells containing pEC135 demonstrated the appearance of a 40.5-kDa protein (Fig. 2, lanes 4, 8, and 12) which was greatly diminished in uninduced cultures (Fig. 2, lanes 3, 7, and 11). These results implied that the 40.5-kDa polypeptide represented the TrpE-UL35 chimeric protein. It should be noted that the TrpE-UL35 polypeptide migrated faster than would be predicted from the calculated molecular weight; however, restriction analysis of pEC135 indicated that the gene fusion was in the correct reading frame (29). The insoluble pellet containing the majority of the TrpE-UL35 protein was solubilized in SDS-PAGE sample buffer and resolved by preparative SDS-PAGE, and the band corresponding to the fusion protein was excised from the gels and used as the immunogen for preparation of rabbit polyclonal antiserum as described in Materials and Methods.

Identification of the UL35 gene product in HSV-infected cells. To examine the reactivity of the TrpE-UL35 antiserum, HEp-2 cells were mock infected or infected with HSV-1 and radiolabeled with 100 μCi of [^{35}S]methionine per ml from 8 to 12 h postinfection. Cell lysates were prepared and immunoprecipitated with preimmune serum or TrpE-UL35 antiserum, and the immunoprecipitated proteins were

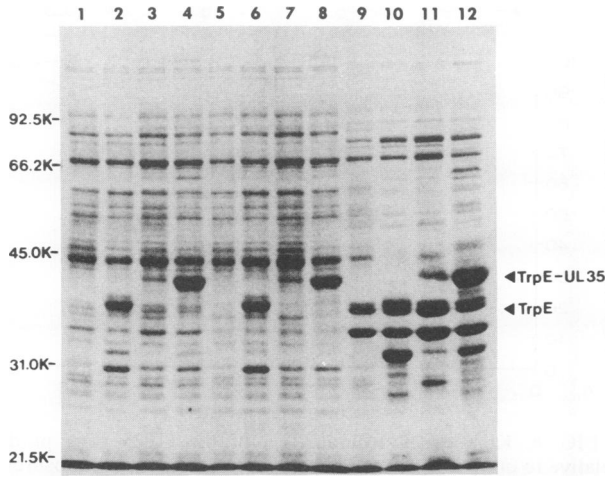

FIG. 2. Expression of the TrpE-UL35 chimeric protein in *E. coli*. Extracts of *E. coli* TB1 grown under inducing (lanes 1, 5, and 9) conditions, cells containing pATH21 grown under inducing (lanes 2, 6, and 10) conditions, and cells containing pEC135 grown under noninducing (lanes 3, 7, and 11) and inducing (lanes 4, 8, and 12) conditions were electrophoresed through an SDS–12% polyacrylamide gel and stained with Coomassie blue. Whole cells extracts are in lanes 1 to 4, and the soluble proteins (lanes 5 to 8) and insoluble proteins (lanes 9 to 12) were isolated as described in Materials and Methods. The migration of the molecular weight markers is shown on the left, and the positions of the TrpE and TrpE-UL35 proteins are shown on the right.

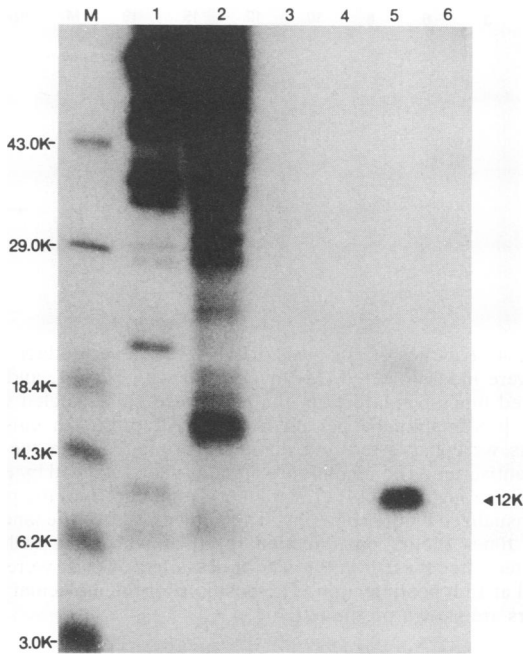

FIG. 3. Identification of the UL35 gene product in HSV-1 infected cells. HEp-2 cells were mock infected or infected with HSV-1 and radiolabeled from 8 to 12 h postinfection with 100 μCi of [^{35}S]methionine per ml. At 12 h postinfection, cell lysates were prepared and immunoprecipitated with preimmune serum or TrpE-UL35 antiserum. The immunoprecipitates were resolved by electrophoresis through an SDS–15% polyacrylamide gel, and the proteins were visualized by fluorography. The odd-numbered lanes are HSV-infected extracts, and the even-numbered lanes are mock-infected extracts. Lanes 1 and 2 are HSV-infected and mock-infected cell extracts, respectively. Lanes 3 and 4 are cell extracts immunoprecipitated with preimmune serum. Lanes 5 and 6 are cell extracts immunoprecipitated with TrpE-UL35 antiserum. The molecular weight markers (M) are shown on the left.

