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Overexpression in Bacteria and Identification in Infected Cells of the Pseudorabies Virus Protein Homologous to Herpes Simplex Virus Type 1 ICP18.5

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The ICP18.5 gene (UL28) of herpes simplex virus type 1 is a member of a well-conserved gene family among herpesviruses and is thought to play a role in localization of viral glycoproteins. We have cloned, sequenced, and expressed the entire pseudorabies virus (PRV) ICP18.5 open reading frame in *Escherichia coli* as a Cro-ICP18.5 fusion protein. Rabbit antiserum against Cro-ICP18.5 immunoprecipitated a 79-kDa protein from PRV-infected cells as well as a 79-kDa protein from in vitro translation of a T7 RNA polymerase transcript of the ICP18.5 gene. ICP18.5 could be detected in infected cells by 2 h postinfection. Analysis by indirect immunofluorescence demonstrated that ICP18.5 became associated with the nucleus. Subcellular fractionation confirmed that ICP18.5 synthesized during a pulse-chase experiment appeared in the nuclear fraction with time and was stable for at least 2.5 h after synthesis. Pulse-chase analysis revealed that ICP18.5 was synthesized as a monomer during a 2-min pulse labeling but formed faster sedimenting complexes which were sensitive to sodium dodecyl sulfate (SDS) treatment. The majority of ICP18.5 appeared in complexes with an antigenically unrelated 70-kDa protein. Immunoblot analysis of total infected-cell extracts using polyvalent anti-ICP18.5 serum demonstrated that a 74-kDa cellular protein in addition to the 79-kDa ICP18.5 was detected. This cellular protein was present at similar levels in uninfected cells and in PRV-infected cells at least 12 h into the infectious cycle.

Herpes simplex virus type 1 (HSV-1) mutants that are defective in location of viral glycoproteins to the cell surface have been identified (24). Cells infected with one of these temperature-sensitive (*ts*) mutants, HSV-1(KOS)*icr ts*78, were not lysed by a polyvalent antiserum to viral glycoproteins in the presence of complement at the nonpermissive temperature. HSV-1(KOS)*icr ts*78 expressed normal levels of glycoproteins gB and gC intracellularly at both permissive and nonpermissive temperatures (24). At the nonpermissive temperature, the infected cell expressed greatly reduced amounts of these glycoproteins on the cell surface (24). Marker rescue analysis localized the *ts* mutation between the ICP8 and gB genes (24). An open reading frame was determined from DNA sequence analysis, and the predicted ICP18.5 protein sequence was used to generate an anti-peptide serum and to identify a 95-kDa protein in HSV-1-infected cells (26). Despite the finding of homologs to ICP18.5 in virtually every herpesvirus genome that has been sequenced (1, 3, 4, 7, 9, 13–15, 20, 23, 25, 30, 35, 41, 43), little is known about the nature or function of the ICP18.5 protein.

Previously we reported the identification and DNA sequence of the pseudorabies virus (PRV) ICP18.5 homolog. In this report, we identify the PRV ICP18.5 gene product as a 79-kDa protein. This was accomplished by expressing the open reading frame in *Escherichia coli* and producing a polyvalent, monospecific antiserum in rabbits. This antiserum immunoprecipitated the 79-kDa protein translated in vitro from ICP18.5-specific RNA as well as a 79-kDa protein from PRV-infected cell extracts.

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MATERIALS AND METHODS

Cells and virus. The PK15 cells and Becker strain of PRV have been described previously (33).

Antibody reagents. The antisera used included goat polyvalent 282 antiserum raised against a denatured *E. coli*-produced Cro-gIII fusion protein (reactive with native and denatured gIII) (36), goat polyvalent 284 antiserum raised against immunoaffinity-purified gII protein (reactive with native and denatured gII) (31), and rabbit polyvalent D71 antiserum raised against *E. coli*-produced Cro-ICP18.5 (described below).

Bacterial strains. The following *E. coli* strains were used: KK2186 [Δ(*lac-pro*) *thi strA endA sbcB15 hsdR4 supE* (F' *traD36 proAB lacI*^{q}*ZΔM15*)], LE392 (37), MBM7060 (37), MC1000 (37), NF1829 (39), XL1-Blue (Stratagene), and NFPU2 (described below).

Construction of pN50. The expression plasmid pN50 (Fig. 1A) is capable of expressing two distinct fusion proteins containing the ICP18.5 gene. The first 23 codons of the lambda *cro* gene are fused to ICP18.5 sequences starting at the second codon. The natural terminator codon of ICP18.5 is followed by an in-frame *lacI-lacZ* gene fusion. In a suppressor-negative host, only the 82-kDa Cro-ICP18.5 protein should be expressed. In a suppressor-positive host, the

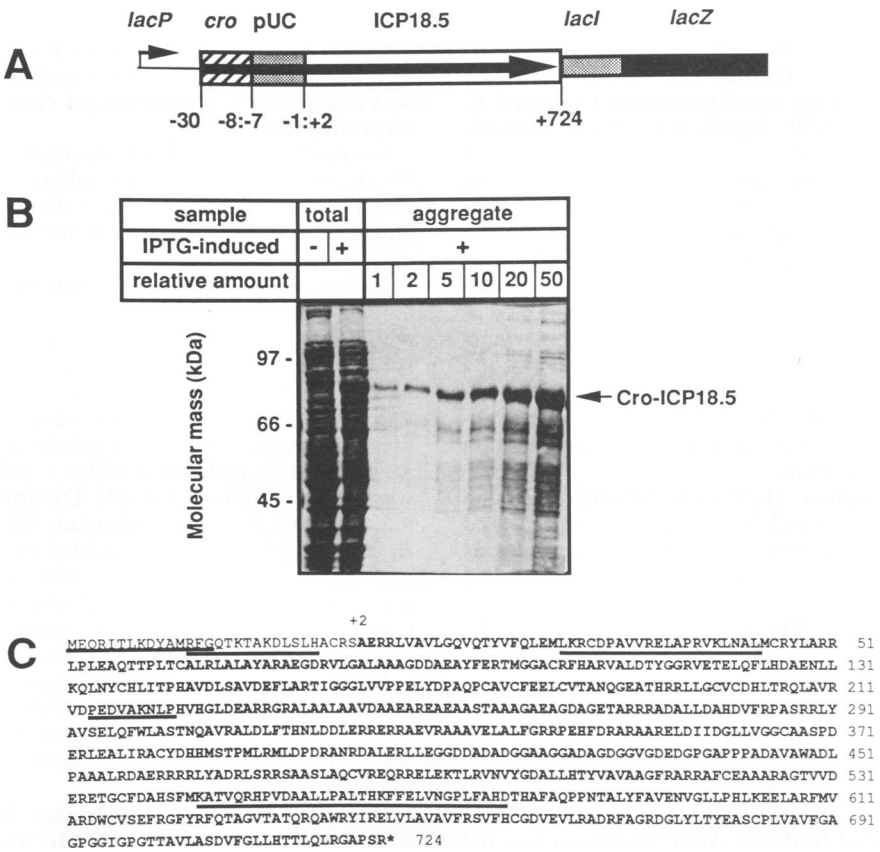

FIG. 1. pN50 and Cro-ICP18.5 expression. (A) The protein expressed from pN50 includes 23 amino acids of Cro (−30 to −8), 7 amino acids from the pUC18 polylinker (−7 to −1), and 723 amino acids of ICP18.5 (+2 to +724). Amino acids are numbered relative to the translation initiation codon of native ICP18.5. Translation of the 753-amino-acid protein terminates at the natural amber codon of ICP18.5, which is in frame with downstream *lacI-lacZ* codons. (B) NFPU2 containing pN50 was incubated in Luria broth media and induced with 1 mM IPTG for 4 h, and aggregate protein was purified as described in Materials and Methods. Whole-cell lysates are from uninduced and induced cultures. Aggregate protein was titrated as indicated. Protein samples were separated by SDS-PAGE and detected by Coomassie staining. (C) The entire amino acid sequence of the Cro-ICP18.5 fusion protein is shown. Amino acids in bold type mark ICP18.5 sequences between +2 and +724 of native ICP18.5. Underlined amino acids were verified by direct amino acid analysis of either the N terminus or internal cyanogen bromide-derived fragments.

