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The Product of the U_L31 Gene of Herpes Simplex Virus 1 Is a Nuclear Phosphoprotein Which Partitions with the Nuclear Matrix

YIJAN E. CHANG AND BERNARD ROIZMAN*

The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 East 58th Street, Chicago, Illinois 60637

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The nucleotide sequence of the U_L31 open reading frame is predicted to encode a basic protein with a hydrophilic amino terminus and a nuclear localization signal. To identify its gene product, we constructed a viral genome in which the thymidine kinase gene was inserted between the U_L31 and U_L32 open reading frames. The thymidine kinase gene was then deleted, and in the process, the 5′ terminus of the U_L31 open reading frame was replaced with a 64-bp sequence in frame with the complete, authentic sequence of the U_L31 open reading frame. The inserted sequence encoded a hydrophilic epitope derived from glycoprotein B of human cytomegalovirus and for which a monoclonal antibody is available. We report that in infected cells, the tagged protein localized in and was dispersed throughout the nucleus. Nuclear fractionation studies revealed that the U_L31 protein partitions with the nuclear matrix. The protein is phosphorylated in infected cells maintained in medium containing $^{32}P_i$.

According to current counts, the herpes simplex virus 1 (HSV-1) genome encodes 77 open reading frames. Of this number, 58 are in the unique sequences of the long component (U_L) (2, 19, 22), 13 are in the unique sequences of the short component (U_S) (12, 23), and 3 map in the inverted repeats *ab* and *ca*, which flank U_L and U_S sequences (1, 11), respectively. Approximately half of the genes encoded by the virus are dispensable for growth in cells in culture, and the functions of many genes, whether dispensable for growth in cells in culture or essential, are not known (36). The focus of this report is on one of a stretch of essential genes mapping between 0.4 and 0.5 map units (Fig. 1). At the left terminus of this stretch are a small portion of the U_L29 gene, which encodes the single-stranded-DNA-binding protein ICP8, and the U_L30 gene, which encodes the viral DNA polymerase. Near the right terminus of this stretch are three genes encoding virion proteins; one of these, encoded by U_L34, is one of the few nonglycosylated, essential membrane phosphoproteins (33, 34). Little is known about the products of the other genes. This report concerns the protein product of the U_L31 open reading frame, which is predicted to contain 306 amino acids. The U_L31 transcript has been mapped by Holland et al. (17), and its synthesis has been shown to be restricted in the presence of 1-β-D-arabinofuranosylthymine, an inhibitor of viral DNA synthesis. Attempts to delete the U_L31 open reading frame have not been successful (1a, 9a). In this report, we provide the first characterization of the gene product. The results presented here indicate that the gene product is a nuclear phosphoprotein which shares properties with proteins known to form the nuclear matrix.

MATERIALS AND METHODS

Cells and viruses. HSV-1 strain F [HSV-1(F)] is the prototype HSV-1 strain used in this laboratory. HSV-1(F)Δ305 was derived from HSV-1(F) by deleting approximately 500 bp from the thymidine kinase (*tk*) gene (30) and is the parent strain for all of the viral recombinants made in this study. The Vero and HEp-2 cell lines were obtained from American Type Culture Collection. The human 143TK$^-$ cell line was obtained from Carlo Croce, whereas the rabbit skin cell line was obtained from John McLaren. All cell lines were grown in Dulbecco's modified Eagle medium supplemented with either 5% newborn calf serum (Vero, HEp-2, and rabbit skin cells) or 5% fetal calf serum and 40 μg of 5′-bromo-2-deoxyuridine per ml (143TK$^-$ cells). Infected cells were maintained in 199V medium, consisting of mixture 199 supplemented with 1% calf serum, unless indicated otherwise.

Buffers, solutions, and antibodies. CSK buffer consisted of 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EDTA, 4 mM vanadyl riboside complex, 1.2 mM phenylmethylsulfonyl fluoride, and 0.5% (vol/vol) Triton X-100. RSB buffer consisted of 10 mM Tris-HCl, (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 4 mM vanadyl riboside complex, 1.2 mM phenylmethylsulfonyl fluoride, 1% (vol/vol) Tween 40, and 0.5% (vol/vol) sodium deoxycholate. Monoclonal antibody CH-28, which is reactive with the cytomegalovirus (CMV) glycoprotein B (gB) epitope described elsewhere (28), was kindly provided by Lenore Pereira.

Enzymes. Restriction endonucleases were from New England Biolabs, T4 DNA polymerase and RNase-free DNase I were from Boehringer Mannheim Biochemicals, and T4 DNA ligase was from U.S. Biochemical.

Construction of plasmids. All plasmids derived in this study were made by standard procedures described elsewhere (37). The structures of plasmids pRB4601, pRB4602, pRB4603, and pRB4606 are shown in Fig. 2. The DNA sequence encoding the CMV epitope used for tagging the DNA is 5′-AAAAGGG ACAGAAGCCCAACCTGCTAGACCGACTGCGACACC GCAAAAACGGGTACCGACATGG-3′.

Construction of recombinant viruses. The recombinant viruses generated for these studies were constructed by double homologous recombination as described by Post and Roizman (31). The selectable marker was the HSV-1(F) *tk* gene. To insert the selectable marker, the α27-*tk* was cloned into the

* Corresponding author.