resolved by SDS-PAGE and visualized by fluorography (Fig. 3). The TrpE-UL35 antiserum recognized a 12-kDa protein which was present in HSV-1-infected cell extracts (Fig. 3, lane 5) but not in mock-infected cells (Fig. 3, lane 6) or extracts which were immunoprecipitated with preimmune serum (Fig. 3, lanes 3 and 4). These results suggest that the UL35 ORF encodes a protein with an apparent molecular weight of 12,000, which is consistent with the predicted molecular weight of 12,095. Furthermore, these data confirm that the predicted UL35 ORF represents a viral gene which is actively expressed during HSV infection.

Time course of expression of the UL35 gene product. The expression of the UL35 gene during lytic HSV infection was analyzed by determining the kinetics of appearance of the UL35 gene product. Initially, pulse-labeling experiments were performed to examine the rate of synthesis of the UL35 gene product during the HSV replicative cycle. HEp-2 cells were mock infected or infected with HSV-1, and at various times postinfection the cells were pulse-labeled for 1 h with 50 μCi of [^{35}S]methionine per ml. Cell lysates were prepared and immunoprecipitated with TrpE-UL35 antiserum, resolved by SDS-PAGE, and visualized by fluorography (Fig. 4). The UL35 gene product was initially detectable at 6 h postinfection, and the rate of synthesis continued to increase until 10 h postinfection, thereafter remaining relatively constant through 18 h postinfection. These results indicate that the UL35 gene product is synthesized late in infection, consistent with being a structural protein of the virion. Furthermore, the increased rate of synthesis of the 12-kDa protein during the course of HSV infection provides additional evidence that the TrpE-UL35 antiserum is recognizing a virally encoded protein.

Studies were also performed to examine the accumulation of the UL35 gene product during HSV infection. HEp-2 cells were mock infected or infected with HSV-1, and at various times postinfection the cells were harvested and the cell lysates were subjected to immunoprecipitation with the TrpE-UL35 antiserum. The proteins were resolved by SDS-PAGE, and Western blot (immunoblot) analysis was performed with the TrpE-UL35 antiserum (Fig. 5B). For comparison and internal controls, a portion of the total cell lysates was separated by SDS-PAGE and Western blot analysis was performed with antisera directed against ICP8 (β gene product), glycoprotein B (γ_1 gene product), and glycoprotein C (γ_2 gene product) (Fig. 5A). The kinetics of accumulation of these proteins during the HSV replicative cycle has been previously reported (10, 41, 43). ICP8 was detectable early after infection (6 h postinfection), reaching maximum levels by 11 to 12 h postinfection and maintaining these levels throughout the replicative cycle. Glycoprotein B was also initially detectable at 6 h postinfection, obtaining maximum levels by 12 h postinfection, and those levels remained constant through 18 h postinfection. Similarly, glycoprotein C was weakly detectable at 6 h postinfection; however, it continued to accumulate through 18 h postinfection. The accumulation of the UL35 gene product was initially detected at 8 h postinfection, and the protein continued to accumulate through 18 h postinfection. Quantitative analysis of these data revealed that the UL35 gene product demonstrated an expression pattern very similar to

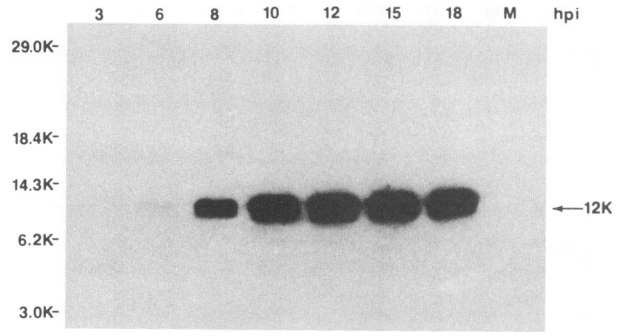

FIG. 4. Kinetics of synthesis of the UL35 gene product. HEp-2 cells were mock infected (M) or infected with HSV-1, and at the indicated times postinfection, the cells were pulse-labeled with 50 μCi of [^{35}S]methionine per ml for 1 h. After the 1-h pulse, cell extracts were harvested and immunoprecipitated with the TrpE-UL35 antiserum. The immunoprecipitates were resolved by electrophoresis through an SDS–15% polyacrylamide gel, and the proteins were visualized by fluorography. The numbers above the lanes refer to the times (hours postinfection [hpi]) at which the cells were harvested after the 1-h pulse. The mock-infected cells were pulse-labeled at 18 h postinfection. The positions of the molecular weight markers are shown on the left.