ICP18.5 terminator should not be translated, and an ~200-kDa Cro-ICP18.5–LacI-LacZ fusion protein should result. Expression is under control of the Lac repressor and is induced by isopropyl-thiogalactoside (IPTG).

The pN50 plasmid was constructed in three steps by using coordinates relative to the A of the translation initiation codon as follows. The ends of an insert spanning from +3 (*Bal* 31 derived) to +1120 (*Xho*I) were filled in with Klenow polymerase (New England BioLabs) and ligated into the *Hin*cII site of pUC18 (44) to create pN8Δ2′5. The 3′ end of ICP18.5 was added by replacing the *Stu*I (+272) to *Xba*I (pUC18) fragment from pN8Δ2′5 with a *Stu*I (+272) to *Nhe*I (+2175) fragment to generate pN40. This ICP18.5 cassette (+3 to +2175) was transferred by flanking *Bam*HI (pUC18) and *Hin*dIII (pUC18) sites into the *Bam*HI and *Hin*dIII sites of the pHK412 expression vector (31) to generate pN50 (Fig. 1A).

Isolation of a spontaneous *E. coli* mutation enabling expression of Cro-ICP18.5. Expression of either Cro-ICP18.5 or Cro-ICP18.5–LacZ fusions in *E. coli* was lethal to the cell. We modified a technique described by Silhavy et al. (37) to select expression of nonlethal fusion proteins. In the pN50 construction (Fig. 1A), the termination codon of ICP18.5 was placed in frame between ICP18.5 and *lacZ*. When this plasmid is introduced into *E. coli* lacking a nonsense suppressor mutation, β-galactosidase should not be produced. Certain spontaneous deletions that fuse Cro-ICP18.5 to LacZ in frame and remove segments of ICP18.5 that lead to lethality should be seen as Lac^+ papillae on lactose indicator media.

NF1829 (39) harboring pN50 was streaked onto lactose MacConkey agar (37) with ampicillin and incubated overnight at 37°C to allow many single colonies to occur. The petri dishes were sealed and incubated at room temperature an additional 10 days. Several dozen red, Lac^+ papillae were then picked and purified on MacConkey plates containing ampicillin. Analysis of the plasmid DNA from each of these Lac^+ isolates revealed that essentially all the PRV ICP18.5 DNA had been deleted, confirming our initial hypothesis that expression of this open reading frame was lethal in *E. coli*.

In addition to red, Lac^+ papillae, white, Lac^- papillae also arose with time. Several dozen were purified, and their plasmid DNA was screened as described above. While most strains contained plasmids with substantial deletions, plasmids from a few of these strains appeared intact by restriction enzyme analysis. One isolate was chosen for further

analysis and was found to produce a novel protein of 82 kDa upon IPTG induction. This was the predicted size of the Cro-ICP18.5 fusion protein. Plasmid DNA from this isolate was purified and assayed for transformation into several *E. coli* strains along with pN50. Significantly, these plasmids would not transform strains lacking the *lacI*^{q} repressor, just as the parent pN50 plasmid would not transform strains lacking the *lacI*^{q} repressor. This suggested that the mutation conferring escape from fusion protein lethality was not in the plasmid but rather in the bacterial chromosome. This was confirmed by curing the plasmid from the new *E. coli* isolate by repeated streaking from single colonies in the absence of ampicillin. The cured strain (NFPU2) was readily transformed with parental pN50. Upon induction with IPTG, the retransformed NFPU2 expressed a novel protein of 82 kDa (Fig. 1B). We concluded that NFPU2 contained a chromosomal mutation permitting overexpression and accumulation of Cro-ICP18.5 fusion protein.

Fusion protein purification. The fusion protein produced in *E. coli* (NFPU2) formed insoluble aggregates. Aggregated protein from induced cultures was isolated essentially as previously described (40). An overnight culture of NFPU2 containing pN50 was diluted into fresh media, shaken for 2 h at 37°C, and induced by addition of IPTG to 1 mM. The induced culture was shaken for 4 h at 37°C, at which time cells were harvested by centrifugation. The cell pellet was frozen on dry ice, thawed, and resuspended in lysis buffer (50 mM Tris [pH 7.9], 200 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol), and lysozyme was added to 100 μg/ml. After 20 min, the cell extract was brought to 1% Triton X-100 and chilled on ice for 10 min, and Zwittergent Detergent 3-14 (Calbiochem) was added to 0.5%. After 10 min on ice, the sample was sonicated briefly, brought to 5 mM $MgCl_2$, and treated with DNase and RNase at 37°C for 25 min. Protein aggregates were pelleted through a 40% sucrose cushion and resuspended in deionized water.

Production of D71 antiserum. Partially purified aggregated proteins were prepared for injection into rabbits as follows. Approximately 100 μg of partially purified insoluble fusion protein was suspended in 200 ml of 50 mM NaOH and solubilized by heating at 65°C for 10 min. The solution was neutralized by the addition of 20 μl of 500 mM HCl and 20 μl of 1 M Tris pH 7.4. This preparation was injected into a rabbit by using the protocol described by Robbins et al. (33).

In vitro transcription. Plasmids encoding sequences for ICP18.5 and gIII were cloned downstream from the T7 RNA polymerase promoter in pBluescript II SK(−) (Stratagene). The plasmid containing the entire ICP18.5 open reading frame was generated by using coordinates relative to the A of the translation initiation codon as follows. The ends of a DNA fragment spanning −123 (*Bal* 31 derived) to +1120 (*Xho*I) were filled in with Klenow polymerase, inserted into the *Hin*cII site of pUC18, and named pN8Δ2′12. This 5′ end of ICP18.5 was transferred to *Pst*I-cleaved pBluescript II SK(−) by restriction of pN8Δ2′12 at a flanking *Pst*I site (pUC18) and the internal *Pst*I site (+361) to create pN56. The 3′ end of ICP18.5 was added by replacing the *Mlu*I (+108) to *Xba*I [pBluescript II SK(−)] fragment of pN56 with the *Mlu*I (+108) to *Nhe*I (+2175) fragment from PRV. This plasmid, pN59, encompasses the entire ICP18.5 gene from −123 to +2175. The entire gIII open reading frame was cloned in plasmid pN61 as follows. A 2.4-kb *Nco*I-*Nco*I fragment of PRV was filled in with Sequenase (U.S. Biochemical) and inserted into the unique *Bam*HI site of pBluescript II SK(−) previously filled in with Sequenase. All restriction recognition sequences were restored. The 5′ *Nco*I site encompasses the translation initiation codon of gIII. Plasmids were linearized and prepared for in vitro transcription and mRNA capping as described by Stratagene. RNA was analyzed by denaturing gel electrophoresis prior to in vitro translation.

In vitro translation. RNA was translated in the presence of 50 μCi of [^{35}S]cysteine with rabbit reticulocytes (New England Nuclear). Aliquots were diluted in radioimmunoprecipitation assay (RIPA) buffer for analysis by immunoprecipitation.