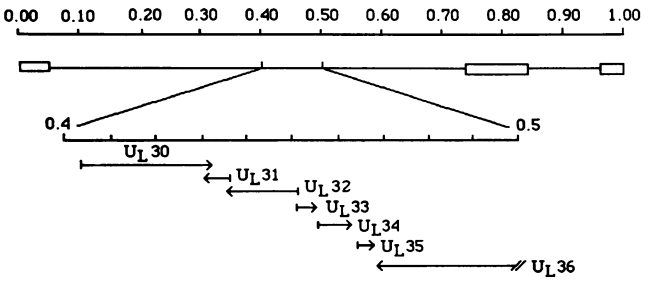

FIG. 1. Schematic representation of HSV-1 genome and the arrangement of open reading frames in HSV-1 genome between 0.4 to 0.5 map units. In the second line, the rectangles represent the inverted repeats flanking the unique sequences of the long and short components. The small portion of the U_L29 open reading frame maps at the left end of U_L30. The arrows indicate the direction of translation of each open reading frame.

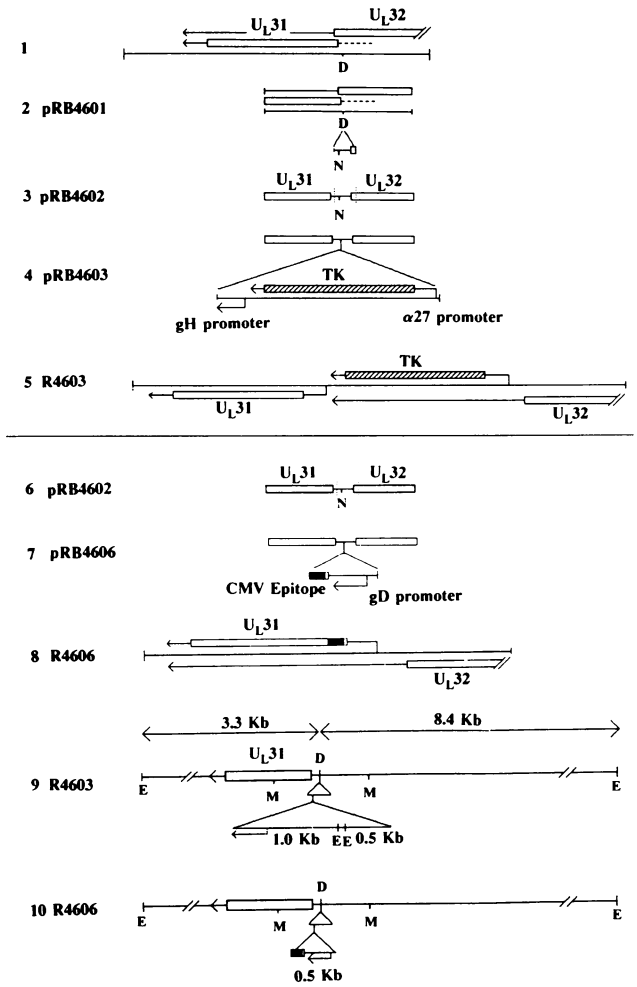

FIG. 2. Diagrammatic representation of the construction of plasmids and recombinant viruses R4603 and R4606. Line 1, the coding domains (rectangles) and 3′-coterminal transcripts of the U_L31 and U_L32 open reading frames. The coding sequences of U_L31 and U_L32 overlap by 5 bp. Line 2, sequence arrangement of the *Msc*I fragment cloned as plasmid pRB4601. This fragment contains the junctions of U_L31 and U_L32 coding domains and extends approximately 500 bp in each direction. The triangle represents the 30-bp oligomer 5′-GTGC CATGGTGTCATACGTAGGTACACAGG-3′, shown antisense to the U_L31 gene, and its complement, which were annealed and inserted into the *Dra*III site as shown in line 3. The oligomer (i) preserved the *Dra*III site cohesive ends, (ii) contained the remaining coding sequence of U_L32 from the *Dra*III site to its stop codon (shown as a small open box) followed by a unique *Nco*I site, and (iii) replaced the initiating methionine codon of the U_L31 gene residing within the 3′-end sequence of U_L32 from ATG to ACG without changing the amino acid sequence of U_L32 (the corresponding codon of the U_L32 gene was changed from TAT to TAC). Line 3, the resulting plasmid, pRB4602, physically separated the coding sequences of U_L31 and U_L32 open reading frames. The inserted oligomer is shown between two dotted lines. Line 4, schematic representation of pRB4603 derived from pRB4602 by insertion of the α27-*tk* gene of HSV-1(F) (30) at the *Nco*I site between the U_L31 and U_L32 genes. This chimeric fragment contained at one end the promoter of the α27 gene fused to the 5′ transcribed noncoding and coding domains of the *tk* gene and at the other end the promoter of the gH gene. Line 5, gene organization of the recombinant virus R4603 obtained by recombination of pRB4603 with HSV-1(F)Δ305 DNAs through homologous flanking sequences. Lines 6 and 7, schematic representation of pRB4606 derived from pRB4602 by insertion at the *Nco*I site of a fragment containing the HSV-1(F) gD promoter and 30 codons consisting of 2 codons derived from the gD gene, 7 additional codons encoded by a linker sequence, and 21 codons encoded by the 64-bp CMV epitope as described in Materials and Methods. The initiating methionine codon of the gD gene (open small rectangle) preceded the sequence encoding the epitope (filled rectangle) and became the initiating codon of the mutated U_L31 coding domain. Line 8, schematic representation of the recombinant virus pRB4606 obtained by recombination between pRB4606 and R4603 viral DNAs through homologous flanking sequences. Lines 9 and 10, genomic organization of the *Eco*RV E fragment of viruses R4603 and R4606, respectively. Open, filled, and hatched rectangles, coding sequences; arrows, transcripts. Restriction sites: D, *Dra*III; N, *Nco*I; E, *Eco*RV; M, *Msc*I.

targeted site. The plasmid containing the inserted *tk* gene was cotransfected with intact tk^- HSV-1(F)Δ305 viral DNA into rabbit skin cells. The progeny of the transfection was then plated on 143TK$^-$ cells in hypoxanthine-aminopterin-thymidine medium to select for tk^+ virus. The virus growing under these conditions was plaque purified and tested for the presence of the *tk* gene at the targeted site as described below. To delete the *tk* gene along with flanking sequences and to substitute them with the desired sequence, the appropriate plasmid was cotransfected into rabbit skin cells with the intact viral DNA from tk^+ recombinant made as described above. The progeny of transfection was plated on 143TK$^-$ cells in the presence of bromodeoxyuridine to select for tk^- virus. The virus replicating under these conditions was plaque purified and tested for the desired sequence arrangement as described below.