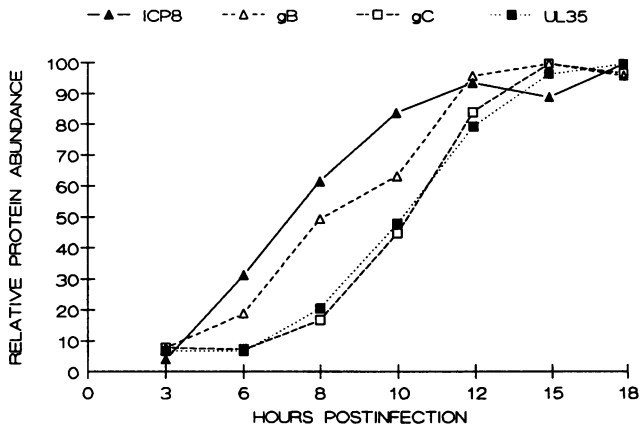

FIG. 6. Kinetics of accumulation of the UL35 gene product relative to other HSV-encoded polypeptides. Multiple exposures of the autoradiographs shown in Fig. 5 were scanned with a laser densitometer. The values shown represent the relative abundance of the detected proteins at various times postinfection. The symbols representing each polypeptide are shown above the graph.

that of glycoprotein C, with detectable levels of synthesis observed at 8 h postinfection and continued accumulation of the 12-kDa protein throughout the 18-h replicative cycle examined (Fig. 6). These studies indicate that the UL35 gene product is expressed as a late gene. It should be noted that the 12-kDa UL35 gene product was observed at 3 and 6 h postinfection; however, this was likely the result of input viral particles since the UL35 gene product was found to be a structural protein of the virion (see Fig. 8). This is supported by two lines of evidence. First, the pulse-labeling studies whose results are shown in Fig. 4 indicate that detectable expression of the UL35 gene product was not observed until 6 h postinfection. Second, the presence of mature glycoprotein C at 3 and 6 h postinfection implies the presence of detectable amounts of input viral proteins.

Expression of the UL35 gene product requires viral DNA replication. The kinetics of synthesis of the UL35 gene product indicated that the protein is expressed late in infection, suggesting that the UL35 gene is regulated as either a quasilate (γ_1) or true late (γ_2) gene. The γ_1 genes are defined as those genes whose products are expressed at reduced levels in the absence of viral DNA replication but are expressed at maximum levels after the onset of DNA replication. In contrast, the expression of γ_2 genes has a stringent requirement for viral DNA synthesis, and the genes are not expressed in the absence of viral DNA replication. To precisely determine the temporal class of the UL35 gene, experiments were performed to examine the expression of

