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The Product of the UL6 Gene of Herpes Simplex Virus Type 1 Is Associated with Virus Capsids

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We report on the analysis of the UL6 and UL7 open reading frames of the herpes simplex virus type 1 (HSV-1) genome. The UL6 and UL7 transcripts were identified in HSV-1-infected cells by Northern blotting and shown to be coterminal at their 3′ ends. Both transcripts were synthesized in the presence of phosphonoacetic acid, although in reduced amounts, indicating that UL6 and UL7 are expressed as delayed-early or γ-1 genes. The 5′ ends of the two transcripts were mapped by S1 nuclease and primer extension analysis. A polyclonal antiserum directed against an *Escherichia coli*-expressed 6× His-UL6 fusion protein identified a protein of approximate M_r 75,000 in cells infected with either HSV-1 or with a vaccinia virus recombinant expressing the HSV-1 UL6 protein. As with the transcript, the UL6 protein was synthesized at reduced levels in the absence of viral DNA replication. Western immunoblotting showed that the UL6 protein was present in purified virions but not in L-particles of HSV-1, and that it was located exclusively in the tegument/capsid fraction of virion. Further analysis of the UL6 protein revealed that this protein was associated with virus capsids.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) virions contain a linear double-stranded DNA genome of approximately 152 kbp, which is enclosed within an icosahedral capsid. The capsid is surrounded by an amorphous layer, termed the tegument, and an external glycoprotein-containing lipid envelope (reviewed by Dargan, 1986). In addition to infectious virions, noninfectious virion-related particles, termed L-particles, have been identified in HSV-1 preparations (Szilágyi and Cunningham, 1991). L-particles contain most of the envelope and tegument components of the virion but lack virus capsids and DNA (Szylágyi and Cunningham, 1991; McLauchlan and Rixon, 1992). Three types of capsid are seen in HSV-infected cells (Gibson and Roizman, 1972). Type A or empty capsids lack viral DNA and internal structure, type B or intermediate capsids also lack viral DNA but possess an internal proteinaceous scaffold, and type C or full capsids contain viral genomic DNA (reviewed by Rixon, 1993). Type B capsids are known to be composed of seven proteins which have been designated VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26 (Gibson and Roizman, 1972; Heilman *et al.*, 1979; Cohen *et al.*, 1980), and the genes to which these capsid proteins have been assigned are UL19, UL38, UL26, UL26.5, UL18, UL26, and UL35, respectively (Preston *et al.*, 1983; Weller *et al.*, 1987; Pertuiset *et al.*, 1989; Rixon *et al.*, 1990; Liu and Roizman, 1991; Davison *et al.*, 1992; McNabb and Courtney, 1992; Preston *et al.*, 1992). With the exception of the internal scaffolding proteins VP21 and VP22a, the protein composition of types A and C capsids is identical to that of type B capsids (see Rixon, 1993).

The genome of HSV-1 encodes at least 75 open reading frames (ORFs) (see McGeoch *et al.* 1993). Many of the ORFs and their potential products remain largely uncharacterized. Two such ORFs, UL6 and UL7, are located near the left end of the long unique (UL) arm of the HSV-1 genome (McGeoch *et al.*, 1988). Nucleotide sequence analysis has shown that the two ORFs partially overlap each other, and it is predicted that the two genes encode transcripts that are coterminal at their 3′ ends (McGeoch *et al.*, 1988). The ORFs UL6 and UL7 code for proteins with predicted M_rs of 74,087 and 33,057, respectively. The UL6 gene is known to be essential for virus growth in tissue culture, whereas the status of UL7 is unknown. Two temperature-sensitive (*ts*) mutants carrying lesions in the UL6 gene have been isolated (Schaffer *et al.*, 1973; Weller *et al.*, 1987). Studies with one such mutant, *ts*F18, have shown that the UL6 gene product is required for the formation of full capsids (Sherman and Bachenheimer, 1987). At nonpermissive temperature (NPT), the *ts*F18 virus synthesizes near wild type levels of viral DNA but fails to cleave and package the replicated concatameric viral DNA, and it accumulates only type B capsids in infected cells (Sherman and Bachenheimer, 1987, 1988). The effect of the mutation is reversible upon shift to permissive temperature but only after new protein and capsids are synthesized following the temperature shift, indicating that the viral DNA cleavage and its encapsidation

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are tightly linked events (Sherman and Bachenheimer, 1987, 1988). In addition to UL6, the gene products of UL25, UL28, and UL33 are required for processing and packaging of nascent viral DNA (Addison *et al.,* 1984, 1990; Al-Kobaisi *et al.,* 1991; Tengelsen *et al.,* 1993), although the mechanisms involved in this process remain obscure.

In the present study, we analyze the transcripts encoded by the UL6 and UL7 genes of HSV-1 and define the precise location of their 5′ ends. We also identify the UL6 gene product in HSV-1-infected cells and show that it is a component of infectious virions but not of L-particles. Further characterization of the UL6 protein reveals that this protein is associated with virus capsids.

MATERIALS AND METHODS

Cells, viruses, and radioisotopic labeling

HSV-1 strain 17 syn$^+$ (Brown *et al.,* 1973) and vaccinia virus strains WR and V-UL6 were propagated in baby hamster kidney (BHK) cells in Eagle's medium supplemented with 2% newborn calf serum (EC2). Purified virions and L-particles of HSV-1 strain F-derived UL46/47 double-deletion mutant virus R(F)UL46/47Δ2 were generously provided by J. McLauchlan (this virus was originally supplied by J. L. C. McKnight; Zhang and McKnight, 1993). HSV-1ambUL8, a viral DNA replication-negative mutant of HSV-1 strain 17 was grown in a complementing cell line A26: the construction of this virus and the cell line A26 will be described elsewhere (Patel and others, unpublished data). To biochemically inhibit viral DNA synthesis, cells were incubated 1 hr prior to and then throughout the infection in medium containing 300 μg/ml phosphonoacetic acid (PAA). Mock-infected and infected cells in 50-mm dishes were labeled with 50 μCi of [^{35}S]-methionine/ml of methionine-free EC2. At the end of the labeling period, the cells were washed with phosphate-buffered saline (PBS), harvested by scraping, lysed in RIPA buffer (50 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40 [NP40], 1% sodium dodecyl sulfate [SDS]), and analyzed either directly or after immunoprecipitation on a 10% SDS–polyacrylamide gel (SDS–PAGE). Immunoprecipitations were performed using Pansorbin cells (suspension of fixed *Staphylococcus aureus* cells, Calbiochem) essentially as described by Zhu and Weller (1992).

Purification of virions and L-particles of HSV-1

BHK cells in 10 roller bottles were infected at a m.o.i. of 0.002 PFU/cell and incubated at 37°C for 4 to 5 days until CPE was complete. Extracellular virus was harvested, subjected to velocity gradient centrifugation on 5 to 15% Ficoll gradients, and bands corresponding to virions and L-particles were collected and concentrated as described previously (Szilágyi and Cunningham, 1991). The envelope components of virions and L-particles were solubilized and separated from the tegument/capsid material by treatment with NP40 as described by McLauchlan and Rixon (1992). Briefly, NP40 was added to the virion and L-particle preparations to a final concentration of 1%. Following incubation at 0°C for 20 min, the samples were centrifuged at 14,000 rpm for 15 min. The supernatants containing envelope proteins were removed and mixed with SDS–PAGE denaturing buffer (0.05 *M* Tris–HCl, pH 6.7, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) and stored at −20°C. The pellets containing tegument and capsid structures were washed three times in culture medium lacking phenol red and calf serum, resuspended in that medium, and stored at −20°C after addition of SDS–PAGE denaturing buffer.

Purification of capsids

BHK cells in roller bottles were infected at a moi of 10 PFU/cell and incubated at 37°C for 24 hr. Virus capsids were isolated and purified from nuclear lysates of infected cells in a 20–50% sucrose gradient as described by Booy *et al.* (1991) and Tatman *et al.* (1994). Following centrifugation, individual capsid bands were removed from the gradient with a syringe, diluted in 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA (NTE), pelleted, and resuspended in a small volume of NTE as described by Booy *et al.* (1991). The capsids used in this study were examined under electron microscope and found to be homogeneous and free from contaminating noncapsid material. HSV-1 type B capsids were extracted with guanidine hydrochloride as described by Newcomb and Brown (1991). To prepare radiolabeled capsids, BHK cells in roller bottles were infected with 10 PFU/cell of wt HSV-1 in EC2. At 6 hr postinfection, the cells were washed with methionine-free EC2 and labeled in that medium with 25 μCi/ml of [^{35}S]methionine for 16 hr. Nuclear lysate of infected cells was subjected to 20–50% sucrose gradient ultracentrifugation as described above. Eight-drop (approximately 300 μl) fractions from below the type C capsid band to the top of the gradient were collected. Aliquots of each fraction were TCA precipitated and radioactivity was measured by scintilation counting.