Southern blot analyses. Viral DNAs extracted from cytoplasmic virions of infected Vero cells were purified, digested with *Eco*RV, electrophoretically separated, and transferred to a nitrocellulose membrane (Schleicher & Schuell) as described elsewhere (24, 39). The hybridization probes were labeled with the DuPont nick translation kit according to the manufacturer's instructions and purified through a Sephadex G-50 (Pharmacia) spin column.

Immunoblot analysis. Infected cells were harvested in 1% deoxycholate–1% Nonidet P-40–0.01 mM tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)–0.01 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) in PBS(A) (phosphate-buffered saline [PBS] supplemented with 0.1 mM $CaCl_2$ and 0.05 mM $MgCl_2$), sonicated briefly, solubilized in sodium dodecyl sulfate, electrophoretically separated in denaturing

polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide, electrically transferred to a nitrocellulose membrane, and reacted first with monoclonal antibody CH-28 to the CMV epitope and then with goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Bio-Rad). The substrate was contained in a kit obtained from Promega. Molecular weight standards were from Bio-Rad or Promega.

Immunofluorescence. HEp-2 cells were grown in chamber slides (Nunc Inc.) to 80% confluence and exposed to 50 PFU of the appropriate virus per cell. The effective multiplicity of infection in these cultures was approximately 5 to 10 PFU per cell. The cultures were removed from the chambers at various times between 8 and 18 h after infection, rinsed in PBS(A) once, and fixed in chilled methanol at −70°C for 20 min. The cells were rehydrated and blocked with PBS containing 0.1% bovine serum albumin at room temperature for 20 min, stained with the monoclonal antibody (CH-28) against the CMV epitope for 2 h at room temperature, extensively rinsed with PBS(A), and counterstained with rabbit anti-mouse immunoglobulin G conjugated to fluorescein (Calbiochem) for 1 h. The slides were washed in PBS(A), air dried, mounted in buffered glycerol, and examined with a fluorescence microscope.

Phosphorylation studies. HEp-2 cells grown in 25-cm^2 flasks were exposed to 10 PFU of the appropriate virus per cell. At 14 h after infection, the cells were rinsed and overlaid with phosphate-free medium for 1 h. At that time, 100 to 200 μCi of $^{32}P_i$ (200 μCi/μmol; Amersham) was added to the medium; the cells were incubated for an additional 4 h and then harvested, solubilized, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and subjected to autoradiography and immunoblotting.

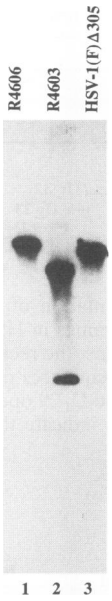

FIG. 3. Autoradiographic images of *Eco*RV digests of HSV-1(F)Δ305, R4603, and R4606 DNAs electrophoretically separated on a 1% agarose gel, transferred to a nitrocellulose sheet, and probed with nick-translated ^{32}P-labeled pRB4601 DNA shown schematically in Fig. 2. The probe hybridized to the *Eco*RV E fragment of HSV-1(F)Δ305 DNA (lane 3) and to two bands in R4603 DNA due to additional *Eco*RV sites in the *tk* gene insert (lane 2) and to one more slowly migrating *Eco*RV band in R4606 inasmuch as the *tk* gene was replaced with a fragment approximately 500 bp in length.

RESULTS

Construction of R4603 and R4606 viruses. The objective of these studies was to tag the U_L31 protein at its amino terminus with a sequence encoding the CMV epitope for which a monoclonal antibody is available. As shown diagrammatically in Fig. 2, line 1, the U_L31 and U_L32 mRNAs are 3′ coterminal and the sequence encoding the carboxyl terminus of the U_L32 protein predicts a 5-bp overlap with the sequence encoding the amino terminus of the U_L31 protein. To achieve this objective, a 30-bp oligonucleotide was synthesized and inserted into the *Dra*III restriction endonuclease site of the *Msc*I HSV-1 fragment cloned in pRB4601 (Fig. 2, line 2). This oligonucleotide contained (i) the remaining coding sequence of the U_L32 gene from the *Dra*III site to its stop codon, (ii) a unique *Nco*I site, and (iii) a replacement of the original start codon of U_L31 residing within the 3′-end sequence of U_L32. Specifically, this codon was mutated from ATG to ACG so as not to change the amino acid sequence of U_L32 (Fig. 2, line 3). The resulting plasmid, pRB4602, physically separated the coding sequences of U_L31 and U_L32. This plasmid was used for the construction of two viruses, R4603 and R4606.

To construct R4603 (Fig. 2, line 5), the α27-*tk* chimeric gene was inserted into the *Nco*I site of pRB4602 such that the gH promoter enabled the transcription of the U_L31 gene (Fig. 2, line 4). The procedure used for selection of this tk^+ recombinant virus, designated R4603, was as described in Materials and Methods. To construct the R4606 recombinant, a fragment containing the sequence −372 to +92 relative to the transcription initiation site at +1 of HSV-1(F) gD (containing the 5′ nontranscribed and 5′ transcribed noncoding sequences and first two codons) and a 64-bp sequence encoding the CMV epitope was inserted into the *Nco*I site of pRB4602 to yield plasmid pRB4606 (Fig. 2, line 7). In this plasmid, the initiation of translation started at the first codon of gD. The additional amino acid sequence obtained by the tagged U_L31 protein included the first two amino acid residues of gD followed by sequence derived from CMV epitope and linker sequence, 5′ to the authentic U_L31 coding sequence. Transfection of rabbit skin cells with pRB4606 plasmid DNA and intact R4603 viral DNAs followed by tk^- selection as described in Materials and Methods yielded the R4606 recombinant virus.