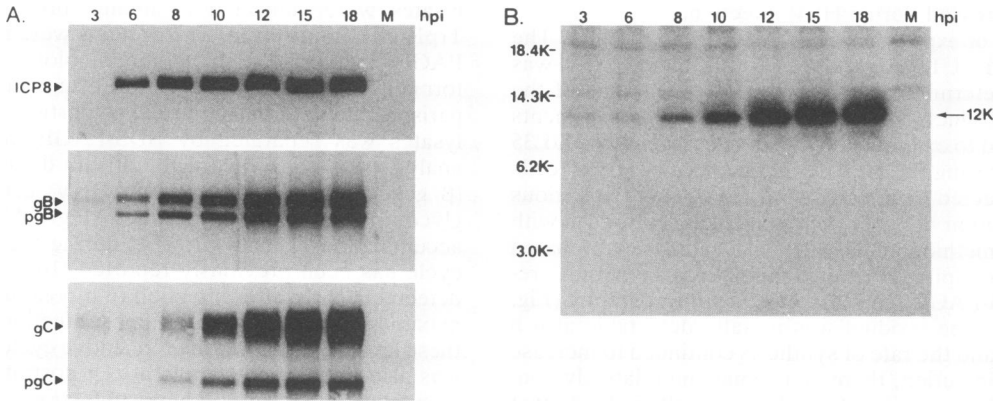

FIG. 5. Time course of the UL35 gene product accumulation in HSV-infected cells. HEp-2 cells were mock infected (M) or infected with HSV-1, and at various times after infection, the cell extracts were harvested and the samples were electrophoresed on SDS–7% polyacrylamide gels (A) or the extracts were immunoprecipitated with TrpE-UL35 antiserum and the immunoprecipitates were resolved on an SDS–15% polyacrylamide gel (B). After electrophoresis, the proteins were transferred to nitrocellulose and immunoblotted with antiserum against ICP8, glycoprotein B (gB), or glycoprotein C (gC) (A) or TrpE-UL35 antiserum (B). The reactive proteins were visualized by incubation with ^{125}I-labeled protein A and subsequent autoradiography. The precursors containing high levels of mannose (pgB and pgC) and the fully processed forms of gB and gC are indicated. The numbers above the lanes refer to the hours postinfection (hpi) that the cells were harvested. The mock-infected cells (M) were harvested at 18 h postinfection. The positions of the molecular weight markers are shown on the left.

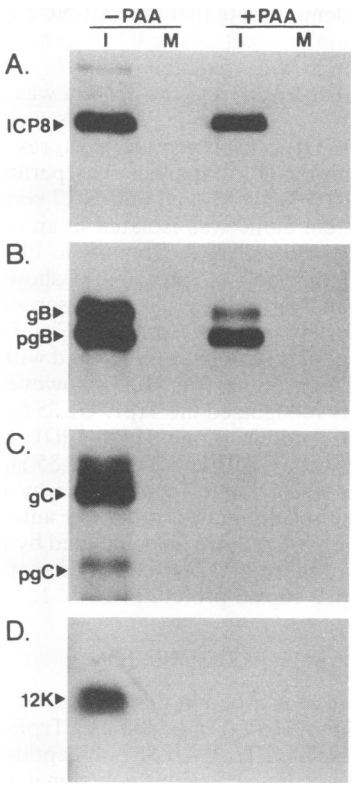

FIG. 7. Synthesis of the UL35 gene product in the presence and absence of viral DNA replication. HEp-2 cells were mock infected (M) or infected with HSV-1 (I) in the presence (+PAA) or absence (−PAA) of 300 μg of PAA per ml as described in Materials and Methods. The cells were radiolabeled with 100 μCi of [^{35}S]methionine per ml between 3 and 18 h postinfection. The cells were lysed in radioimmunoprecipitation assay buffer, and aliquots were reacted with antiserum against ICP8 (A), glycoprotein B (gB) (B), glycoprotein C (gC) (C), or the UL35 gene product (D). The precursors containing high levels of mannose (pgB and pgC) are also indicated. The immunoprecipitated proteins were resolved by electrophoresis through SDS–7% polyacrylamide (A, B, and C) or SDS–15% polyacrylamide (D) gels and visualized by fluorography.

the UL35 gene in the absence of viral DNA replication. In experiments whose data are not shown, it was determined that 300 μg of PAA added 2 h prior to infection and maintained throughout infection was sufficient to block viral DNA synthesis. To study the effect of viral DNA replication on the synthesis of the UL35 gene product, HEp-2 cells were mock infected or infected with HSV-1 in the presence or absence of PAA, incubated in the presence of 100 μCi of [^{35}S]methionine per ml between 3 and 18 h postinfection, and harvested at 18 h postinfection. The proteins were immunoprecipitated from the extracts, resolved by SDS-PAGE, and visualized by fluorography. The synthesis of ICP8, a known early (β) gene product (17, 18), was only slightly diminished in the absence of viral DNA replication (Fig. 7A), indicating that the DNA synthesis inhibitor was not suppressing viral gene expression in a nonspecific manner. Synthesis of the control protein, glycoprotein B, was diminished approximately threefold in the absence of viral DNA synthesis (Fig. 7B), which is consistent with its assignment as a γ_1 gene product (22, 37). The expression of the second control protein, glycoprotein C, was completely abolished in the absence of viral DNA replication (Fig. 7C), which is consistent with its classification as a γ_2 gene product (22, 23). The synthesis of the UL35 gene product was also completely abolished in the absence of viral DNA replication (Fig. 7D). These results demonstrate that the expression of the UL35 gene is absolutely dependent on viral DNA replication, indicating that UL35 represents a γ_2 (true late) gene.