Radioimmunoprecipitation analysis. Preparation of infected-cell extracts in RIPA buffer and precipitation of immune complexes were performed essentially as previously described (33). RIPA buffer is 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% deoxycholate, 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS). Immune complexes were adsorbed to *Staphylococcus aureus* (ICN) on ice for 15 min, pelleted at 6,000 × *g* for 2 min, and serially washed with buffer B (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% NP-40), buffer B containing 500 mM LiCl, and buffer B. The complex was resuspended in sample buffer (60 mM Tris [pH 6.8], 3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.001% bromphenol blue) and heated to boiling. Bacteria were pelleted, and the immune complexes in the supernatant were separated by denaturing electrophoresis.

PAGE. Immunoprecipitates were fractionated by polyacrylamide gel electrophoresis (PAGE) on SDS-polyacrylamide slab gels of various concentrations (31). Fluorography was performed with 1 M sodium salicylate (6) and was followed by autoradiography.

Quantitation of autoradiographs. Regions of dried gels were quantitated with an AmBis radioanalytical imaging system (AmBis Systems, San Diego, Calif.).

Amino acid sequence determination. Aggregate protein in deionized water was dissolved in 50% acetic acid. The amino-terminal sequence was determined by Edman degradation chemistry on an Applied Biosystems, Inc. 470A gas-vapor sequencer interfaced with an Applied Biosystems 120A PTH analyzer. All fragments were run with a phenylthiohydantoin (PTH) standard and blank. Cyanogen bromide-derived fragments were generated by overnight digestion (38). Fragments were resolved on a Hewlett-Packard 1090 reversed-phase high-performance liquid chromatography (HPLC) column. Selected fractions were sequenced as described above.

Precipitation of total protein. Samples were incubated with 3 volumes of acetone at −20°C for at least 30 min. The precipitate was pelleted by centrifugation at 14,000 × *g* for 5 min. The supernatant was aspirated and discarded, and the pellet was dried, resuspended in sample buffer, and heated to boiling prior to separation by SDS-PAGE.

Affinity purification of serum. Serum was purified by binding to immobilized protein as described by Harlow and Lane (16). Cro-ICP18.5 was separated from minor components of the aggregate preparation by SDS-PAGE, transferred to Immobilon-P, and incubated with D71 antiserum or preimmune serum. The filters were washed with phosphate-buffered saline (PBS) and PBS with 0.4% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma). Antibodies were eluted with two washes of 100 mM glycine, pH 2.5, and the eluate was neutralized with 1 M Tris-Cl, pH 8.0, and dialysis against PBS. The purified serum was then used for immunofluorescence.

Indirect immunofluorescence. The protocol described by Larjava et al. (21) was followed. PK15 cells were seeded on coverslips and infected with the Becker strain of PRV at a

multiplicity of infection (MOI) of 10. At various times postinfection the cells were washed with PBS and fixed for 20 min in 3.7% formaldehyde and 4% sucrose in PBS at room temperature. The cells were washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. The cells were incubated with primary antisera in PBS with 2.5% bovine serum albumin (BSA) and washed with PBS, and the secondary antisera were incubated in PBS with 2.5% BSA. Coverslips were washed with PBS and mounted. Immunofluorescence and phase-contrast fields were visualized with a Zeiss microscope and documented with Tri-X Pan 400 film (Eastman Kodak Co., Rochester, N.Y.).

Pulse-chase analysis. The pulse-chase analyses were performed essentially as described previously (36). PK15 cells were infected at an MOI of 10, and at 4.5 h postinfection the cells were starved for cysteine. At 5 h postinfection the cells were labeled for 2 min with 100 μCi of [^{35}S]cysteine, the monolayer was then washed, and the cells were incubated in fresh media containing an excess of nonradioactive cysteine for various times.

Subcellular fractionation. Cells were scraped in 4 ml of ice-cold PBS, pelleted, and resuspended in buffer L (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM $MgCl_2$) with 0.5% NP-40. Nuclei were pelleted at 1,000 × *g* for 2 min and set aside. The supernatant was saved, and an equal volume of 2× RIPA buffer was added. Remaining debris was removed by centrifugation at 100,000 × *g* for 45 min. This supernatant was defined as the cytoplasmic fraction. The nuclei previously set aside were resuspended in buffer L and washed by being sedimented through a sucrose cushion (buffer L, 5% sucrose, 0.5% NP-40) at 1,000 × *g* for 2 min. The supernatant was discarded, and the pellet containing nuclei was lysed by resuspension in RIPA buffer and briefly sonicated. Debris was removed by centrifugation at 100,000 × *g* for 45 min. This supernatant was defined as the nuclear fraction.

Sucrose gradient sedimentation. Infected-cell extracts were prepared, and sucrose gradient sedimentation was as previously described (42). Samples layered on 12-ml gradients in Beckman Ultraclear tubes (14 by 95 mm) were sedimented in an SW40Ti rotor at 196,000 × *g* for 18 h. Twenty 600-μl fractions were collected and analyzed as described in the text.

Western immunoblot. Proteins were electroblotted onto Immobilon-P (Millipore) membranes with an Attoblot (Atto Corp., Tokyo, Japan) semidry apparatus. Two sheets of filter paper saturated with 300 mM Tris, pH 10.4, were placed on the anode, and two sheets saturated with 25 mM Tris, pH 10.4, were layered next and were followed by hydrated Immobilon-P and the polyacrylamide gel. Three filter paper sheets saturated with cathode buffer (25 mM Tris [pH 10.4], 40 mM 6-hexanoic acid) were layered on top. Electrotransfer was conducted for 30 min at a current of 1.5 mA/cm^2. Filters were then incubated with diluted antisera in PBS (137 mM NaCl, 2.7 mM KCl, 1.6 mM Na_2HPO_4, 1.5 mM KH_2PO_4) with 5% BSA and washed as previously described (33). Antigen-antibody interactions were visualized by incubating the filters with 1 μCi of ^{125}I-protein A (New England Nuclear) in PBS with 5% BSA, washing them as before, and exposing them to Kodak X-Omat AR film.

RESULTS

Expression of ICP18.5 in *E. coli*. Our objective was to identify the protein product of the PRV ICP18.5 open reading frame. Our first approach was to produce the ICP18.5 protein in *E. coli* by using the expression plasmid pN50 described in Materials and Methods (Fig. 1A). After several experiments, it was readily apparent that expression of both Cro-ICP18.5 and Cro-ICP18.5-LacI-LacZ fusion proteins were lethal. For example, pN50 was readily propagated in either nonsense suppressor-positive (LE392 with F′/*lacI*^{q}, KK2186, XL1-Blue) or nonsense suppressor-negative (NF1829) strains of *E. coli* as long as they carried the *lacI*^{q} mutation. pN50 would not transform either nonsense suppressor-positive (LE392, MBM7060) or nonsense suppressor-negative (MC1000) strains lacking the *lacI*^{q} mutation. Furthermore, despite a variety of protocols, we could never detect any fusion protein after IPTG induction of suppressor-positive or -negative strains carrying the *lacI*^{q} mutation. Cross-streaking experiments with IPTG on plates suggested that cells with pN50 were killed even with low levels of IPTG. To overcome the apparent lethality of Cro-ICP18.5, a second strategy was designed. As a result of the approach described in Materials and Methods, we isolated a novel *E. coli* strain (NFPU2) that allowed significant production of the 82-kDa Cro-ICP18.5 fusion protein from pN50 (Fig. 1B). Upon induction, the 82-kDa protein formed insoluble aggregates in *E. coli* that were partially purified as described in Materials and Methods.

TABLE 1. Revision of the PRV ICP18.5 DNA sequence[a]

Sequence	
Reported	Revised
A V T R	A L T H
GCC GTC ACG CGC	GCG CTC ACG CAC

[a] The PRV ICP18.5 DNA sequence from 2026 to 2037 (lower sequence) and the predicted protein sequence of ICP18.5 from 560 to 563 (upper sequence) are shown. Underlined nucleotides have been revised, and underlined amino acids have been affected. Coordinates are from Pederson and Enquist (25).