Figure 3 shows the results of studies designed to verify the sequence arrangement of the R4603 and R4606 recombinant viruses. The parent HSV-1(F)Δ305, R4603, and R4606 viral DNAs were digested with *Eco*RV, electrophoretically separated on a 1% agarose gel, transferred to a nitrocellulose sheet, and hybridized to the nick-translated *Msc*I fragment cloned as pRB4601 (Fig. 2, line 2). The autoradiographic images shown in Fig. 3 indicate that the positions of the *Eco*RV bands hybridizing with this probe correspond to predicted sizes. In the parent virus (Fig. 3, lane 3), the probe hybridized with the *Eco*RV E fragment. The α27-*tk* sequence inserted into R4603 contains two closely positioned *Eco*RV cleavage sites; therefore, two bands hybridized with the probe sequence. In R4606 DNA, the *tk* gene was replaced with sequence encoding the CMV epitope and the HSV-1 gD promoter. The new *Eco*RV E fragment was approximately 500 bp larger than the authentic *Eco*RV E fragment in HSV-1(F)Δ305 DNA.

Studies of the CMV-tagged U_L31 protein by immunoblotting. Several plaque-purified isolates of the R4606 recombinant virus were tested for the capacity to encode a protein reactive with the CMV antibody. In this series of experiments, replicate HEp-2 cell cultures were infected with the wild-type virus and each of isolates 4, 5, and 6. The cells were harvested 18 h after infection, solubilized, electrophoretically separated

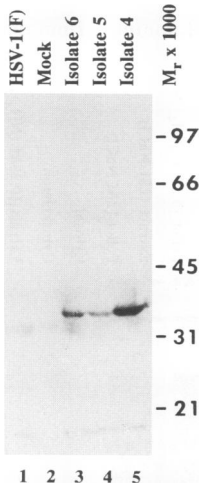

FIG. 4. Photograph of proteins from mock-infected or infected cells electrophoretically separated in a denaturing 12% polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and reacted with monoclonal antibody CH-28, which recognizes specifically the CMV epitope. Protein molecular weight standards (Bio-Rad), whose positions are shown on the right in thousands, were phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), bovine carbonic anhydrase (31,000), and trypsin inhibitor (21,500). Isolate 4 (lane 5) was amplified and designated R4606.

in denaturing gels, and reacted with the anti-CMV mouse monoclonal antibody as described in Materials and Methods. In the experiments shown in Fig. 4, the antibody reacted with a protein band whose electrophoretic mobility corresponds to a protein approximately 37,000 in apparent molecular weight (Fig. 4, lanes 3 to 5). The antibody did not react with electrophoretically separated lysates of mock-infected cells or of cells infected with wild-type virus (Fig. 4, lanes 2 and 1).

Localization of the U_L31 protein in infected cells. Infected cells were fixed and stained with antibody at 8, 13, and 18 h after infection. Photomicrographs of HEp-2 cells fixed and stained with monoclonal antibody to the CMV epitope at 13 and 18 h postinfection, respectively, are shown in Fig. 5. The results were as follows. At 13 h postinfection, the antibody reacted with very fine, punctate forms accumulating in nuclei of infected cells. At 18 h postinfection, the antigen reacting with the anti-CMV monoclonal antibody was intense and dispersed throughout the nucleus. The antibody did not react with uninfected cells or with cells infected with wild-type virus (not shown). In another experiment (not shown), the tagged U_L31 protein could be detected in cells as early as 8 h after infection with the R4606 recombinant virus. This is not surprising inasmuch as the expression of the tagged U_L31 protein was governed by the promoter of the gD gene.

Cofractionation of the U_L31 protein with the nuclear matrix proteins in infected cells. Preliminary studies showed that U_L31 protein was relatively insoluble in nonionic detergent extracts of intact or sonicated nuclei. The distribution of the U_L31 protein in the nuclei of infected cells raised the possibility that the protein is associated with the nuclear matrix fraction. To test this hypothesis, infected cells were fractionated as described by Penman et al. (9, 16) and shown diagrammatically in Fig. 6. Briefly, HEp-2 cells were exposed to 10 PFU of the appropriate virus per cell. The cells were labeled from the time of infection with [^{35}S]methionine (>1,000 mCi/μmol; Amersham) to a final concentration of 10 μCi/ml of medium. At 15 h postinfection, the cell monolayers were