Association of the UL35 gene product with purified HSV virions and capsids. Our original hypothesis was that the UL35 ORF may encode the p12 capsid protein. The classification of UL35 as a γ_2 gene further suggests that the UL35 gene product may be a structural protein. Since previous studies have shown that p12 is associated with both type A and B intranuclear capsids (7, 11, 20), and by inference should also be associated with whole virions, we wondered whether the 12-kDa product of the UL35 ORF was also associated with all three types of particles. If the product of the UL35 ORF is associated with the three types of particles, the UL35 ORF may encode p12.

To address this possibility, HSV-1 virions and type A and B capsids were isolated as described in Materials and Methods. In experiments whose results are not shown, the purified virions or A and B capsids were resolved by SDS-PAGE and visualized by Coomassie blue or silver staining to assess the purity of each preparation. The structural protein profiles were similar to those described by Spear and Roizman (44) and Gibson and Roizman (16), with minimal contamination by cellular proteins. To determine whether the UL35 gene product was associated with the purified virus particles, the proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose, and immunoblot analysis was performed with the TrpE-UL35 antiserum (Fig. 8A). These results demonstrated that the 12-kDa protein encoded by the UL35 gene is present in purified virions as well as type A and B capsids, implying that the UL35 gene may encode the p12 capsid protein. It should be noted that the SDS-PAGE gels were loaded quantitatively in terms of virus particles by using the amount of VP5 (the major capsid protein) present in each particle preparation as the common reference. To confirm that each sample contained equivalent amounts of viral particles the same blot was reprobed with anti-VP5 antiserum, and the results are shown in Fig. 8B. Unexpectedly, these studies demonstrated that less of the 12-kDa protein was present in isolated type A and B capsids than in purified virions. By using densitometric scanning of the autoradiograms, these data were examined quantitatively (Table 1). These results indicate that isolated virions contained 2.5-fold more p12 than did purified type A and B capsids. At present, we cannot provide an explanation for this observation; however, experiments to define the precise location of the 12-kDa protein within the viral capsid are currently under way, and these studies may provide some insights into this finding.

Anti-NC7 and anti-TrpE-UL35 antisera recognize the same polypeptide. The association of the 12-kDa UL35 gene product with purified virions as well as type A and B capsids strongly suggests that the UL35 gene encodes the p12 capsid protein. To confirm this assignment, we obtained antiserum prepared against the purified p12 capsid protein (designated anti-NC7, kindly provided by G. Cohen and R. Eisenberg) and performed studies to determine whether anti-NC7 would cross-react with the same polypeptide recognized by the TrpE-UL35 antiserum. If the protein product of the UL35 ORF is p12, the antiserum prepared to the purified p12 capsid protein should react with the same 12-kDa protein recognized by the TrpE-UL35 antiserum.

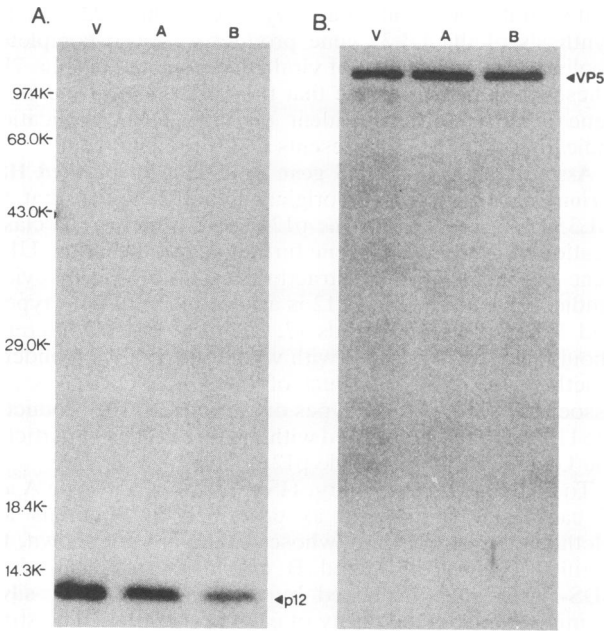

FIG. 8. Association of the UL35 gene product with HSV virions and intranuclear capsids. HSV-1 virions and intranuclear capsids (types A and B) were purified as described in Materials and Methods. The viral polypeptides present in each preparation were resolved by electrophoresis through an SDS–12% polyacrylamide gel, and the proteins were transferred to nitrocellulose and immunoblotted with either the TrpE-UL35 antiserum (A) or anti-VP5 serum (B). The reactive proteins were visualized by incubation with ^{125}I-labeled protein A and subsequent autoradiography. Panels A and B show autoradiographs of the same nitrocellulose sheet separately probed with each antiserum. The positions of the molecular weight markers are shown on the left, and the positions of p12 and VP5 are shown on the right. Abbreviations: V, purified virions; A, type A capsids; B, type B capsids.