We confirmed the authenticity of the fusion protein produced in this manner by direct protein sequencing. The amino-terminal sequence of the 82-kDa fusion protein agreed with that predicted for the Cro leader (Fig. 1C). Cyanogen bromide cleavage of the 82-kDa protein resulted in a number of fragments which were separated by HPLC and sequenced. Several peptides were sequenced and identified within the predicted sequence of Cro-ICP18.5 (Fig. 1C). These results confirmed that the 82-kDa fusion protein isolated from IPTG-induced NFPU2 cells containing pN50 was the expected Cro-ICP18.5 protein.

A secondary result of peptide sequencing revealed a discrepancy between the determined protein sequence and the predicted protein sequence. The DNA sequence from this region of the ICP18.5 gene contained a number of band compressions that were particularly difficult to resolve. The DNA sequence of this region originally was determined five independent times from both strands. The number of nucleotides was correctly interpreted, but the sequence was not. The original and corrected DNA and protein sequences are listed in Table 1.

Aggregates of Cro-ICP18.5 were partially purified, solubilized, and injected into two rabbits as described in Materials and Methods. D71 serum is from one of the rabbits and reacted strongly with the Cro-ICP18.5 fusion protein in a Western blot (data not shown). This serum was used in further analysis of the ICP18.5 protein, as described below.

The primary protein product of the ICP18.5 open reading frame is a 79-kDa protein. Expression vectors for in vitro

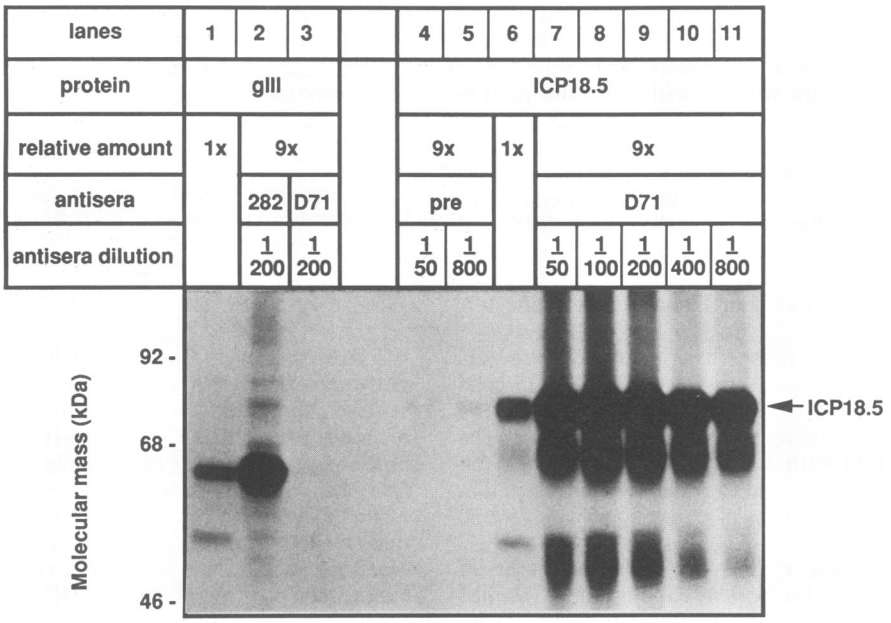

FIG. 2. In vitro protein synthesis. In vitro-transcribed RNA from pN59 and pN61 was translated in vitro with rabbit reticulocytes in the presence of [^{35}S]cysteine. Relative amounts of protein extracts are given above each lane. Dilutions of antisera were incubated with constant volumes of extracts. Immunoprecipitated proteins were separated by SDS-PAGE.

transcription of ICP18.5 and a control PRV gene, glycoprotein gIII, were constructed as described in Materials and Methods. Sense strand RNA was synthesized and capped by using linearized plasmid DNA as the template. An aliquot of RNA from each in vitro reaction was added to a rabbit reticulocyte in vitro translation system in the presence of [^{35}S]cysteine, as described in Materials and Methods. Aliquots were either added to loading buffer or diluted in RIPA buffer and immunoprecipitated. Proteins were then separated by SDS-PAGE.

Translation of gIII RNA produced one prominently labeled species in the absence of immunoprecipitation (Fig. 2, lane 1). The in vitro-translated gIII product was 57 kDa, as previously described (34), and was immunoprecipitated with 282 antiserum essentially quantitatively, as determined by radioanalytical imaging on an AmBis system (Fig. 2, lane 2). Translation of ICP18.5 RNA (Fig. 2, lane 6) produced a prominently labeled protein of 79 kDa, as predicted from DNA sequence analysis (25), and several smaller species of 65 and 52 kDa. D71 quantitatively immunoprecipitated the 79- and 65-kDa proteins at dilutions of up to 1:200 (Fig. 2, lanes 7 to 11) but did not react with in vitro-translated gIII (Fig. 2, lane 3). Pooled rabbit preimmune sera did not immunoprecipitate either the ICP18.5 or the gIII proteins (Fig. 2, lanes 4 and 5). We conclude that the primary translation product of the ICP18.5 open reading frame is a 79-kDa protein. The smaller species may result from premature translation termination products or from strong pause sites within the ICP18.5 message. Although D71 antiserum efficiently immunoprecipitated ICP18.5 at dilutions of 1:200, significant amounts of ICP18.5 were still immunoprecipitated at a dilution of 1:800, indicating that the antiserum had a high titer.

Identification of ICP18.5 in PRV-infected cell extracts. PK15 cells were infected with PRV, and [^{35}S]cysteine was added at 4 h postinfection. At 10 h postinfection, cells were harvested and lysed, and labeled proteins were analyzed by immunoprecipitation (Fig. 3). The D71 serum immunoprecipitated a major species of 79 kDa (Fig. 3, lane 2) as well as two minor species at 92 and 120 kDa. The preimmune serum immunoprecipitated only the minor species at 92 and 120 kDa. Control 284 serum immunoprecipitated the predicted gII family (110, 100, 68, and 55 kDa) of glycoproteins (Fig. 3, lane 4), and control 282 serum immunoprecipitated the

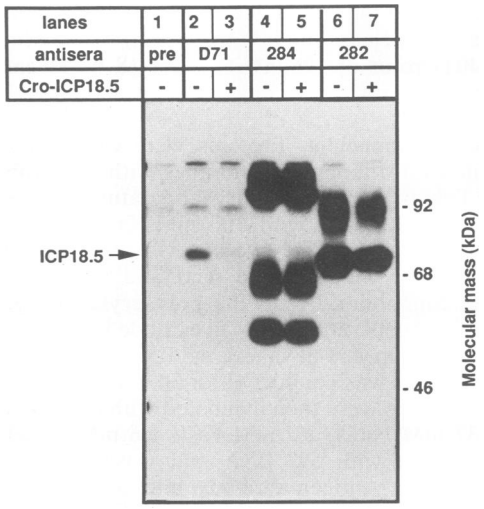

FIG. 3. Competition of infected-cell extracts with Cro-ICP18.5. PK15 cells were infected with PRV at an MOI of 10 and were continuously labeled from 4 to 10 h postinfection with 100 μCi of [^{35}S]cysteine. The cells were lysed at 10 h postinfection in RIPA buffer. Solubilized Cro-ICP18.5 was added to half of the samples prior to antisera addition. Polyvalent ICP18.5-specific D71 antiserum, polyvalent gII-specific 284 antiserum, or polyvalent gIII-specific 282 antiserum was then added. Immunoprecipitated proteins were resolved by SDS-PAGE and imaged by fluorography.