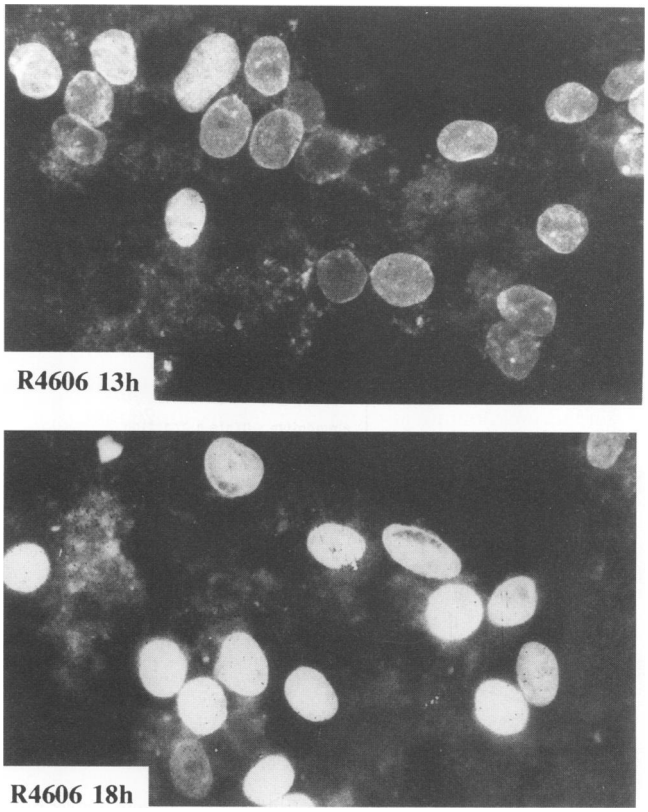

FIG. 5. Photographs of HEp-2 cells grown in chamber slides and exposed to approximately 50 PFU of R4606 for 13 and 18 h. The cells were fixed and stained with monoclonal antibody CH-28 as described in Materials and Methods.

chilled to 4°C, rinsed once with PBS, and extracted with CSK buffer to remove the cytosolic proteins. In the next step, the cytoskeleton was extracted with RSB buffer at 4°C for 5 min. Following these procedures, the nuclei and intermediate filaments remained attached to the solid substrate and were removed by gentle scraping. Next, chromatin was digested with 20 to 40 U of RNase-free DNase I at room temperature for 1 h in digestion buffer consisting of CSK buffer but with only 50 mM NaCl. Chromatin was removed by subsequent extraction with 0.25 M $(NH_4)_2SO_4$. The pellet containing the nuclear matrix fraction obtained by low-speed centrifugation was then extracted with 2 M NaCl. The insoluble material expected to contain the core filaments of the nuclear matrix was pelleted by low-speed centrifugation. The salient features of the results shown in Fig. 7 were as follows. (i) A large number of polypeptide species partitioning with the nuclear matrix were labeled after infection (lanes 4 and 6). The distinctive electrophoretic profile shown in this figure was reproducible. (ii) The monoclonal antibody to the CMV epitope reacted with a prominent protein band present predominantly in the pellets obtained after the ammonium sulfate and 2 M NaCl extractions, indicating that the U_L31 protein partitioned with the nuclear matrix (Fig. 7, lanes 4, 6, 10, and 12).

The CMV-tagged U_L31 protein is phosphorylated in infected cells. The amino acid sequence of the U_L31 protein predicted several potential phosphorylation sites for casein kinase II, cyclic AMP (cAMP)-dependent kinase, and protein kinase C. In preliminary experiments, HEp-2 cell proteins labeled with

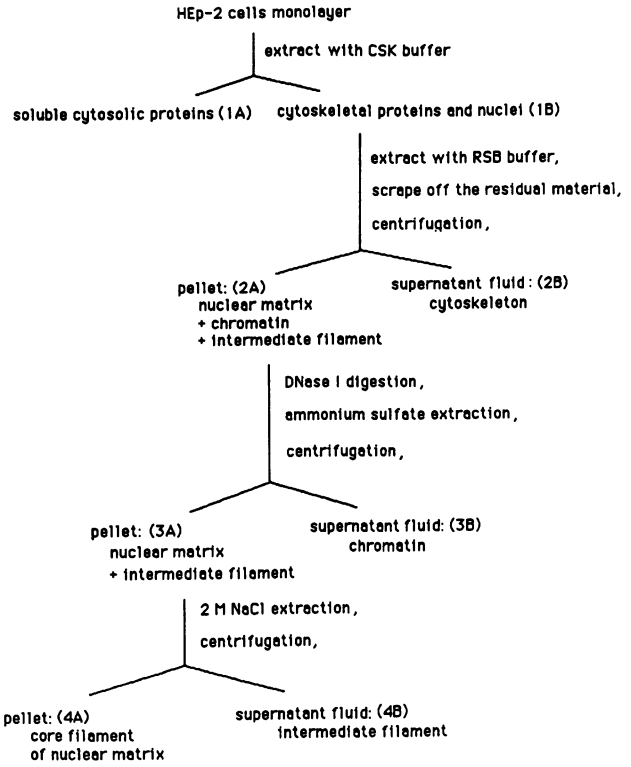

FIG. 6. Schematic diagram of the isolation of nuclear matrix proteins as described by Penman et al. (9, 16). The buffers are described in Materials and Methods.