DNA manipulations

The UL6 ORF is located between nucleotide positions 15132 to 17160 of the HSV-1 genome (Fig. 1a; McGeoch *et al.,* 1988). Nucleotide sequences carrying the UL6 gene and a portion of UL7 gene (position 14590 to 17675; Fig. 1a) were inserted between the *Eco*RI and the *Bam*HI sites of pAT153 forming pAS21. A 256-bp *Nae*I fragment

(position 15756 to 16011; Fig. 1a) located within the UL6 ORF was deleted from pAS21 to give pAS213.

For expression in *Escherichia coli*, nucleotide sequences from 10 bp upstream (position 15122) from the ATG start codon of UL6 to the *Sal*I site at position 17323 (Fig. 1a) were cloned in frame into the coding sequences of six histidine residues of the *E. coli* expression vector pQE30 (QIAGEN Inc.) to form pAS20. This gene fusion construct consists of 6 codons of histidine residues (6 × His), 6 codons from the 5′ nontranslated region of the UL6 gene, and all 676 codons of the UL6 gene. The expression of 6×His-tagged UL6 protein is regulated by isopropyl-*β*-*D*-thiogalactopyranoside (IPTG)-inducible *lac* operator sequences and a phage T5 promoter. The translation is expected to terminate at the UL6 TGA stop codon. Plasmid pAS20 was transformed into *E. coli* strain M15[pREP4] (QIAGEN Inc.) which, following induction with IPTG, expressed large quantities of 6×His-tagged UL6 fusion protein in an insoluble form. Purification of the fusion protein under denaturing conditions by metal chelate chromatography according to manufacturer's instruction resulted in extremely low recovery of the protein. Therefore, inclusion bodies of *E. coli* strain M15[pREP4] (QIAGEN Inc.) carrying pAS20 were prepared as described (Harlow and Lane, 1988). This preparation consisting predominantly of 6×His-UL6 protein (approximately 80% of the total protein) was used for immunization purposes (see below).

To express UL6 in vaccinia virus, the HSV-1 sequences present in pAS20 were inserted into the vaccinia virus vector pMJ601 (Davison and Moss, 1990) such that the UL6 gene was placed under the control of the synthetic late promoter of vaccinia virus. Here the translation is expected to be initiated at the UL6 ATG start codon. This construct was inserted into the thymidine kinase gene of the vaccinia virus strain WR by homologous recombination, and three independent plaque isolates of recombinant vaccinia virus were selected as described by Davison and Moss (1990). All three isolates expressed HSV-1 UL6 polypeptide, and one such virus, V-UL6, was used in experiments described in this paper.

RNA isolation and Northern blot analysis

BHK cells were mock-infected or infected with HSV-1 in the presence or absence of PAA at 10 PFU/cell and total cellular RNA was extracted at 6 hr postinfection as described (Maniatis *et al.*, 1982). For Northern blot analysis, the RNA was fractionated on a 1.2% agarose–formaldehyde gel and transferred to Hybond-N membrane (Amersham) according to Maniatis *et al.* (1982). Hybridization was performed using appropriate ^{32}P-labeled probes as described (Patel and Elliott, 1992). The double-stranded DNA probes used for hybridization were nick translated using [^{32}P]dCTP. To prepare a single-stranded probe, a 5′ end-labeled oligonucleotide complementary to sequences coding for the N-terminal amino acids of UL7 gene was hybridized to denatured pAS213 and extended as described below. The DNA was cleaved with *Nco*I (Fig. 1a), and the labeled single-stranded fragment was isolated from alkaline agarose gel (Greene, 1988).

S1 nuclease and primer extension analysis

This was performed as described by Kingston (1987). To prepare probes for S1 mapping, 5′ end-labeled 30-mer oligonucleotides complementary to nucleotide sequences coding for the N-terminal 10 amino acids of the genes UL6 and UL7 of HSV-1 were synthesized. The oligonucleotides were hybridized to denatured plasmid pAS213 and extended using a Klenow fragment of *E. coli* DNA polymerase I. The DNA was cut to appropriate lengths using *Bsp*HI (for UL6-specific probe; Fig. 1a) or *Dra*I (UL7-specific probe; Fig. 1a) and the labeled single-stranded DNA fragments were isolated using alkaline agarose gel electrophoresis (Greene, 1988). For primer extension analysis, 5′ end-labeled 30-mer oligonucleotides complementary to nucleotides 49 to 78 (coordinates 14980 to 15009; UL6) and 56 to 85 (coordinates 17014 to 17043; UL7) downstream from the initiation site (as determined by S1 mapping) of UL6 and UL7 mRNAs were used. The reaction products were analyzed on a 6% polyacrylamide urea sequencing gel. To determine the exact sizes of the bands, dideoxy sequencing ladders of the bacteriophage M13 DNA were electrophoresed on adjacent lanes.

Preparation of antiserum

Antisera were produced in two rabbits by immunization with an emulsion containing approximately 0.7 mg of *E. coli*-expressed 6×His-tagged UL6 in the form of insoluble inclusion body preparation (see above) in Freund's complete adjuvant. Subsequent boosts used Freund's incomplete adjuvant and 0.7 mg of the inclusion body preparation. A total of five booster injections were given each at 3-week intervals after primary injection. Eleven days after the last boost, rabbits were bled out and antisera prepared. The experiments described in this paper used one antiserum, 2C2. Both the preimmune and immune antisera were extensively adsorbed against acetone powder of uninfected BHK cells prior to use as described by Harlow and Lane (1988).

SDS–PAGE and Western blotting

Proteins were fractionated on 9% (or as indicated) polyacrylamide gels cross-linked with 2% (wt/wt) *N,N′*-methylenebisacrylamide, and detected by either autoradiography or silver staining as described (McLean *et al.*, 1990). For Western immunoblotting, proteins were electrophoretically transferred to Hybond ECL membranes (Amer-