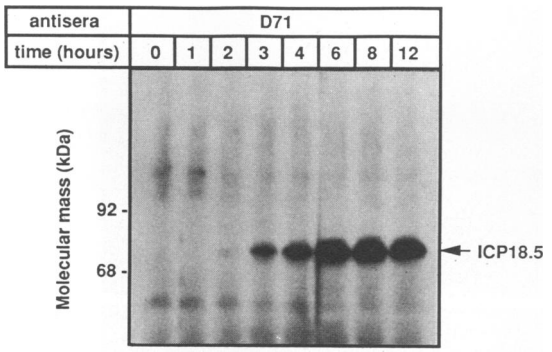

FIG. 4. Time course of ICP18.5 accumulation in PRV-infected cells. PK15 cells were infected with PRV at an MOI of 10 and harvested at various times by lysis in RIPA buffer. Samples were incubated with D71 antiserum, and immunoprecipitated proteins were separated by SDS-PAGE and transferred to Immobilon-P. The filter was then reacted with D71 antiserum in a Western blot format and identified by ^{125}I-protein A. The time postinfection is shown above each lane.

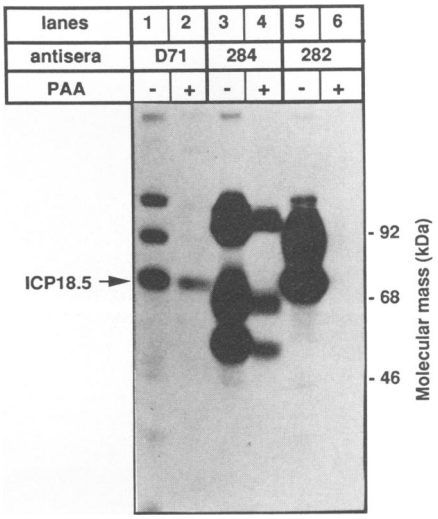

FIG. 5. Protein expression in the presence of a DNA synthesis inhibitor. PK15 cells were infected with PRV at an MOI of 10 with either 0 or 300 μg of PAA per ml present at the start of infection. [^{35}S]cysteine (100 μCi) was added to the media between 4 and 10 h postinfection. The cells were lysed in RIPA buffer, and aliquots were incubated with ICP18.5-specific polyvalent D71 antiserum, gII-specific polyvalent 284 antiserum, or gIII-specific polyvalent 282 antiserum. Immunoprecipitated proteins were resolved on a 10% SDS gel and visualized by autoradiography.

expected precursor (74 kDa) and mature (92 kDa) forms of gIII (Fig. 3, lane 6).

To demonstrate that the 79-kDa protein immunoprecipitated by D71 serum corresponded to bona fide ICP18.5, immunoprecipitation of ^{35}S-labeled PRV-infected extracts was competed with unlabeled *E. coli*-derived Cro-ICP18.5 fusion protein. The Cro-ICP18.5 aggregate was solubilized in 9.5 M urea with 800 μM β-mercaptoethanol and heated to 100°C for 5 min. Denaturants were removed by dialysis, and the dialysate was used in subsequent experiments.

The extracts in Fig. 3 indicated with a plus (+) were incubated with denatured Cro-ICP18.5 prior to addition of the antisera. The Cro-ICP18.5 fusion protein did not compete in the control immunoprecipitations with 284 and 282 sera. However, specific competition was observed for the 79-kDa species precipitated by D71 serum. The 92- and 120-kDa species present in both preimmune and D71 immunoprecipitations remained unchanged in intensity, confirming that similar quantities were loaded and that these are unrelated to ICP18.5. Moreover, ICP18.5 from infected cells has the same mobility as the in vitro-synthesized ICP18.5, suggesting that no posttranslational modifications that may affect electrophoretic mobility had occurred.

Time course of expression of ICP18.5. PK15 cells were infected with PRV and harvested at various times after infection. Extracts were immunoprecipitated with D71 antiserum, separated by SDS-PAGE, transferred to Immobilon-P, and reacted with D71 antiserum in a Western blot format (Fig. 4). No protein reacting with D71 antiserum was detected in uninfected cells or at 1 h after infection. A specific band at 79 kDa predicted to be ICP18.5 that increased in intensity as the infection proceeded was detectable by 2 h postinfection.

When the experiment shown in Fig. 4 was performed with total uninfected- and infected-cell protein on the membrane rather than immunoprecipitated protein, an additional cellular protein of approximately 74 kDa reacted with D71 antiserum and was observed at all times. Both uninfected and infected cells contained this protein (data not shown, but see Fig. 8, lane 1). The cross-reactivity of D71 antiserum with this 74-kDa cellular protein is discussed later.

Viral DNA replication is not required for ICP18.5 synthesis. In experiments not shown, we determined that 300 μg of phosphonoacetic acid (PAA; Aldrich) per ml added at the onset of infection was sufficient to block viral DNA replication. To study the effect of viral DNA synthesis on expression of ICP18.5, cells were infected in the presence or absence of PAA, incubated with 100 μCi of [^{35}S]cysteine between 4 and 10 h postinfection, and harvested in RIPA buffer. Proteins were immunoprecipitated from the extracts and separated by SDS-PAGE (Fig. 5). Expression of the viral control protein, glycoprotein gIII, was not detected in the presence of PAA (Fig. 5, lanes 5 and 6), which is consistent with the premise that gIII is a late gene similar to HSV-1 gC (32; unpublished data). Accumulation of the second control viral protein, glycoprotein gII, was slightly diminished in the presence of PAA (Fig. 5, lanes 3 and 4), consistent with its assignment as an early gene similar to HSV-1 gB (31). ICP18.5 accumulation was also diminished but not abolished in the presence of PAA (Fig. 5, lanes 1 and 2). These results are consistent with ICP18.5 being an early gene whose synthesis does not require DNA synthesis. The two bands above ICP18.5 seen previously with both preimmune and D71 sera in Fig. 3 appear sensitive to PAA but not relevant to ICP18.5.

Indirect immunofluorescence. We determined the intracellular localization of ICP18.5 in infected cells by indirect immunofluorescence with immunoaffinity-purified antiserum to reduce background. The purified antibodies were prepared by eluting bound antibodies from Cro-ICP18.5 fusion protein attached to Immobilon-P (16). PRV-infected PK15 cells were fixed and permeabilized at 1-h intervals after infection and incubated with affinity-purified antisera as indicated in Fig. 3. Bound rabbit immunoglobulin G antibodies were identified by incubation with rhodamine tetrachloride-conjugated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, Pa.).