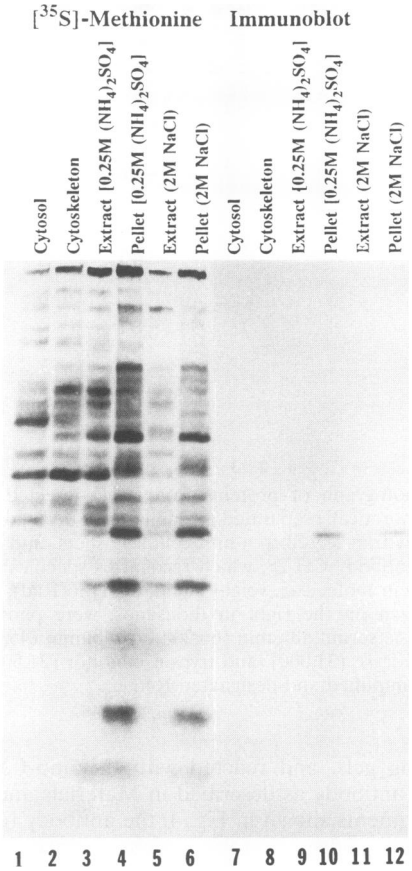

FIG. 7. Autoradiographic images and photograph of infected cell proteins fractionated according to the protocol shown in Fig. 6. HEp-2 cells were exposed to 10 PFU of R4606 per cell and labeled with [^{35}S]methionine immediately after infection. At 15 h postinfection, the cells were harvested, fractionated as described for Fig. 6, electrophoretically separated on denaturing polyacrylamide gels, and reacted with anti-CMV monoclonal antibody CH-28. Lanes 1 to 6 are autoradiographic images of the [^{35}S]methionine-labeled proteins; lanes 7 to 12 show reactivities of the separated polypeptides with the anti-CMV monoclonal antibody. Lanes 1 and 7 contain fraction 1A (according to the procedure shown in Fig. 6), lanes 2 and 8 contain fraction 2B, lanes 3 and 9 contain fraction 3B, lanes 4 and 10 contain fraction 3A, lanes 5 and 11 contain fraction 4B, and lanes 6 and 12 contain fraction 4A.

$^{32}P_i$ were solubilized, electrophoretically separated in denaturing polyacrylamide gels, transferred electrically to a nitrocellulose sheet, and reacted with the anti-CMV antibody as described in Materials and Methods. Although the monoclonal antibody reacted with a protein band which incorporated ^{32}P, a labeled band was also apparent in mock-infected cells at the same position (data not shown). To separate the U_L31 gene product from the host phosphoprotein, we took advantage of the insolubility of the U_L31 protein. In the second series of experiments, labeled cell extracts were sonicated and centrifuged at 12,000 rpm for 5 min. The electrophoretically separated proteins from the pellets obtained from cells mock infected or infected with R4606 or HSV-1(F) were transferred to a nitrocellulose sheet, reacted with antibody to the CMV epitope, and subjected to autoradiography. The results shown in Fig. 8 indicate that a unique band identified by the arrow and present in the pellet of cells infected with R4606 (lane 2) was labeled with ^{32}P and reacted with anti-CMV antibodies. This band was not present in lysates of HSV-1(F), as could be expected since the U_L31 protein made in cells infected with the R4606 recombinant had an additional 30 amino acids. The corresponding HSV-1(F) phosphoprotein could be expected to migrate faster than that made in R4606-infected cells and in this instance may comigrate with another phosphoprotein also present in lysates of R4606-infected cells and migrating just behind the 30,000-molecular-weight marker.

A limited number of HSV-1 proteins in addition to U_L31 are copurified with the nuclear matrix. The objective of this series of experiments was to evaluate the extent to which the nuclear matrix becomes altered after infection. Specifically, uninfected HEp-2 cells were labeled with [^{35}S]methionine and either fractionated immediately thereafter (Fig. 9, lanes 1 to 4) or infected with R4606 and incubated in label-free medium for an additional 18 h (Fig. 9, lanes 5 to 8). In addition, the HEp-2 cells were infected with R4606 and labeled at 6 h postinfection with [^{35}S]methionine until 18 h postinfection (Fig. 9, lanes 9 to 12). The results were as follows.

(i) The nuclear matrix of infected cells contained most of the abundant proteins present in the nuclear matrix of uninfected cells labeled throughout a 24-h period (Fig. 9; compare lanes 1 to 4 with lanes 5 to 8), suggesting that these proteins turn over very slowly after infection. The exceptions are a few slowly migrating proteins which exhibited a reduced intensity of labeling with [^{35}S]methionine after infection and 18 h of incubation in label-free medium. (ii) The nuclear matrix of cells labeled between 6 and 18 h postinfection contained, in addition to some of the polypeptide bands found in uninfected cells, novel bands which could be of viral origin (lanes 9 to 12, arrowheads). One of these bands (Fig. 9A, lanes 10 and 12)

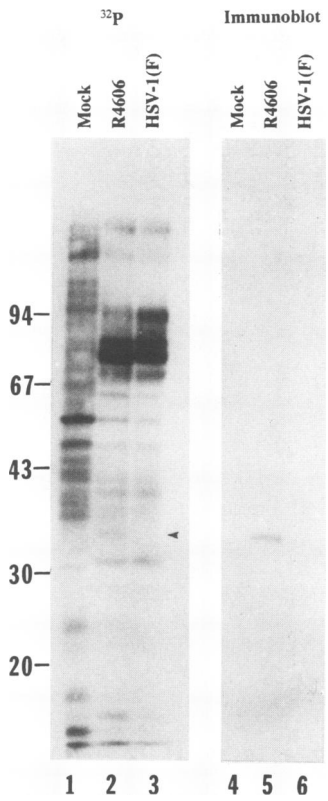

FIG. 8. Autoradiographic images (lanes 1 to 3) and photograph of immunoblot (lanes 4 to 6) of proteins from precipitates of mock-infected or infected HEp-2 cell lysates labeled with ^{32}P as described in Materials and Methods. Proteins were electrophoretically separated on denaturing polyacrylamide gels, transferred to a nitrocellulose sheet, and reacted with the anti-CMV monoclonal antibody CH-28 (lanes 4 to 6) and subjected to autoradiography (lanes 1 to 3). The arrowhead indicates the position of a ^{32}P-labeled band which corresponds in position to the band which reacted with anti-CMV monoclonal antibody CH-28. Molecular weight markers (Promega), whose positions are shown on the left in thousands, were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

comigrated with the polypeptide band which reacted with the anti-CMV monoclonal antibody (Fig. 9B, lanes 10 and 12), indicating that it contained the CMV-tagged U_L31 protein.

We conclude the following. (i) There was no significant turnover of cellular proteins contained in the nuclear matrix fraction. We should note that subtle changes would not be detected by one-dimensional electrophoresis in denaturing polyacrylamide gels, and the kinds of changes in the morphology of nuclear chromatin and the redistribution of ribonuclear protein antigens reported by Martin et al. (21) in HSV-1-infected cells may not be demonstrable by the procedures used in our studies. (ii) The nuclear matrix extracted from infected cells contained infected cell proteins in addition to the U_L31 protein.