To test the cross-reactivity of these antisera, HEp-2 cells were mock infected or infected with HSV-1 and cell lysates were prepared at 18 h postinfection. The lysates were immunoprecipitated with either anti-NC7 or anti-TrpE-UL35 serum, and the immunoprecipitates were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose. The samples were then subjected to immunoblot analysis with the opposite antiserum. The results for these analyses are shown in Fig. 9 and clearly demonstrate that the anti-NC-7 antiserum and the TrpE-UL35 antiserum recognize the same 12-kDa protein. As a control, purified HSV-1 virions were also analyzed in parallel to confirm the reactivity of each antiserum.

TABLE 1. Relative amounts of p12 associated with HSV-1 virions and type A and B capsids[a]

Particle type	Amt of:		p12/VP5 ratio
	VP5	p12	
HSV-1 virions	1.3	3.2	2.5
Type A capsids	1.7	2.0	1.2
Type B capsids	1.4	1.2	0.9

[a] The procedures for gel electrophoresis and immunoblotting are described in Materials and Methods. The autoradiographs shown in Fig. 8 were quantitated by using an LKB Ultroscan XL laser densitometer. The relative values and the ratio of p12/VP5 are shown.

To further demonstrate that the anti-NC7 antiserum reacts with the protein product of the UL35 gene, the TrpE-UL35 chimeric protein was isolated from *E. coli* extracts by preparative SDS-PAGE and the protein was eluted from the gel fragments as described in Materials and Methods. The isolated TrpE-UL35 polypeptide was resolved by SDS-PAGE, and immunoblot analysis was performed with preimmune, anti-TrpE-UL35, and anti-NC7 sera. As a control, the TrpE protein alone was isolated in an identical manner and analyzed in parallel. A Coomassie blue-stained SDS-PAGE gel of the isolated proteins is shown in Fig. 10A. Immunoblot analysis with preimmune serum failed to demonstrate reactivity to either protein (Fig. 10B). As anticipated, the TrpE-UL35 antiserum reacted with both the TrpE and TrpE-UL35 proteins (Fig. 10C); however, the anti-NC7 antiserum only recognized the TrpE-UL35 fusion protein but not the TrpE protein alone (Fig. 10D). Since the only difference between TrpE and TrpE-UL35 is the addition of the 91 amino acid residues contributed by the UL35 gene product, these results indicate that the anti-NC7 antiserum recognizes the viral polypeptide encoded by the UL35 gene. On the basis of these data, we conclude that the UL35 gene encodes the p12 capsid protein of HSV-1.

DISCUSSION

In the studies described in this report, we have expressed the UL35 ORF of HSV-1 in *E. coli* as a TrpE-UL35 chimeric protein. The isolated TrpE-UL35 polypeptide was used as an immunogen to produce a rabbit polyclonal antiserum which would specifically react with the putative protein product of the UL35 ORF in HSV-infected cells. We found that this antiserum reacted specifically with a 12-kDa protein present only in HSV-infected cells. The apparent size of the protein was in excellent agreement with the molecular weight of 12,095 predicted for the UL35 gene product (28). The identification of the predicted product of the HSV-1 UL35 ORF confirms the reading frame of this ORF and demonstrates that UL35 represents an actively expressed gene during HSV infection. Analysis of the kinetics of synthesis of the UL35 gene product indicated that the protein is synthesized late in infection, with an accumulation pattern similar to that of glycoprotein C. Additional studies revealed that the synthesis of the UL35 gene product was absolutely dependent on prior viral DNA replication, implying that UL35 is a true late (γ_2) gene.

The association of the UL35 gene product with purified virions as well as type A and B capsids suggested that this polypeptide might represent the previously identified 12-kDa capsid protein variably designated p12, NC7, and VP26 (11, 20, 32). This assignment was confirmed by using two approaches. First, immunoprecipitation and immunoblotting studies were performed to demonstrate that antiserum prepared against the purified 12-kDa capsid protein (anti-NC7) cross-reacted with the same 12-kDa protein recognized by the TrpE-UL35 antiserum. Second, the anti-NC7 antiserum was also shown to react with the isolated TrpE-UL35 polypeptide synthesized in *E. coli*. With the identification of UL35 as the gene encoding p12, the only remaining capsid proteins which have not been assigned to a specific gene are VP21 and VP24. As stated previously, VP21 may represent a processed form of VP22a (8), thereby also being encoded by the UL26 gene (35). Thus VP24, a 25-kDa capsid protein, may represent the only remaining capsid protein which has not been assigned to a specific gene.