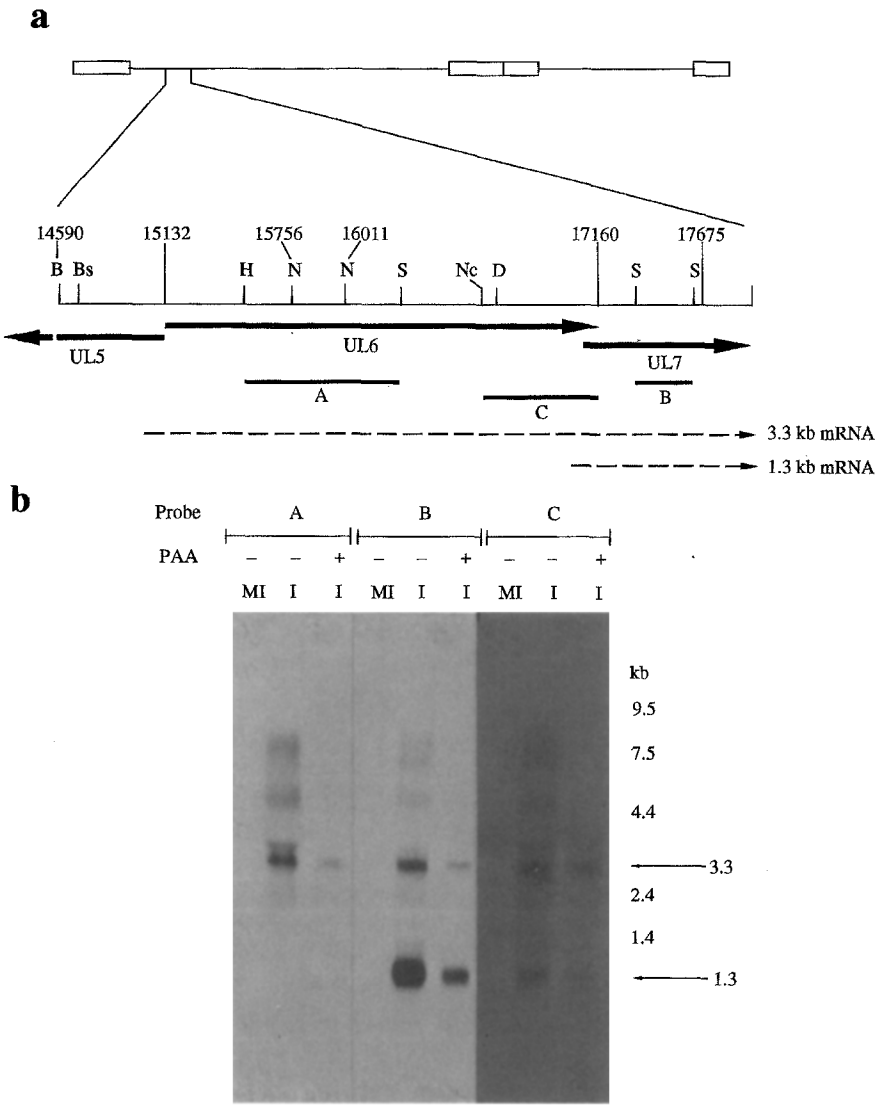

FIG. 1. (a) Schematic representation of the HSV-1 genome showing the location of the UL6 and UL7 genes. The relevant nucleotide positions and restriction sites are shown. B, Bs, D, H, N, Nc, and S refer to restriction sites *Bgl*II, *Bsp*HI, *Dra*I, *Hin*dIII, *Nae*I, *Nco*I, and *Sal*I, respectively. The probes used in Northern blot analysis (b) are indicated by solid lines. Probes A and B correspond to the *Hin*dIII to *Sal*I fragment of the UL6 gene and the *Sal*I fragment of the UL7 gene, respectively. Probe C represents the single-stranded probe complementary to the coding strands of the UL6 and UL7 genes. The location and direction of the 3.3- and 1.3-kbp transcripts are shown. (b) Northern blot analysis of the UL6 and UL7 transcripts. Total cellular RNA from BHK cells mock-infected (MI) or infected with HSV-1 (I) in the presence (+) or absence (–) of PAA was hybridized with ^{32}P-labeled probes A, B, or C (see Fig. 1a). The positions of the 3.3- and 1.3-kbp transcripts are shown.

sham) as described by Towbin *et al.* (1979). Membranes were incubated with 1:200 dilution of serum, and the immunoreactive proteins were detected using protein A conjugated to horseradish peroxidase (Sigma) and enhanced chemilumenescence detection reagents (Amersham).

RESULTS

Northern blot analysis

HSV-1 genes UL6 and UL7 are located near the left end of the unique long region of HSV-1 genome (Fig. 1a; McGeoch *et al.*, 1988). The putative UL7 ORF overlaps UL6 at its 5′ end; the translational start site of UL7 ORF is 23 bp upstream of the UL6 stop codon. The translational start sites of genes UL5 and UL6 are located within one nucleotide of each other, but are coded by opposite strands (Fig. 1a; McGeoch *et al.*, 1988). Two hypothetical transcripts coterminal at their 3′ ends have been predicted for UL6 and UL7 genes from nucleotide sequence analysis (McGeoch *et al.*, 1988). To identify and analyze the expression of the UL6 and UL7 transcripts, total cell RNA was isolated from mock-infected or HSV-1-infected cells at 6 hr postinfection and subjected to Northern blot analysis. As shown in Fig. 1b, a UL6-specific double-stranded DNA probe (probe A) identified a transcript of

approximately 3.3 kb, whereas a UL7-specific double-stranded DNA probe (B) hybridized to transcripts of 3.3 and 1.3 kb. The same two transcripts were detected when infected cell RNA was probed with a single-stranded DNA probe (C) complementary to the coding sequences of both the UL6 and the UL7 genes, confirming the rightward orientation of the two transcripts (Fig. 1b). These results show that the 1.3-kb transcript is colinear with the 3′ portion of the 3.3-kb transcript, and they are consistent with the hypothetical transcripts predicted from the nucleotide sequence (McGeoch *et al.*, 1988). The larger transcripts seen hybridizing with all three probes probably represent readthrough products of the UL6 and UL7 mRNAs occurring due to inefficient utilization of the polyadenylation site located at the 3′ end of the UL7 gene (McGeoch *et al.*, 1988). The 1.3- and 3.3-kb transcripts were also found in RNA made from cells infected with HSV-1 in the presence of PAA, although in reduced quantities (Fig. 1b), indicating that viral DNA replication is required for maximum gene expression.

S1 nuclease and primer extension analysis

To map the 5′ initiation sites of the UL6 and UL7 mRNAs, S1 nuclease analysis was carried out using radiolabeled probes that spanned the putative 5′ end of the respective genes. As shown in Fig. 2a, a single DNA band of 230 nucleotides (nt) for the UL6-specific probe and three bands of 205, 207, and 208 nt for UL7-specific probe were protected with RNA isolated from HSV-1-infected cells (Fig. 2a). These results are consistent with transcription beginning at 200 nt (UL6) and 175, 177, and 178 nt (UL7) upstream from the ATG start codons of the UL6 and UL7 ORFs (Figs. 2a and 2c).

Primer extension analysis to map the 5′ termini of these two transcripts confirmed the results obtained in S1 nuclease experiments. As shown in Fig. 2b, several extended products of between 78 to 81 nt (UL6) and 83 to 87 nt (UL7) were identified. The largest of these products is consistent with transcription beginning at 203 (UL6) and 180 (UL7) nt upstream from the ATG start codons (Figs. 2b and 2c). These results are in close agreement with the results obtained by S1 nuclease analysis (Fig. 2a). The possible TATA box for the two genes are shown (Fig. 2c).

Expression of UL6 in vaccinia virus

To express the HSV-1 gene UL6 in a heterologous system, a recombinant vaccinia virus, V-UL6, was constructed. The synthesis of viral polypeptides in BHK cells infected with wild type (wt) HSV-1, V-UL6, and the parental vaccinia virus strain WR was examined. Infected cells were labeled with [^{35}S]methionine and analyzed on SDS–PAGE. The results show that V-UL6 synthesized a novel protein with an approximate M_r of 75 kDa (Fig. 3, lane 3). This is in close agreement with the M_r of 74,087 predicted for the UL6 ORF (McGeoch *et al.*, 1988). This protein was not present at a detectable level in HSV-1-infected cell extracts (Fig. 3, lane 2). A slightly slower migrating protein band was seen in HSV-1-infected cells (lane 2), but it is probably a host protein as it was also found in mock-infected cells (lane 1).

Detection of the UL6 gene product in HSV-infected cells

To identify the UL6 gene product in infected cells, a polyclonal antiserum (2C2) was raised against an *E. coli*-expressed 6×His-tagged UL6 protein (see Materials and Methods). The [^{35}S]methionine-labeled infected cell proteins described above were immunoprecipitated with preimmune or immune antisera 2C2 and analyzed on SDS–PAGE (Fig. 3, lanes 5 to 12). Immune serum 2C2 precipitated the 75-kDa UL6 protein synthesized by the vaccinia virus recombinant V-UL6 (lane 7). A minor protein of the same mobility was also immunoprecipitated from HSV-1-infected cell extracts (lane 6) but not from vaccinia strain WR-infected (lane 8) or mock-infected (lane 5) cells. The results show that the UL6 protein is expressed at comparatively low levels in HSV-1-infected cells. The preimmune 2C2 serum failed to recognize the UL6 protein in HSV-1-infected cells (lane 10), although it reacted weakly with the overexpressed UL6 in V-UL6-infected cells (lane 11). The cross-reactive bands seen in lanes 6 and 7 may be due to nonspecific precipitation by the antiserum. Alternatively, at least in the case of HSV-1-infected cell extract (lane 6), some of the cross-reacting proteins may be coimmunoprecipitated possibly because they interact with the UL6 protein.