Several infected cell proteins which cofractionated with the nuclear matrix comigrate with nucleocapsid proteins. Maturation of the HSV-1 nucleocapsid takes place in the nuclei of infected cells. Early electron microscopic studies showed that empty nucleocapsids were enriched in the nuclear matrix fraction, whereas full nucleocapsids were very rarely found in the same fraction (8). This observation suggests that the nuclear matrix may facilitate capsid maturation at a late stage. To determine whether capsid proteins were associated with nuclear matrix, HEp-2 cells were infected with HSV-1(F) at a multiplicity of infection of 10 and labeled with [^{35}S]methionine (6 μCi/ml) from 6 to 24 h postinfection. Nucleocapsids were isolated 24 h postinfection from a 10 to 40% (wt/wt) sucrose gradient as described by Gibson and Roizman (13, 14), solubilized, and subjected to electrophoresis in denaturing polyacrylamide gels along with nuclear matrix fractions isolated from HSV-1(F)- or R4606-infected HEp-2 cells (Fig. 10). Lane 3 of Figure 10 was the same sample as that shown in lane 12 of Fig. 9. The sample in lane 1 of Fig. 10 was isolated the same way as that shown in lane 3 but from HEp-2 cells infected with HSV-1(F). The protein compositions of these two samples were identical except for two protein species with molecular weights of about 34,000 and 37,000 (arrowheads in Fig. 10). These two proteins on the nitrocellulose blot reacted with polyclonal antibodies to U_L31 (9a, work in progress) and the CMV monoclonal antibody, respectively (data not shown). Capsid proteins were numbered on the right according to Gibson and Roizman (13, 14). It is noteworthy that the patterns obtained with [^{35}S]methionine label differ significantly from those obtained with [^{14}C]leucine, [^{14}C]isoleucine, and [^{14}C]valine described by Gibson and Roizman (13, 14). In contrast to the results reported previously, in the patterns shown in Fig. 10, VP21 is more prominent, whereas VP23 and VP24 were labeled with least intensity. We conclude from this experiment that a significant fraction of the infected cell proteins which fractionated with the nuclear matrix also comigrated with capsid proteins.

DISCUSSION

In this paper, we report the properties of one of a series of apparently essential gene products mapping in the middle of the L component of HSV-1 DNA. The salient features of the experimental design and results are as follows.

(i) For a preliminary characterization of the protein, we inserted a CMV epitope into the coding domain of the gene. The site of insertion was based on the predicted structure of the protein. The amino acid sequence of U_L31 of HSV-1(17) published by McGeoch et al. (22) predicted a highly basic protein with molecular weight of 34,000. Kyte-Doolittle hydropathic analysis predicts a hydrophilic amino terminus of the protein. The rationale of inserting a hydrophilic CMV epitope at the amino terminus was therefore based on the similarity of their hydropathic properties and the expectation that this insertion would not significantly distort the structure or alter the function of the protein. The hypothesis that the CMV epitope does not grossly alter the properties of the protein is supported by the following observations. The R4603 recombinant virus shown in Fig. 2, line 5, was constructed for two purposes, i.e., to substitute in place of the *tk* gene the recombinant U_L31 sequence containing the epitope and, eventually, to delete the *tk* gene along with most of the U_L31 open reading frame. Although, as noted in the introduction, attempts to delete the gene were not successful, we cannot conclude that the U_L31 gene is essential without additional tests. However, on the basis of previous experience in this laboratory, the probability that the gene is dispensable is low. In contrast, the recombinant R4606 was readily obtained, and furthermore, to date we have not been able to differentiate between the growth properties of HSV-1(F) and R4606 in any of the cell lines tested. Further evidence supporting the conclusion that the function of the U_L31 protein was not significantly impaired by the insertion of the epitope is based