With the general structure of the herpesvirus capsid now

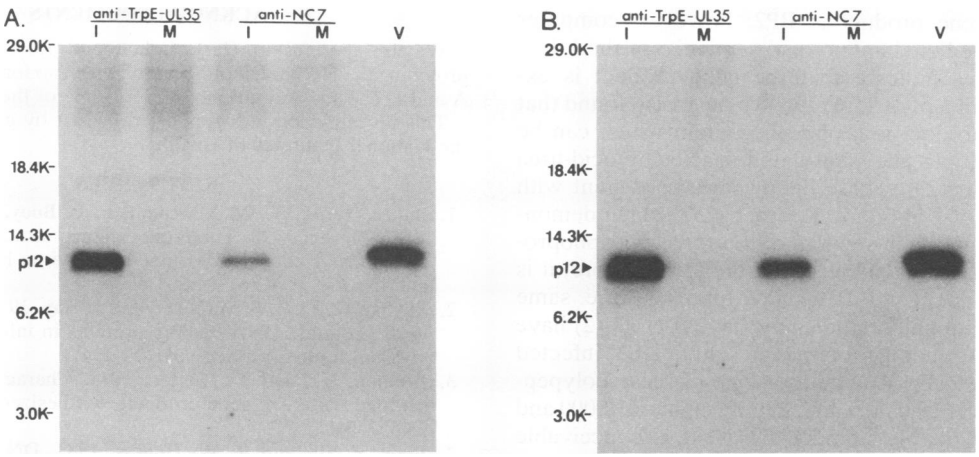

FIG. 9. Cross-reactivity of anti-TrpE-UL35 and anti-NC7. HEp-2 cells were mock infected (M) or infected with HSV-1 (I), and cell lysates were prepared at 18 h postinfection. Aliquots of the lysates were reacted with either anti-TrpE-UL35 or anti-NC7, and the immunoprecipitates were resolved by electrophoresis on SDS–15% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes and immunoblotted with the TrpE-UL35 antiserum (A) or anti-NC7 (B). The reactive proteins were visualized by incubation with ^{125}I-labeled protein A and autoradiography. The antisera used for the immunoprecipitations are indicated above each pair of lanes. Purified HSV-1 virions (V) were analyzed in parallel as a control. The positions of the molecular weight markers are shown on the left of each panel.

reasonably well defined (1, 7, 40), the current challenge is to understand the precise location and function(s) of these seven proteins which comprise the HSV capsids and to understand their role in the assembly and maturation of the herpesvirion. In our studies, we have observed a 2.5-fold increase in the amount of p12 associated with HSV virions versus intranuclear type A and B capsids (Fig. 8 and Table 1). Studies to explain this observation are currently in progress, but the presence of more p12 associated with purified virions induced us to raise some hypotheses about the possible location and function of p12 within the virion.

It has been previously demonstrated that preparations of type A capsids appear to contain little or no viral DNA (7, 16), and preparations of type B capsids appear to be a mixture of DNA-containing and empty capsids (27, 42). These findings differ from those for isolated virions, which represent a population highly enriched for DNA-containing particles. If p12 is somehow associated with the viral DNA, then the presence of less p12 in isolated type A and B capsids could be explained by the variable amounts of viral DNA in these particle preparations. The possibility of p12 being associated with the viral DNA is supported by several independent studies. Knopf and Kaerner (25) have demonstrated that an acid-soluble 12-kDa basic phosphoprotein, designated BP2, was found to be associated with the chromatin isolated from HSV-infected cells extracted with 0.25 M HCl and with similarly acid-extracted HSV virions. On the basis of current information, we cannot directly conclude