We next examined the reactivity on Western blot of the antiserum 2C2 with infected cell proteins. As shown in Fig. 4a, the immune serum detected the UL6 polypeptide from V-UL6- and HSV-1-infected cells but not from mock- or WR-infected cells. The UL6 protein was not recognized by the preimmune serum (Fig. 4a). As with the immunoprecipitation experiment above, the low intensity of the UL6 band in HSV-1-infected cells suggests that this protein is expressed at relatively low levels in infected cells. The 2C2 serum cross-reacted with proteins that migrate close to but at a slower rate than the UL6 protein (Fig. 4a). To minimize these nonspecific reactions, a combination of immunoprecipitation and immunoblotting experiments was performed. Cell extracts from mock-infected cells or cells infected with V-UL6, vaccinia virus strain WR, wt HSV-1 (in the presence or absence of PAA), or a viral DNA replication-negative mutant of HSV-1, HSV-1ambUL8, were immunoprecipitated with antiserum 2C2. The immune complexes were subjected to SDS–PAGE and analyzed on a Western immunoblot using the same antiserum (Fig. 4b). A series of twofold dilutions of the

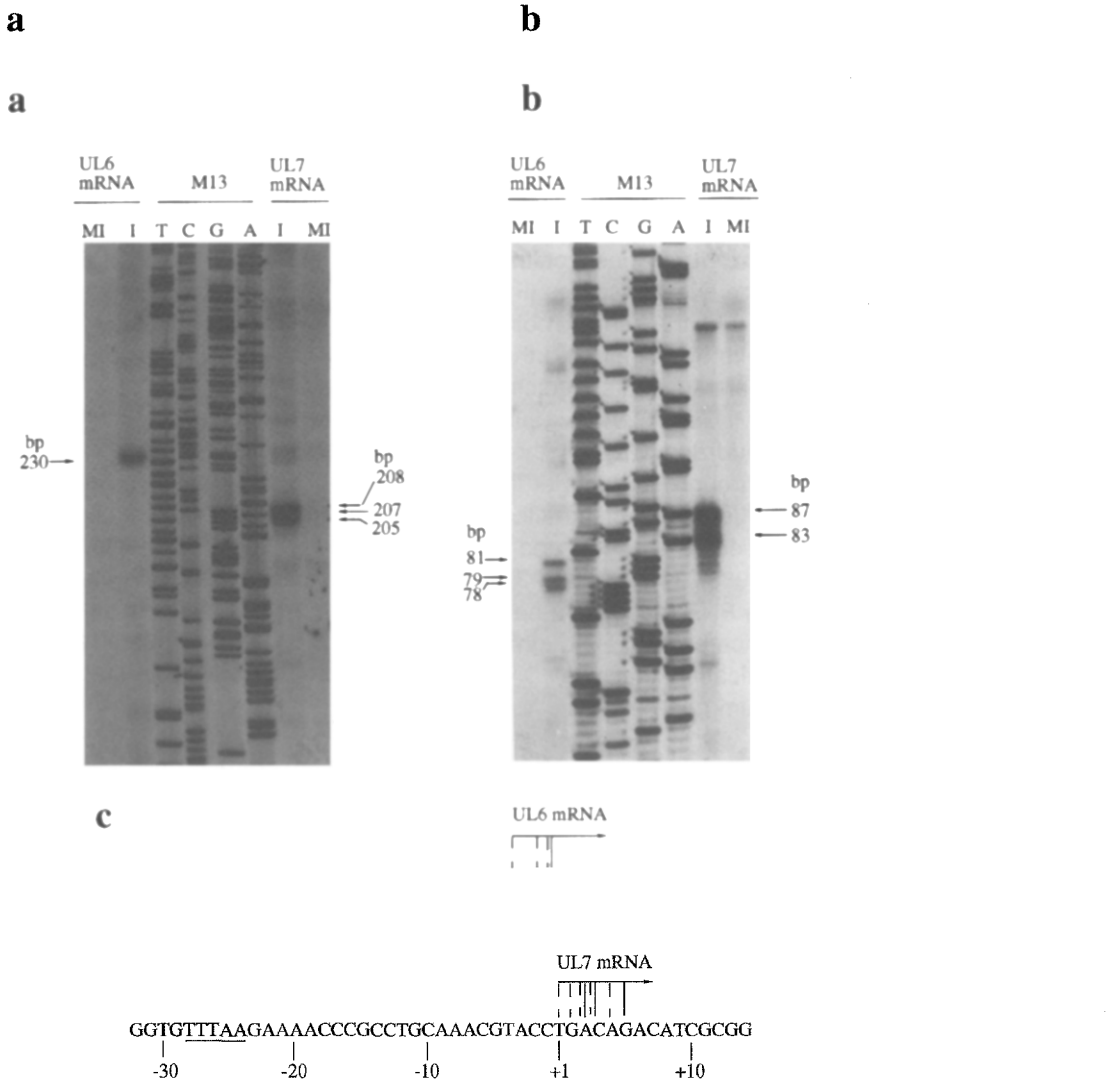

FIG. 2. Mapping of the 5′ end of the UL6 and UL7 transcripts. (a) S1 nuclease analysis was performed using appropriate 5′ end-labeled probes (see Materials and Methods) that were hybridized to total cellular RNA isolated 6 hr postinfection from mock-infected (MI) or HSV-1-infected (I) BHK cells. The hybrids were digested with S1 nuclease and the protected fragments were fractionated on a 6% polyacrylamide/9 *M* urea sequencing gel. T, C, G, A, dideoxy sequencing ladder of bacteriophage M13 DNA used as size markers. Numbers indicated by arrows represent the size of S1 nuclease protected fragments. (b) Primer extention analysis was carried out by hybridizing appropriate 5′ end-labeled primers (see Materials and Methods) to total cellular RNA from mock-infected (MI) or HSV-1-infected (I) BHK cells. The primers were elongated using AMV reverse transcriptase. The extended products were analysed in parallel with the M13 dideoxy sequencing ladders (T, C, G, A) as described in (a). The sizes of the extended products are indicated. (c) The nucleotide sequence adjacent to the 5′ termini of the UL6 and UL7 mRNAs. The position of the potential TATA box relative to the first transcription initiation site of the UL6 and UL7 mRNAs is underlined.

immune complexes of HSV-infected cell extracts were analyzed in this experiment. The UL6 protein was detected not only in V-UL6- and wt HSV-1-infected cells, but also in cells infected under conditions in which the viral DNA synthesis was blocked either by adding PAA to the medium, or by using the mutant virus HSV-1amb-UL8 (Fig. 4b). The second band seen in this experiment is the immunoglobulin protein (IgG) from the immune complexes. HSV-1ambUL8 virus carries an amber nonsense stop codon inserted in frame by site-directed mutagenesis at codon 267 of gene UL8 (unpublished data) which encodes a protein that is essential for viral DNA replication in infected cells (Carmichael and Weller, 1989). In noncomplementing cells this virus does not synthesize viral DNA and therefore is unable to replicate (data not shown). These results are consistent with the Northern blot data shown above where the UL6-specific transcript was detected in cells infected in the presence of PAA (Fig. 1b). As with the transcript, the quantity of the UL6 protein was somewhat reduced (Fig. 4b) under these conditions, suggesting that viral DNA synthesis is required for maximum gene expression. Analysis of the

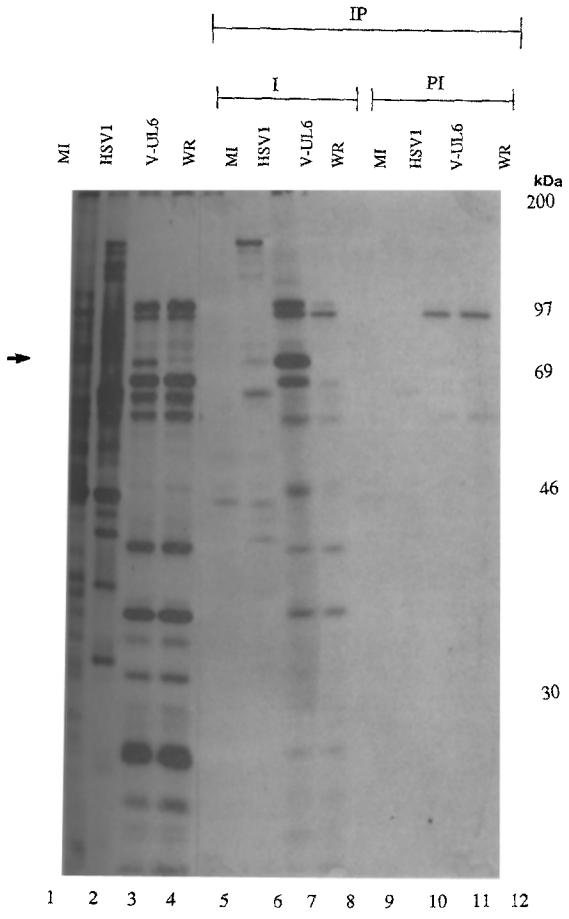

FIG. 3. SDS–PAGE analysis of the proteins synthesized in infected cells. BHK cells were mock-infected (MI) or infected with HSV-1, V-UL6, or vaccinia virus strain WR at 10 PFU/cell. Proteins were labeled with [^{35}S]methionine from 6 to 24 hr postinfection (mock- or HSV-1-infected cells) or 6 to 8 hr postinfection (WR- or V-UL6-infected cells). Cell extracts were analyzed either directly or after immunoprecipitation (IP) with preimmune (PI) or immune (I) 2C2 sera on a 10% polyacrylamide gel. The position of the UL6 proteins is indicated by an arrow. The migration of protein size markers is shown at the right.

time course of UL6 synthesis in HSV-1-infected cells showed that the protein could be detected as early as 2 hr postinfection (data not shown). We have recently isolated an HSV-1 mutant carrying a lacZ insertion in the UL6 gene. As expected, the UL6 protein was not detected by antiserum 2C2 in the mutant-infected cells (unpublished data).