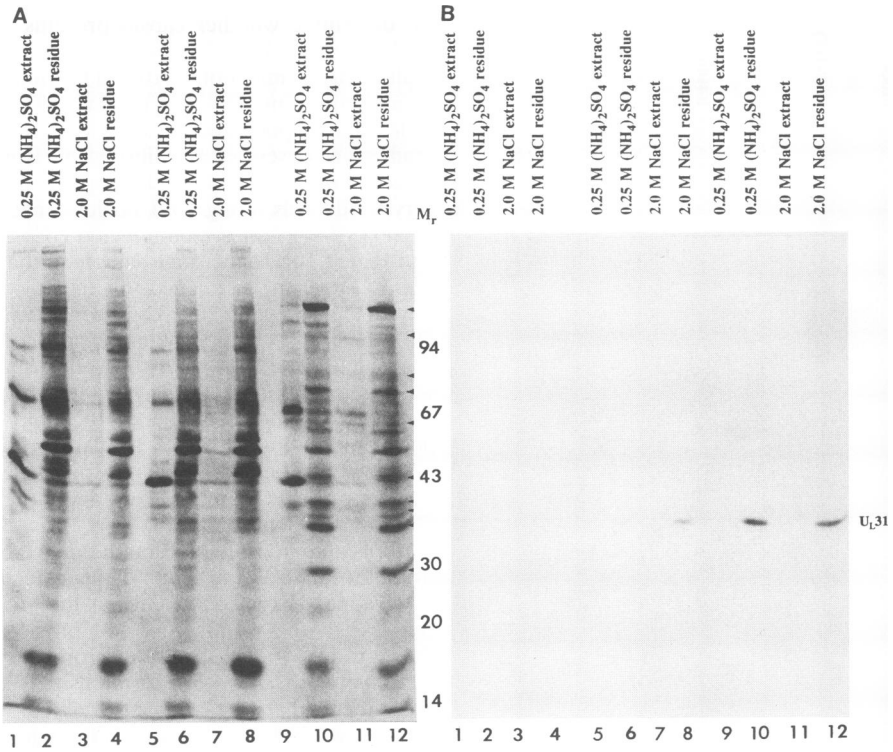

FIG. 9. Autoradiographic images and photographs of proteins partitioning with nuclear matrix proteins extracted from cells mock infected or infected with R4606 recombinant virus. (A) HEp-2 cells were labeled with [^{35}S]methionine for 24 h and either harvested immediately (lanes 1 to 4) or infected with R4606 (10 PFU per cell) and maintained in medium containing unlabeled methionine for 18 h (lanes 5 to 8); alternatively, HEp-2 cells were infected with R4606 (10 PFU per cell) and incubated in medium containing [^{35}S]methionine from 6 to 18 h postinfection (lanes 9 to 12). The cells were harvested after labeling of mock-infected cells (lanes 1 to 4) or at 18 h postinfection. The protocol for extraction of the nuclear matrix proteins is shown in Fig. 6. The fractionated proteins were solubilized, subjected to electrophoresis in denaturing gels, and transferred electrically to a nitrocellulose sheet. Lanes 1, 5, and 9 contain fraction 3B of Fig. 6, lanes 2, 6, and 10 contain fraction 3A, lanes 3, 7, and 11 contain fraction 4B, and lanes 4, 8, and 12 contain fraction 4A. Molecular weight markers (Promega), whose positions are shown between panels A and B in thousands, were those used for Fig. 8 plus α-lactalbumin (14,400). Arrowheads point to abundant infected cell proteins which were not detected in lysates of mock-infected cells. (B) The same blot was probed with the anti-CMV monoclonal antibody CH-28. The position of U_L31 is indicated.

on current studies in which we tested early bleedings from a rabbit immunized with a U_L31 fusion protein (9a). There was only one protein species, with a molecular weight of about 37,000, from R4606-infected cell lysate reacting with this serum on immunoblot (data not shown). The immunofluorescence pattern observed with that serum could not be differentiated from those shown in Fig. 5 (data not shown).

(ii) The coding sequence of U_L31 predicts several casein kinase II, cAMP-dependent protein kinase, and protein kinase C phosphorylation consensus sites. In this report, we show that the chimeric U_L31 protein was phosphorylated. The phosphorylated amino acids, their locations, and their functions remain to be determined.

(iii) The studies reported here suggest that U_L31 encodes an abundant nuclear protein consistent with the predicted nuclear localization signal (Lys-Glu-Arg-Arg-Arg) (32) located 18 amino acids past the amino terminus. We have not detected evidence of cytoplasmic localization even late in infection. The nuclear distribution was punctate; the U_L31 protein was dispersed throughout the nucleus except for the nucleoli at early times after infection. The protein was insoluble in the buffers tested, and its behavior is similar to that of nuclear matrix proteins.

(iv) The technical definition of nuclear matrix is the matter remaining insoluble after extraction with high-salt buffers. The procedures followed in our studies was that described by Penman and colleagues (9, 16). On the basis of its reactivity with the CMV antibody, the quantity of U_L31 chimeric protein which copurified with the nuclear matrix could account for almost all of its expression in infected cells.

The nuclear matrix is the skeleton which maintains the physical shape and integrity of the nucleus (4, 26). It has been suggested that the nuclear matrix plays an active role in the regulation of mRNA maturation and transport, and even gene expression, by mechanisms which are largely unknown (4, 5, 18, 20, 27, 40). It is noteworthy that a number of viruses investigated to date have been shown to encode proteins which partition with the nuclear matrix. These include adenoviruses, human papillomavirus, human immunodeficiency virus, and simian virus 40 (10, 15, 25, 38, 41). Inasmuch as the nucleus is the site of HSV DNA transcription and replication and of virion assembly, the existence of viral proteins which associate with the nuclear matrix and which could be expected to facilitate the interaction of protein-viral nucleic acid complexes with the nuclear matrix would come as no surprise. Indeed, there have been previous attempts to define the role of nuclear matrix in the replication of herpesviruses (3, 6–8, 29, 35). In this report, we show that proteins with properties similar to

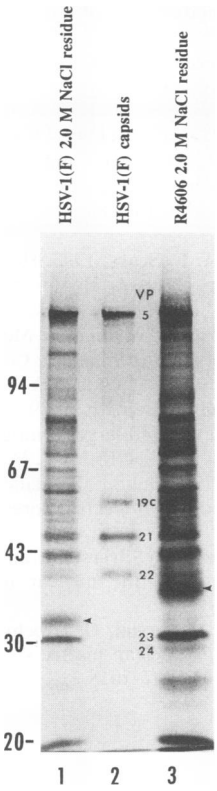

FIG. 10. Autoradiographic images of electrophoretically separated capsid proteins and nuclear matrix proteins. The [^{35}S]methionine-labeled proteins contained in the 2.0 M NaCl residues obtained during fractionation of the nuclear matrix from HSV-1(F) (lane 1)- and R4606 (lane 3)-infected HEp-2 cells were separated in a 9.5% denaturing polyacrylamide gel. The arrowheads identify the authentic and CMV epitope-tagged U_L31 proteins. The capsid proteins in lane 2 are numbered on the right according to Gibson and Roizman (13, 14). The migration of molecular weight markers is shown on the left in thousands.

those of the constituents of the HSV capsid were also reproducibly associated with and cofractionated with the nuclear matrix. The function of the U_L31 protein and the nature of the association of the other viral proteins with the nuclear matrix remain to be determined.

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