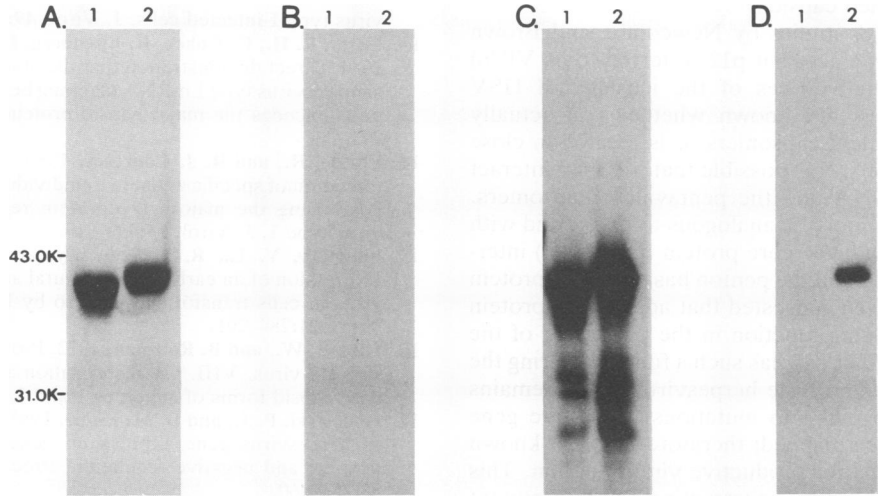

FIG. 10. Reaction of anti-NC7 with the TrpE-UL35 chimeric protein synthesized in *E. coli*. The TrpE and TrpE-UL35 proteins synthesized in *E. coli* were isolated from preparative SDS-polyacrylamide gels as described in Materials and Methods. The isolated proteins were electrophoresed on SDS–10% polyacrylamide gels and stained with Coomassie blue (A) or transferred to nitrocellulose. Immunoblot analyses were subsequently performed with rabbit preimmune serum (B), anti-TrpE-UL35 serum (C), or anti-NC7 serum (D), and the reactive proteins were visualized by incubation with ^{125}I-labeled protein A and autoradiography. Lanes 1 and 2 of each panel contain TrpE and TrpE-UL35, respectively. The positions of molecular weight markers are shown on the left of panel A.

that the UL35 gene product is BP2; however, computer analysis of the predicted amino acid sequence of the UL35 gene product does indicate that the gene product is extremely basic, with a pI of 11.6 (29). We have also found that the UL35 gene product is a phosphoprotein which can be resolved into multiple phosphorylated species by acid-urea gel electrophoresis (29). These findings are consistent with the observations of Knopf and Kaerner (25), which demonstrated that BP2 could be separated into three phosphoprotein species by acid-urea gel electrophoresis; therefore, it is conceivable that p12 and BP2 may represent the same polypeptide. Additionally, studies by Bayliss et al. (2) have identified 16 DNA-binding proteins, present in HSV-infected cells, which bind to DNA-cellulose. One of these polypeptides, designated BP15, had a molecular weight of 12,000 and appeared to bind double-stranded DNA. It is conceivable that this 12-kDa protein may represent the p12 capsid protein; however, similar DNA-binding studies and assays for p12 will have to be performed to demonstrate this definitively. Finally, studies of the DNA-binding proteins of herpesvirus saimiri by Blair and Honess (4) have demonstrated the presence of a 12-kDa basic nucleocapsid protein which appears to bind to herpesvirus saimiri DNA in vivo and in vitro. We do not know whether the 12-kDa protein of herpesvirus saimiri represents a homolog to p12 of HSV; however, the proteins have several properties which are strikingly similar. First, both proteins are extremely basic and have been described as histonelike (4, 11, 25). Second, they are both components of the viral nucleocapsids (4, 11, 20). Finally, both proteins appear to be true late (γ_2) gene products, requiring viral DNA replication for their synthesis (4 and Fig. 7 of this report).

At present, we have no direct proof that p12 interacts with the viral DNA within the nucleocapsid; however, these observations indicate that this possibility warrants further investigation. Current studies in our laboratory are focused on determining whether p12 can bind to HSV DNA in vitro and in vivo. If p12 can bind to viral DNA, then p12 may function in the condensation and/or packaging of the viral DNA into the preformed capsids.

As stated previously, studies by Newcombe and Brown (32) have provided evidence that p12 (referred to as VP26) may be located at the vertices of the icosahedral HSV capsids; however, it is not known whether p12 actually comprises the pentavalent capsomers or is located in close proximity to the pentons. It is possible that p12 may interact with both the viral DNA and the pentavalent capsomers. Such an arrangement would be analogous to that found with adenovirus, in which a basic core protein (protein V) interacts with the viral DNA and the penton base protein (protein III) (24). It has also been suggested that adenovirus protein V may have a positioning function in the packaging of the virion DNA (24). Whether p12 has such a function during the assembly and maturation of the herpesvirus virion remains to be determined. To date, no mutations within the gene encoding p12 have been mapped; therefore, it is not known whether p12 is essential for productive viral infection. This problem can be addressed by constructing an HSV mutant containing a null allele of UL35. Such a mutant would be valuable in addressing the function of p12 in the assembly and maturation of HSV. We are currently performing studies to further characterize p12 in an attempt to understand its function in the assembly pathway of HSV.

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