Analysis of purified virus particles

To determine if the UL6 protein is a component of virus particles, virions and L-particles released from BHK cells infected with wt HSV-1 were purified in a 5–15% Ficoll gradient using the method of Szilágyi and Cunningham (1991). Approximately 2 × 10^9 purified particles were analyzed on a Western immunoblot with antiserum 2C2. As shown in Fig. 5a, a protein that comigrated with the UL6 protein expressed in V-UL6-infected cells was found in virions, but not in L-particles, of HSV-1 (lanes 1 to 3). The virion location of the UL6 protein was further confirmed by analysis of fractions across a Ficoll gradient containing HSV-1 preparation by Western immunoblotting which showed that this protein cofractionated with the virions, but not L-particles (data not shown). The antiserum cross-reacted mainly with larger proteins of HSV-

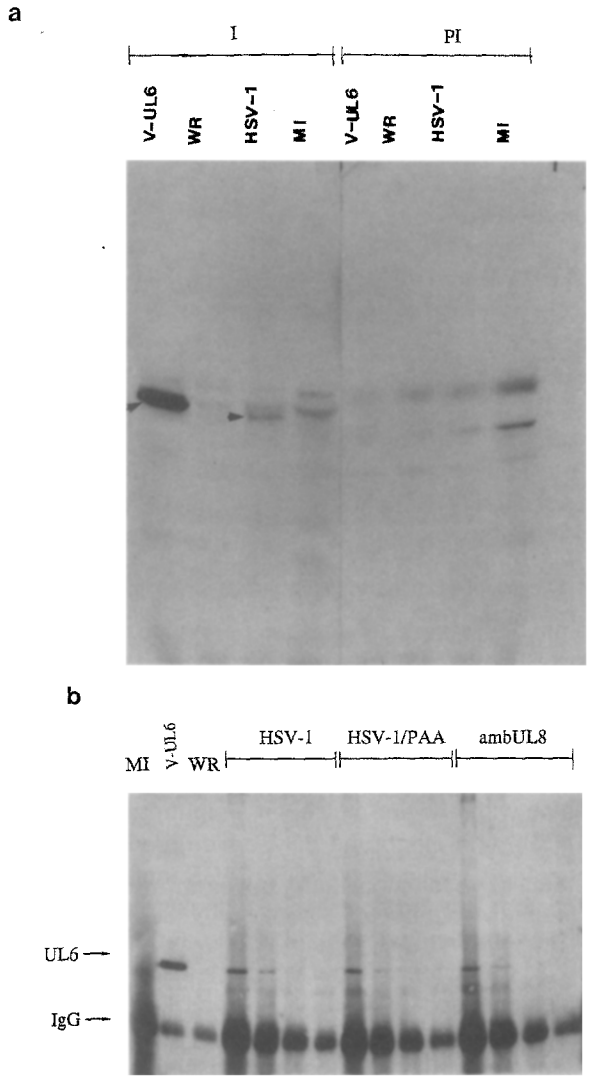

FIG. 4. Western immunoblotting of infected cell proteins. (a) BHK cells were mock-infected (MI) or infected with HSV-1, V-UL6, or vaccinia virus strain WR at 10 PFU/cell. At 18 hr postinfection the medium was removed and the cells were suspended in SDS–PAGE denaturing buffer. Proteins from each sample were subjected to SDS–PAGE and analyzed by immunoblotting with preimmune (PI) or immune (I) 2C2 sera. Arrows indicate the position of the UL6 protein. (b) Expression of the UL6 protein in the absence of viral DNA replication. BHK cells were mock-infected (MI) or infected with V-UL6, vaccinia strain WR, wt HSV-1 (in the presence or absence of PAA), or ambUL8 virus at 10 PFU/cell for 18 hr. Infected cell proteins were immunoprecipitated with antiserum 2C2. The immune complexes were subjected to SDS–PAGE and analyzed by immunoblotting with antiserum 2C2. The positions of the UL6 protein and IgG are indicated.

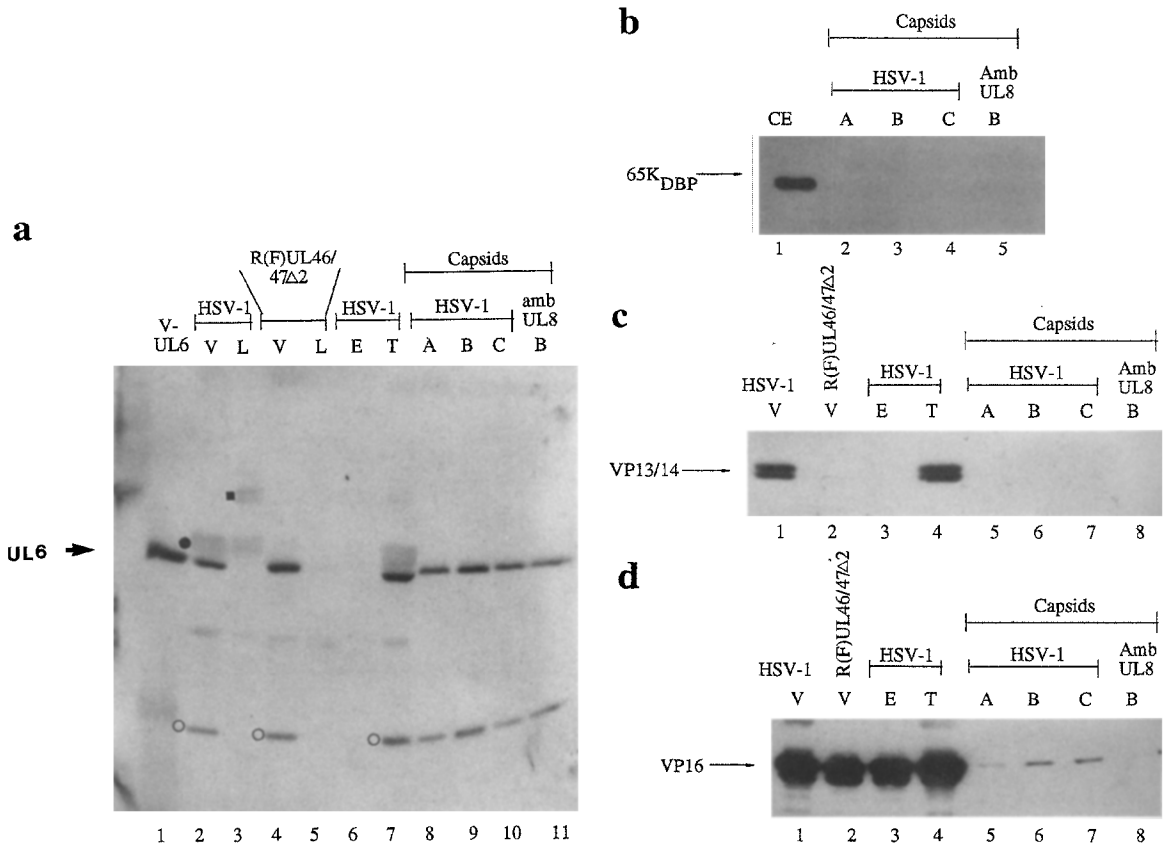

FIG. 5. Immunoblot analysis of virus particles. Proteins were subjected to SDS–PAGE and analyzed by immunoblotting. As indicated, immunoprecipitated cell extracts (with antiserum 2C2) from V-UL6-infected cells, wt HSV-1-infected cell extract (CE), purified virions (V) and L-particles (L) of wt HSV-1 and strain R(F)UL46/47Δ2, envelope (E) and tegument (T) components of wt HSV-1 virions, purified type A, B, C capsids of wt HSV-1, and type B capsids of ambUL8 virus were analyzed using antisera (a) 2C2 (UL6), (b) Z1F11 ($65K_{DBP}$), (c) 94497 (VP13/14), or (d) LP1 (VP16). The relative cross-reactive proteins in (a) are highlighted.