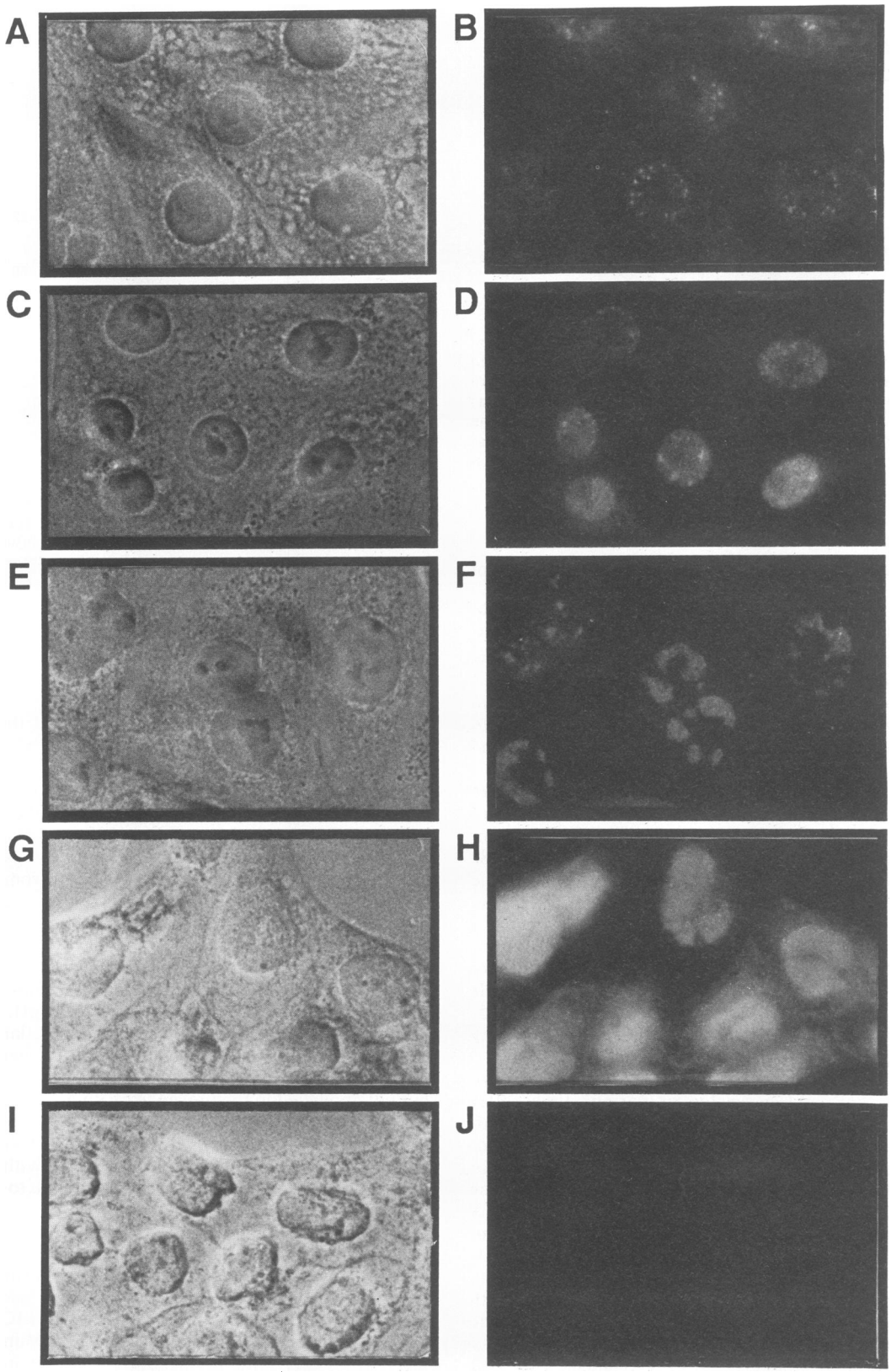

FIG. 6. Intracellular localization of ICP18.5 in PRV-infected cells. PK15 cells were infected with PRV at an MOI of 10 and then fixed and permeabilized at 1 h (A and B), 2 h (C and D), 3 h (E and F), 4 h (G and H), or 6 h (I and J) postinfection. The primary antiserum used was either D71 antiserum (1:250) immunopurified to Cro-ICP18.5 (B, D, F, and H) or preimmune serum (1:250) immunopurified to Cro-ICP18.5 (J). The secondary antiserum was rhodamine tetrachloride-conjugated goat anti-rabbit immunoglobulin G (1:50). Each field was visualized by phase contrast (A, C, E, G, and I) or fluorescence (B, D, F, H, and J).

Visible immunofluorescence from D71 antiserum could be detected by 1 h postinfection in PRV-infected cells as a punctate pattern of fluorescence in the nucleus of most cells (Fig. 6B). By 2 h postinfection, the nucleus was fairly well delineated by diffuse fluorescence in addition to the punctate pattern (Fig. 6D). At 3 h postinfection the punctate regions had grown to sizeable segments within the nucleus (Fig. 6F). From 4 h onward all portions of the nucleus except the nucleolus contained D71-reactive material. The cytoplasm of infected cells at 1 to 3 h postinfection exhibited slightly increased fluorescence. The cytoplasmic pattern was diffuse, and the fluorescence was not as striking as that in the nucleus. Preimmune serum produced background levels of fluorescence when incubated with infected cells (Fig. 6J), as did D71 and preimmune serum on uninfected cells (data not shown).

Kinetic analysis of ICP18.5 synthesis and subcellular fractionation. PRV-infected PK15 cells were pulse-labeled with [^{35}S]cysteine and chased for various times as indicated in Fig. 7. Cells were lysed in the presence of NP-40, and the cytoplasmic fraction was separated from intact nuclei. Nuclei were washed and lysed, and each fraction was incubated with antiserum. Immune complexes were analyzed by SDS-PAGE (Fig. 7A). ICP18.5 protein immunoprecipitated by D71 antiserum was made in the 2-min pulse and found only in the cytoplasmic fraction. Moreover, the protein was stable for at least 2.5 h after synthesis. ICP18.5 made in the 2-min pulse was undetectable in the nuclear fraction; however, after 30 min of chase, ICP18.5 could easily be detected in the nuclear fractions.

As controls for this fractionation, two PRV glycoproteins were examined by using the same infected-cell lysates. The precursor forms of these glycoproteins are synthesized in the rough endoplasmic reticulum which is contiguous with the outer nuclear membrane and should be found in the nuclear fractions. As the glycoproteins are exported from the endoplasmic reticulum to the Golgi apparatus for final processing, we would expect the precursors to disappear from the nuclear fractions. These experiments with gII and gIII are shown in Fig. 7B and C, respectively. The gII precursor is 100 kDa and the gIII precursor is 74 kDa. Immediately after the 2-min pulse, the gII and gIII precursor forms and incompletely synthesized forms are found in the nuclear fraction. Full-length precursor products appear in the cytoplasmic fraction. The precursor forms in the cytoplasm chase to the mature forms with kinetics previously described (36, 42). Mature gII is 110 kDa, and mature gIII is 92 kDa. No mature glycoproteins were found in the nuclear fractions, and all precursor forms disappear from the nuclear fraction between 30 and 45 min after synthesis. This time coincides with the predicted time of export of the 74-kDa gIII and 100-kDa gII precursors to the Golgi apparatus (36, 42). These control experiments suggest that ICP18.5 is synthesized in the cytoplasm and then becomes associated with the nucleus.

Subcellular fractionation of unlabeled ICP18.5. Infected cells were harvested and separated into nuclear and cytoplasmic fractions as described in Materials and Methods. Aliquots of each fraction were either immunoprecipitated with D71 antiserum (Fig. 8, right 2 lanes) or precipitated with acetone (Fig. 8, left 2 lanes). Proteins were separated by SDS-PAGE and transferred to Immobilon-P. The filter was incubated with D71 antiserum, and reactive proteins were identified with ^{125}I-protein A. ICP18.5 is found in both the cytoplasm and the nuclear fractions in all four lanes. Recall that a 74-kDa cellular protein is also recognized by D71 antiserum (Fig. 8, lane 1). This protein remained in the cytoplasm and was not immunoprecipitated by D71. These results are consistent with the pulse-chase experiments which showed that ICP18.5 is synthesized in the cytoplasm and becomes associated with the nucleus.