1 virions and L-particles (Fig. 5a, lanes 2 and 3, ■, ●) and also with a lower molecular weight protein (lanes 2 and 4, ○). Analysis of the polypeptide profiles of HSV-1 virions and L-particles on SDS–PAGE (not shown) suggested that the larger cross-reacting proteins may be VP11/12 (also known as 92/91K; Fig. 5a, ■) and VP13/14 (82/81K; Fig. 5a, ●) tegument proteins of virion and L-particles encoded by genes UL46 and UL47, respectively (McLean *et al.*, 1990; McLauchlan and Rixon, 1992; Zhang and McKnight, 1993). To test this possibility, virions and L-particles of a double-deletion mutant of HSV-1 strain F, R(F)UL46/47Δ2, carrying deletions which remove genes UL46 and UL47 (Zhang and McKnight, 1993), were analyzed on the same immunoblot (Fig. 5a, lanes 4 and 5). The antiserum recognized the UL6 protein in the virions, but not L-particles, of strain R(F)UL46/47Δ2 although, as expected, the cross-reacting proteins VP11/12 and VP13/14 were not detected (lanes 4 and 5). The continued presence of the lower molecular weight protein (lane 4, ○) shows that it is not a degradation product of VP11/12 or VP13/14. The small amount of UL6 protein seen in L-particle preparations in Fig. 5a (lanes 3 and 5) is due to virion contamination; Ficoll gradient produced virions and L-particle preparations are known to be cross-contaminated at low levels (Szilágyi and Cunningham, 1991; McLauchlan and Rixon, 1992).

UL6 is associated with virus capsids

The fact that the UL6 protein is a component of virions and not L-particles suggested a possible association of this protein with the virus capsid. To test this possibility, NP40-treated purified wt HSV-1 virion preparations, and virus capsids (approximately 2×10^9) purified from BHK cells infected with either the wt HSV-1 or the HSV-1amb-UL8 virus were analyzed on the same immunoblot (Fig. 5a, lanes 6 to 11). In contrast to the three types of capsids (types A, B, and C; see Introduction) synthesized by wt HSV-1 in infected cells, viral DNA replication-negative mutants (such as ambUL8) synthesize only type B capsids under nonpermissive growth conditions (Sherman and Bachenheimer, 1988). The location of the UL6 protein within the virion was examined by treatment of wt HSV-1 virions with NP40 followed by centrifugation to separate

envelope proteins from the tegument and capsid structures (see Materials and Methods). As shown in Fig. 5a (lanes 6 and 7), the UL6 protein was exclusively present in the tegument/capsid fraction. Furthermore, all three types of wild type HSV-1 capsids and type B capsids made by the DNA-negative mutant ambUL8 under nonpermissive conditions (i.e., when grown on noncomplementing BHK cells) were found to contain the UL6 protein (Fig. 5a, lanes 8 to 11). The quantity of the UL6 protein found in the capsids was comparable to that in the virions. The slightly faster mobility of the UL6 protein in the tegument/capsid fraction (lane 7) compared to that seen in the virion and capsid preparations is due to local distortion in the gel which was not observed in other experiments. The lower molecular weight protein seen in the capsid and virion preparations probably represents a breakdown product of the UL6 protein (Fig. 5a, ○, lanes 2, 4, and 7 to 11).

To rule out the possibility that UL6 is a tegument (or a nonstructural) contaminant present in the capsid preparations, the presence in the capsids of three "noncapsid" proteins was examined: 65 K DNA binding protein ($65K_{DBP}$), a major protein found predominantly in infected cell nuclei (Schenk *et al.,* 1988), and VP13/14 and VP16, both abundant virion tegument proteins (McLean *et al.,* 1990; Roizman and Furlong, 1974; Szilágyi and Cunningham, 1991; McLauchlan and Rixon, 1992). HSV-1-infected cell extract or approximately 2×10^9 capsids or virions used in Fig. 5a were analyzed by Western immunoblotting using antibodies against the $65K_{DBP}$ (monoclonal Z1F11, Schenk *et al.,* 1988), VP13/14 (rabbit antiserum 94497, McLean *et al.,* 1990), or VP16 (monoclonal LP1, McLean *et al.,* 1982). The antibodies Z1F11 and 94497 failed to detect the $65K_{DBP}$ and VP13/14, respectively, in all the capsid preparations tested (Fig. 5b, lanes 2 to 5; Fig. 5c, lanes 5 to 8). As expected, Z1F11 detected the $65K_{DBP}$ in infected cells (Fig. 5b, lane 1), whereas antibody 94497 recognized VP13/14 in the virions (Fig. 5c, lane 1), and the tegument/capsid fraction of virions of wt HSV-1 (lane 4), but not in the virions of strain R(F)UL46/UL47Δ2 (lane 2). The antibody LP1 identified VP16 in abundance in virions of strain R(F)UL46/UL47Δ2 as well as wt HSV-1 and also in the tegument/capsid and envelope fractions of wt virions (Fig. 5d, lanes 1 to 4). The presence of VP16 in the envelope fraction of virus particles has been observed previously and is thought to be due to partial solubilization of this protein with NP40 (McLaughlan and Rixon, 1992; Visalli and Brandt, 1993). Surprisingly, VP16 was found in the wt HSV-1 capsid preparations (Fig. 5d, lanes 5 to 7), although in vastly reduced quantities as compared to that in the virions (lanes 1 to 4), and it was barely detectable in the type B capsids synthesized in BHK cells by strain ambUL8 (lane 8).

The results of Fig. 5 were verified by fractionation of a capsid gradient. [^{35}S]methionine-labeled nuclear lysate of wt HSV-1-infected cells was subjected to sucrose density gradient centrifugation and fractions were collected as described under Materials and Methods. The radioactivity profile and SDS–PAGE analysis of these fractions identified the position of types A, B, and C capsid bands in the gradient (Figs. 6a, 6b). Thus, fractions 3, 10, and 14 corresponded to peaks consisting of types C, B, and A capsids, respectively (Figs. 6a, 6b). Western immunoblotting of the fractions using antiserum 2C2 revealed that the UL6 protein was predominantly present in fractions 3 to 5 (representing C capsids), 9 to 13 (B capsids), and fraction 14 (A capsids) (Fig. 6c). The UL6 protein was not detected beyond fraction 14, although a small amount was seen at the top of the gradient in fraction 28 (Fig. 6c). The fractions were also analyzed by Western immunoblotting for $65K_{DBP}$, VP13/14, and VP16. Consistent with the results in Figs. 5b and 5c, antibodies Z1F11 and 94497 failed to detect $65K_{DBP}$ and VP13/14, respectively, in fractions 1 to 21, although these proteins were found in minute quantities in fractions 22 to 28 at the top of the gradient (not shown). Interestingly, probing for VP16 with antibody LP1 revealed that this protein was present throughout the gradient (Fig. 6d). These data strongly indicate that the small quantity of VP16 found in the purified capsid preparations in Fig. 5c represent not a tegument contaminant, but residual VP16 present due to this protein smearing through the gradient.

Treatment of purified capsid with guanidine hydrochloride (GuHCl) has been shown to result in selective solubilization of some of its proteins (Newcomb and Brown, 1991; Newcomb *et al.,* 1993). To test the effect on the UL6 protein of GuHCl treatment of capsids, wt HSV-1 type B capsids were extracted with various concentrations of GuHCl and analyzed by SDS–PAGE and Western immunoblot. As shown in Fig. 7a, proteins VP21, VP22a, VP24, and VP26 were removed either completely or nearly so by treatment with 2 *M* GuHCl; capsids were completely solubilized at 3 *M* GuHCl (Fig. 7a). This is in close agreement with the results obtained by Newcomb and Brown (1991). Interestingly, Western immunoblotting of the same GuHCl-treated capsid preparations showed that the UL6 protein could be detected at or below 2 *M* GuHCl without significant loss, if any, in quantity, indicating that this protein is tightly associated with the capsids (Fig. 7b).