Sedimentation analysis of ICP18.5. PRV-infected PK15 cells were pulse-labeled for 2 min at 6 h postinfection with [^{35}S]cysteine. Extracts were made after a 2- or 90-min chase period. Cell extracts were solubilized with 1% Triton X-100 and loaded onto a 5 to 15% sucrose gradient. ICP18.5 was immunoprecipitated from each fraction and separated by SDS-PAGE. The primary ICP18.5 translation product made in the 2-min pulse sedimented initially to a position consistent with a monomer of 79 kDa (Fig. 9A). However, after 90 min of chase, ICP18.5 sedimented more rapidly, suggesting that ICP18.5 formed a complex after synthesis (Fig. 9B). An additional protein of approximately 70 kDa was also present in complex fractions and may be responsible for this shift in sedimentation. When the 90-min chase sample was treated with SDS prior to sedimenting the extract on the sucrose gradient, the ICP18.5 complex was disrupted (Fig. 9C). ICP18.5 was detected exclusively in fractions consistent with ICP18.5 monomers. The smaller protein was not detected after SDS treatment, indicating that it does not react directly with D71 antiserum. We suggest that the 70-kDa protein complexes with ICP18.5 in a noncovalent fashion soon after synthesis.

Steady-state analysis of monomer and complex forms of ICP18.5. The steady-state distribution of ICP18.5 in monomer and complex forms was examined as follows. Unlabeled PRV-infected extracts (5 h postinfection) were prepared and sedimented through sucrose gradients. Total protein from each fraction was separated by SDS-PAGE, transferred to Immobilon-P, and probed with D71 antiserum (Fig. 10). The 70-kDa protein in the complex fraction was not recognized by the D71 antiserum. Moreover, ICP18.5 was detected exclusively in the complex form; no monomeric protein could be detected. This is consistent with the idea that virtually all ICP18.5 forms a complex. As noted above, D71 antiserum reacted with a 74-kDa cellular protein that sedimented as predicted for its molecular mass and was a useful internal standard.

DISCUSSION

The HSV-1 ICP18.5 gene is highly conserved among herpesviruses for which DNA sequences are currently available. Comparison of the PRV ICP18.5 predicted protein sequence with other sequenced herpesviruses reveals significant conservation in regions throughout the protein (Fig. 11). Complete and partial sequence information was aligned with the assistance of the University of Wisconsin Genetics Computer Group programs (10). BESTFIT and LINEUP were used reiteratively to produce the protein alignments shown in Fig. 11. Every amino acid identical among all the alpha-herpesvirus sequences was marked. Those residues which were also identical in the beta- or gamma-herpesvirus sequences were marked as well. The most highly conserved regions are shown with this stringent scheme. Comparison of these sequences indicates that the ICP18.5 proteins from neurotropic herpesviruses (bovine herpesvirus, equine herpesvirus, herpesvirus saimiri, infectious laryngotracheitis virus, PRV, and varicella-zoster virus) are more closely related to each other than any of them are to the ICP18.5 proteins of lymphotropic herpesviruses (Epstein-Barr virus, human cytomegalovirus, and herpesvirus saimiri) with the

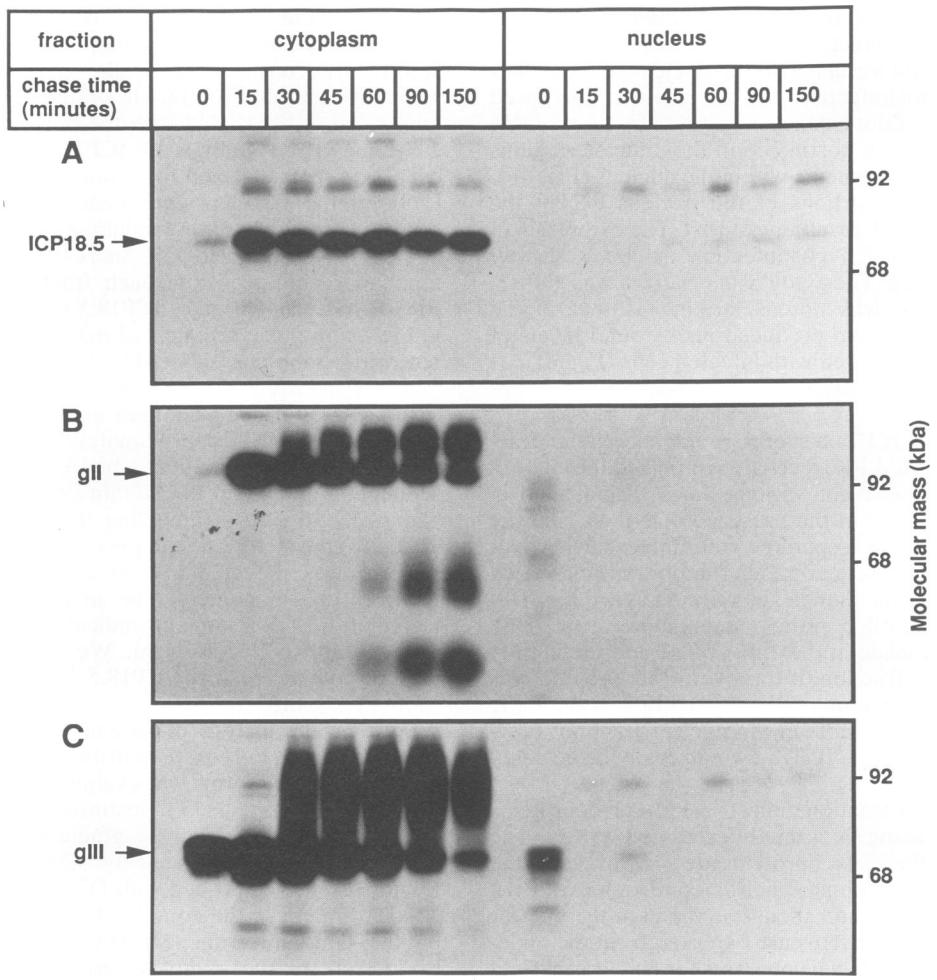

FIG. 7. Kinetics of ICP18.5 synthesis and subcellular fractionation. PK15 cells were infected with PRV at an MOI of 10. Infected cells were pulse-labeled at 5 h postinfection for 2 min with [^{35}S]cysteine and chased for the times indicated. Cells were processed into nuclear and cytoplasmic fractions. Each fraction was immunoprecipitated with D71, 282, or 284 antiserum. Immunoprecipitated proteins were resolved on an SDS–7% polyacrylamide gel and visualized by autoradiography.

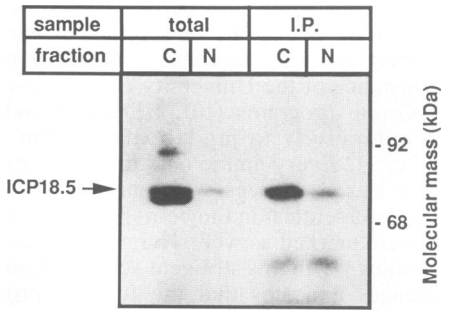

FIG. 8. Subcellular fractionation of ICP18.5. PK15 cells were infected with PRV at an MOI of 10 and subjected to subcellular fractionation as described above at 6 h postinfection. Fractions were precipitated with acetone or immunoprecipitated with D71 antiserum, separated by SDS–7% PAGE, transferred to Immobilon-P, reacted with D71 antiserum, and identified with ^{125}I-protein A.

exception of Marek's disease virus. The clusters of amino acids which are conserved in all herpesviruses may mark domains that provide clues to the function of ICP18.5. It seems likely that most herpesvirus ICP18.5 proteins function in much the same way, considering their high degree of similarity. A consideration of ICP18.5 homology concerns the upstream major DNA binding protein, ICP8 (HSV-1 UL29), and downstream gB (HSV-1 UL27) genes. Both of these genes are essential. While there is no evidence that the upstream HSV-1 ICP8 gene affects ICP18.5, transcriptional mapping places the promoter and mRNA start site of HSV-1 gB within the domain of the ICP18.5 coding region (5, 18; unpublished data). In some herpesviruses (bovine herpesvirus type 1, Epstein-Barr virus, equine herpesvirus types 1 and 4, infectious laryngotracheitis virus, and PRV) the coding regions of gB and ICP18.5 overlap, suggesting that these regions of conservation among ICP18.5 genes are likely to be the result of features required for expression of gB. However, it is clear from Fig. 11 that ICP18.5 is conserved in many different regions not believed to influence gB expression. Comparisons of the DNA sequences suggest that only one region of protein homology is due to gB