The silver-stained protein profiles of the same purified virions and L-particles of wt HSV-1 and capsids of wt HSV-1 and ambUL8 virus used in Fig. 5a were compared. Also included in this experiment were NP40-soluble and -insoluble fractions of virions and L-particles. The protein profiles of virions and L-particles (Fig. 8) were consistent with those described previously (Szilágyi and Cunningham, 1991; McLauchlan and Rixon, 1992). Thus, the HSV glycoproteins (e.g., VP7, VP8, and VP17, better known as

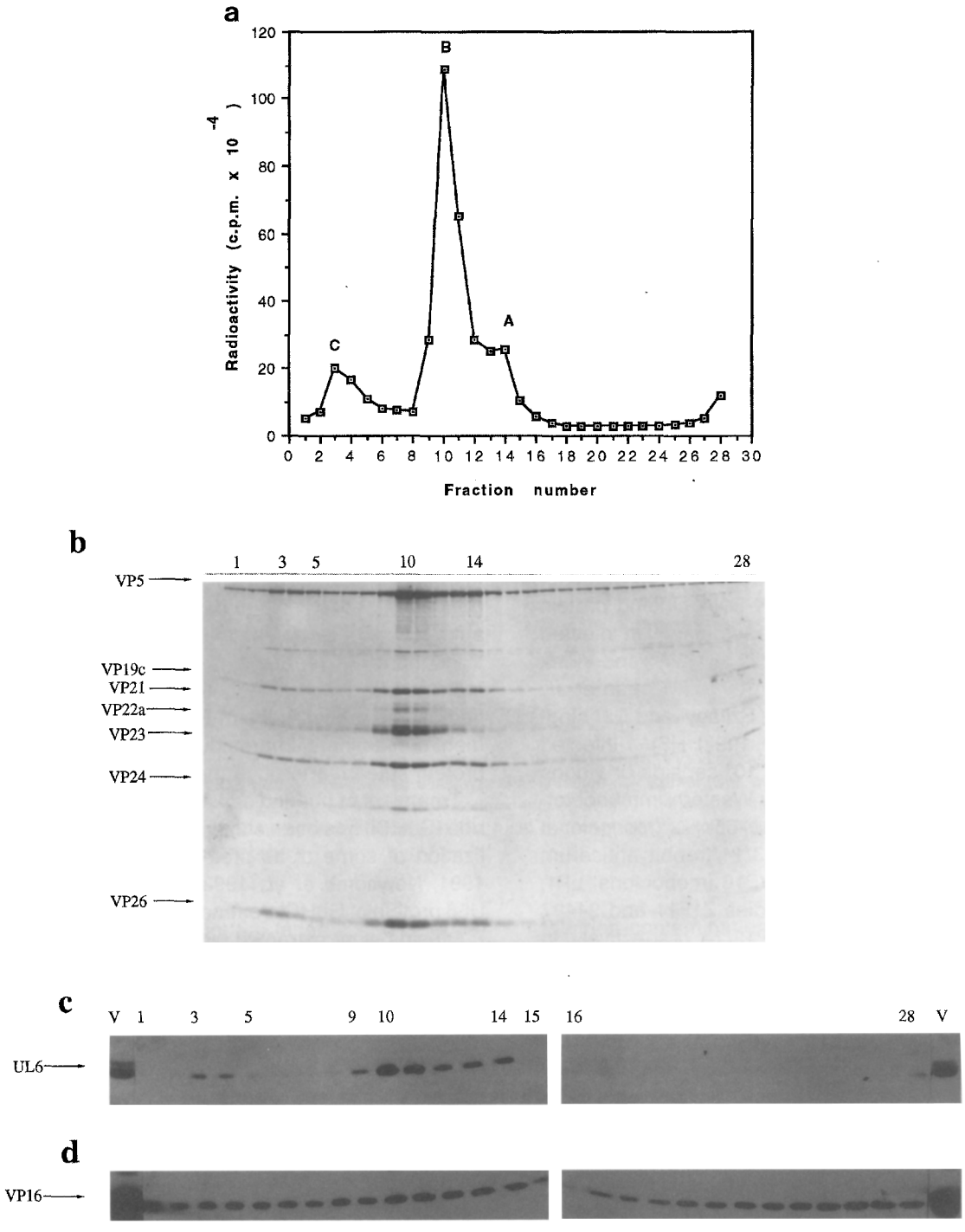

FIG. 6. Sucrose gradient ultracentrifugation of intranuclear capsids. (a) Nuclear lysate of [^{35}S]methionine-labeled wt HSV-1-infected cells was subjected to 20–50% sucrose gradient centrifugation. Twenty-eight fractions were collected, and radioactivity in each was measured as described under Materials and Methods. The positions of A, B, and C capsids are indicated. (b) Polypeptide profiles of fractions 1 to 28 shown in (a) on SDS–PAGE. The positions of capsid proteins are indicated. (c, d) Immunoblot analysis of fractions 1 to 28 shown in (a) using antiserum 2C2 (c) or LP1 (d). V, Ficoll-purified HSV-1 virions.

gB, gC, and gD, respectively) were found exclusively in the NP40-soluble fractions of virions and L-particles (Fig. 8, lanes 3 and 6), whereas the tegument and capsid proteins were found in the insoluble fraction [e.g., VP13/14 (82/81K) in the tegument of both virions and L-particles, VP4 (or Vmw175) in L-particles tegument, and VP5 and VP19C capsid proteins predominantly in the virions; lanes 2 and 5; McLean *et al.*, 1990; Szilágyi and Cunning-

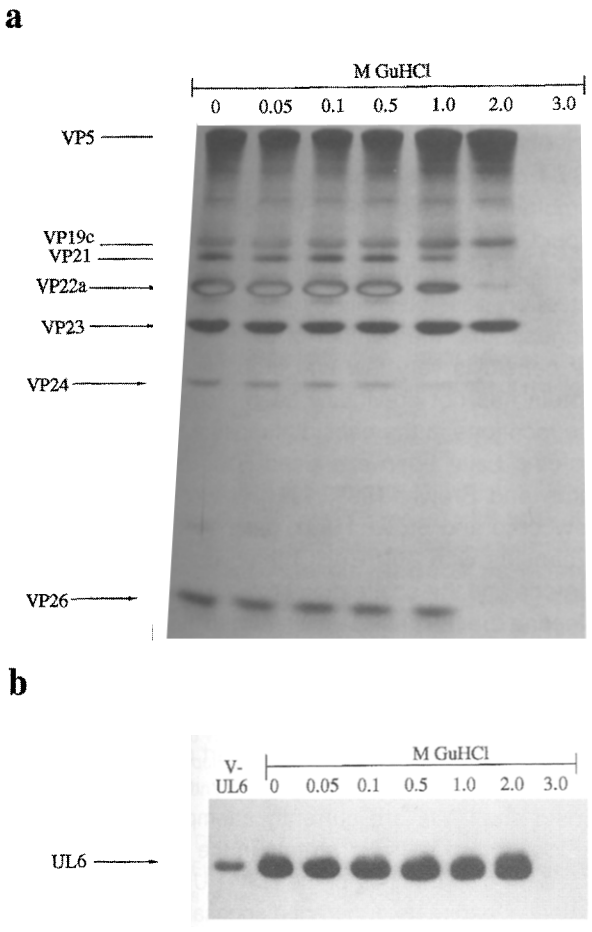

FIG. 7. Analysis of GuHCl-extracted capsids. HSV-1 B capsids were extracted with various concentrations of GuHCl and subjected to (a) 12% SDS–PAGE and (b) immunoblotting using antiserum 2C2. The positions of the capsid proteins in (a) and of UL6 in (b) are indicated. V-UL6, UL6 immunoprecipitated with antiserum 2C2 from V-UL6-infected cells.

ham, 1991; McLauchlan and Rixon, 1992; Zhang and McKnight, 1993)]. The protein profiles of the capsids corresponded to those described previously (see Introduction). VP16 was not present at detectable levels in the capsid preparations (lanes 7 to 10), although small amounts of this protein were found in them by Western immunoblotting (Fig. 5d). The UL6 protein, which migrates slightly ahead of VP13/14 virion proteins (Figs. 5a), was not present at a detectable level either in the virions or in the capsid preparations (Fig. 8, lanes 1 and 7 to 10). A protein band was seen below the VP13/14 bands in virions (Fig. 8, lane 1, ●), but this is unlikely to be UL6 as it was also found in the L-particles (lane 4, ●). Furthermore, this protein appears to be a component of the envelope of both virion and L-particles (Fig. 8, lanes 3 and 6). Interestingly, a protein of approximately 70 kDa unrelated to UL6 was seen in A, B, and C capsids of wt HSV-1 (Fig. 8, lanes 7 to 9, ◀), although it was not present, at least at a detectable level, in the B capsids made under nonpermissive growth conditions by ambUL8 virus (Fig. 8, lane 10). Like UL6, this protein was consistently found not in the L-particles, but in the tegument of the virions (lanes 1 and 2, ▶). One possibility is that it is a degradation product of the major capsid protein, VP5. However, at present we cannot completely rule out that it is a separate protein also associated with the capsids.

DISCUSSION

We describe the transcriptional analysis of the UL6 and UL7 genes of HSV-1. We have also identified and characterized the UL6 gene product of HSV-1 in infected cells. The transcripts of the UL6 and UL7 genes were shown to be synthesized in HSV-1-infected cells, and their 5′ ends were mapped by S1 nuclease and primer extension analysis.

A vaccinia virus recombinant (V-UL6) carrying the HSV-1 UL6 gene synthesized a novel protein with an approximate M_r of 75 kDa in infected cells, which is close to the M_r value of 74.087 kDa predicted from the UL6 ORF (McGeoch *et al.*, 1988). A rabbit polyclonal antiserum

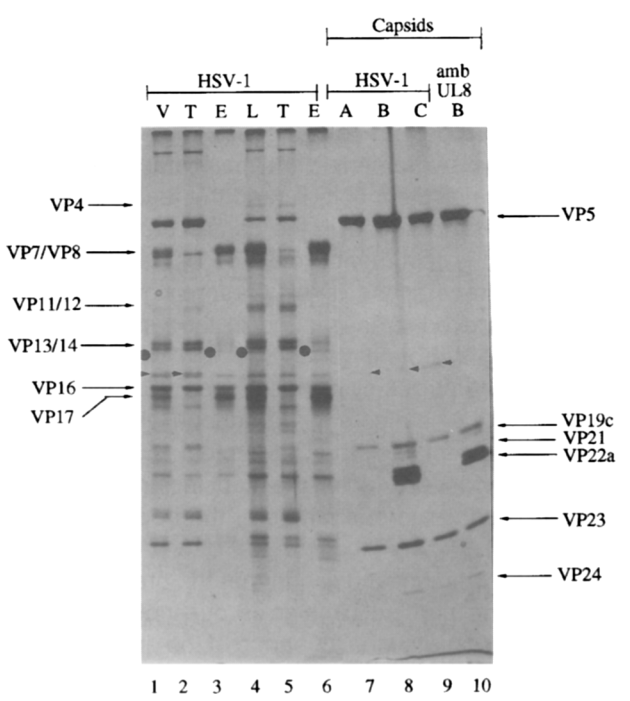

FIG. 8. Polypeptide profiles of virions, L-particles, and capsids of HSV-1. Purified wt HSV-1 virions (V, lane 1), L-particles (L, lane 4) and their envelope (E) and tegument (T) components (lanes 2 and 3 and 5 and 6, respectively), and purified types A, B, and C capsids of wt HSV-1 (lanes 7 to 9) and type B capsids of ambUL8 virus (lane 10) were fractionated on a polyacrylamide gel. The proteins were visualized by silver staining. Some of the known polypeptides of virions and L-particles are indicated on the left, whereas those of the capsids are shown on the right. The relevant bands are highlighted by arrowheads or closed circles.

raised against an *E. coli*-expressed 6×His-UL6 fusion protein precipitated the UL6 protein expressed in V-UL6- and HSV-1-infected cells. The reactivity of the antiserum was more apparent in Western immunoblot analysis where it specifically identified the UL6 gene product in both HSV-1- and V-UL6-infected cells. The UL6 and UL7 mRNAs, and the UL6 protein were expressed suboptimally in the absence of viral DNA replication, indicating that viral DNA synthesis was required to achieve maximum levels of expression. Thus, the UL6 and UL7 genes can be classified as delayed-early or γ-1 genes, although a more detailed analysis of the kinetics of gene expression is required to support this observation.

Analysis of the purified virus particles showed that the UL6 protein was a component of virions but not of L-particles and that it was located exclusively in the tegument/capsid fraction of the virion. As L-particles are known to lack virus capsids and DNA, and they share the tegument and envelope proteins with the virions, it seemed plausible that the UL6 protein was associated with virus capsids. Western immunoblot analysis of the purified types A, B, and C capsids of HSV-1, and the type B capsids synthesized by HSV-1ambUL8 virus under nonpermissive growth conditions showed that this was the case.

The possibility that the UL6 protein is a tegument or a nonstructural contaminant present in the capsid preparations can be strongly discounted for the following reasons. The UL6 protein cofractionated with the viral capsids in a sucrose gradient. Noncapsid viral proteins such as the nuclear protein $65K_{DBP}$, and the tegument protein VP13/14 were not found in the capsid preparations. Although another tegument protein VP16 was detected in the capsid preparations, it was present in minute quantities as compared to that found in the virions, and the amount of this protein varied with the type of capsids used. In the ambUL8-synthesized type B capsids VP16, which, like UL6, is also a γ-1 product (Hall *et al.,* 1982), was barely detectable. The UL6 protein, in contrast, was present in virions and all the capsids in comparable amounts. Furthermore, analysis of the fractionated capsid gradient showed that, unlike UL6, VP16 smeared through the gradient, indicating that the presence of this protein in the purified capsids is due not to tegument contamination, but to the smearing of the gradient by this protein. Finally, the UL6 protein was not removed from the capsids by treatment with 2 *M* GuHCl, although the capsid proteins VP21, VP22a, VP24, and VP26 were selectively removed. Taken together these observations strongly indicate that the UL6 protein is a minor component of HSV-1 capsids.

Apart from the seven known proteins that are thought to make up the capsid (see Introduction), to our knowledge no other virus (or cellular) proteins have so far been implicated with capsid structure. In this report, we unambiguously show that the UL6 gene product of HSV-1 is associated with the virus capsid. The nature of its association with the capsid is not clear. However, it is not required for capsid assembly. Recently, Tatman *et al.* (1994) and Thomsen *et al.* (1994) have demonstrated HSV-1 capsid assembly in insect cells infected with recombinant baculoviruses expressing only the known capsid proteins. Our results show that the UL6 protein occurs in HSV-infected cells in very small amounts, with detection possible only by immunological means. This suggests that the number of UL6 molecules associated per capsid is very low and probably explains why this protein has not previously been recognized in capsids. The locations in the capsid of most of the known capsid proteins have been examined (Trus *et al.,* 1992; Newcomb and Brown, 1989, 1991; Newcomb *et al.,* 1993). Newcomb and Brown (1991) have shown that treatment of capsids with 2 *M* GuHCl removed most, if not all, of pentons and the scaffolding proteins without significantly affecting the hexons. Consistent with these observations, our 2 *M* GuHCl-treated capsids were also found to lack the pentons (data not shown). The fact that the UL6 protein was not removed from the capsids at 2 *M* GuHCl suggests that this protein is not part of the penton subunit. It appears to be more tightly integrated into the capsid shell. We are currently attempting to determine the location of the UL6 protein in the capsid.

The precise function of the HSV-1 UL6 protein remains unclear. Despite its apparent dispensability for capsid assembly, studies with the UL6 *ts* mutant, *ts*F18, show that this protein is required for cleavage and packaging of nascent viral DNA (Sherman and Bachenheimer, 1987). One line of circumstantial evidence that the UL6 gene product may associate with the virus capsid comes from the observations that in temperature-shift experiments comprising *ts*F18-infected cells, only the capsids assembled after the shift to permissive temperature participate in encapsidation of *ts*F18 DNA synthesized at NPT (Sherman and Bachenheimer, 1988). Our results are consistent with this hypothesis, and taken together they strongly indicate that the association of functional UL6 protein with the virus capsid is required for viral DNA cleavage and encapsidation. Furthermore, the results show that the interaction of the UL6 protein with the capsid is not a transient one, as this protein is found in mature virions. Exactly how the UL6 protein mediates its functions is not understood. We have recently constructed transformed cell lines expressing the UL6 protein for isolation of virus carrying site-specific mutations in the UL6 gene. Such mutants will be valuable in elucidating the role of UL6 in viral morphogenesis and in determining the nature of its association with the capsids.

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