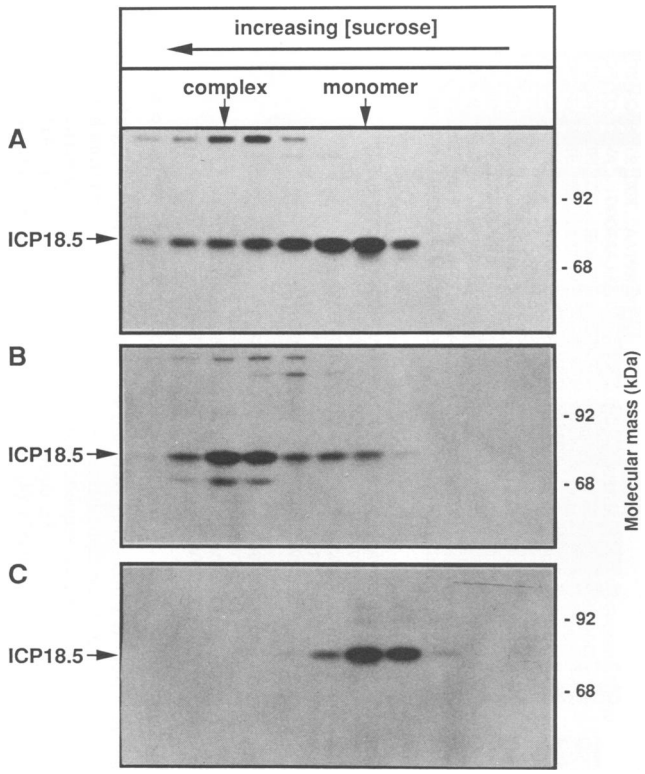

FIG. 9. Kinetics of ICP18.5 synthesis and sedimentation analysis. PK15 cells were infected with PRV at an MOI of 10 and pulse-labeled for 2 min at 5 h postinfection. (A) The cells were chased for 2 min, solubilized in 1% Triton X-100, sedimented through a 5 to 15% sucrose gradient, fractionated, immunoprecipitated with D71, and separated by SDS–7% PAGE. (B) The cells were chased for 90 min and treated exactly as described above. (C) The cells were chased for 90 min and harvested as described above. The extract was brought to 1% SDS and incubated at 4°C for 30 min prior to sucrose gradient sedimentation. Fractions were then treated exactly as described above.

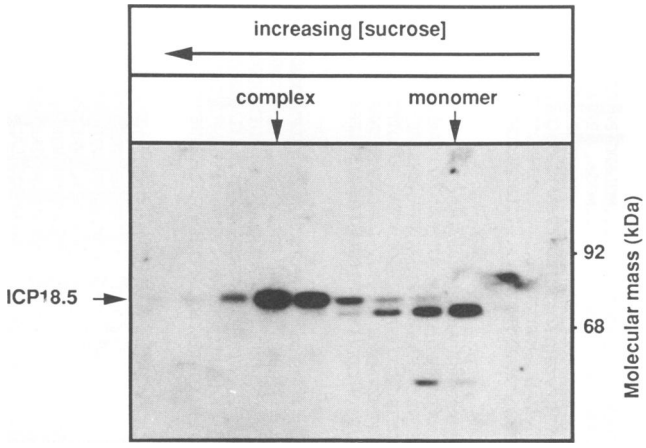

FIG. 10. Sedimentation analysis of ICP18.5. PRV-infected cells were lysed at 6 h postinfection in 1% Triton X-100 and layered on 5 to 15% sucrose gradients. Fractions were collected, acetone precipitated, resuspended in sample buffer, and heated to boiling. Treated samples were separated by SDS–7% PAGE, transferred to Immobilon-P, reacted with D71 antiserum, and identified with ^{125}I-protein A.

promoter elements. This is the sequence of PRV (641-YIREL-645) immediately to the right of the bovine herpesvirus type 2 carboxy terminus.

Despite the level of conservation, the product of this conserved gene has not been studied in any detail in any herpesvirus. HSV-1 ICP18.5 is essential, as demonstrated with *ts* mutants (24), and HSV-2 *ts* mutants have been assigned to two complementation groups within the ICP18.5 locus (12). ICP18.5 thus appears to be essential in at least two human herpesviruses, although the nature of these mutations is unknown. Pellett et al. (27) used an anti-peptide serum to identify the 95-kDa ICP18.5 protein in HSV-1(F), which was 10 kDa larger than predicted from DNA sequence analysis. In this report we expressed the PRV ICP18.5 open reading frame as a fusion protein in *E. coli* and made a polyclonal serum specific for ICP18.5.

Expression of the fusion protein in bacteria was an important part of our analysis. However, our initial efforts suggested that even small quantities of the Cro-ICP18.5 fusion protein were lethal to *E. coli*. We subsequently isolated a spontaneous *E. coli* mutant that enabled expression of significant amounts of Cro-ICP18.5. We have taken care to demonstrate that the fusion protein produced by this mutant strain is that predicted from the DNA sequence. The nature of the spontaneous mutation(s) which enabled expression of Cro-ICP18.5 is intriguing but beyond the scope of this report. We do know that the strain may be specific for alleviating the lethal phenotype of PRV ICP18.5, since it does not appear to be of general use in the expression of foreign proteins in *E. coli* (unpublished data). We note that H.-J. Rziha (Federal Research Center for Virus Diseases of Animals, Tübingen, Germany) (36a) has expressed portions of PRV ICP18.5 in a different *E. coli* expression system with no problems of lethality. Further work is necessary to characterize our strain and the lethality phenotype of Cro-ICP18.5.

We have also taken care to demonstrate that the reactivity of the antiserum directed against the Cro-ICP18.5 fusion protein is specific for PRV ICP18.5. We transcribed ICP18.5 RNA in vitro and translated the RNA in vitro to demonstrate that the primary product of the PRV ICP18.5 open reading frame migrated in SDS-PAGE as a 79-kDa protein, as expected from the DNA sequence. We used the polyclonal serum D71 and the *E. coli*-produced Cro-ICP18.5 fusion protein to demonstrate that the major product of the ICP18.5 gene in PRV-infected cells is also a 79-kDa protein. Thus, PRV ICP18.5 does not appear to be posttranslationally modified in any way that affects its electrophoretic mobility.

We detected PRV ICP18.5 protein in infected cells within 2 h after infection even in the presence of the viral DNA synthesis inhibitor PAA. This is consistent with the finding that HSV-1 ICP18.5 mRNA was detected at early times (17). ICP18.5 accumulated throughout infection and was stable for at least 2.5 h after synthesis. The kinetics of appearance of ICP18.5 were confirmed by indirect immunofluorescence. Some ICP18.5 was detected in most cells at 1 h postinfection and in greater amounts in virtually all cells by 2 h postinfection. ICP18.5 was localized to the nucleus, as measured by indirect immunofluorescence and by subcellular fractionation. The appearance of ICP18.5 at discrete locations within the nucleus was striking. As the infection progressed, these localized regions diffused from the initial sites of reactivity until the entire nucleus contained ICP18.5. It is noteworthy that the nucleolus was devoid of ICP18.5 immunofluorescence during the entire infection.

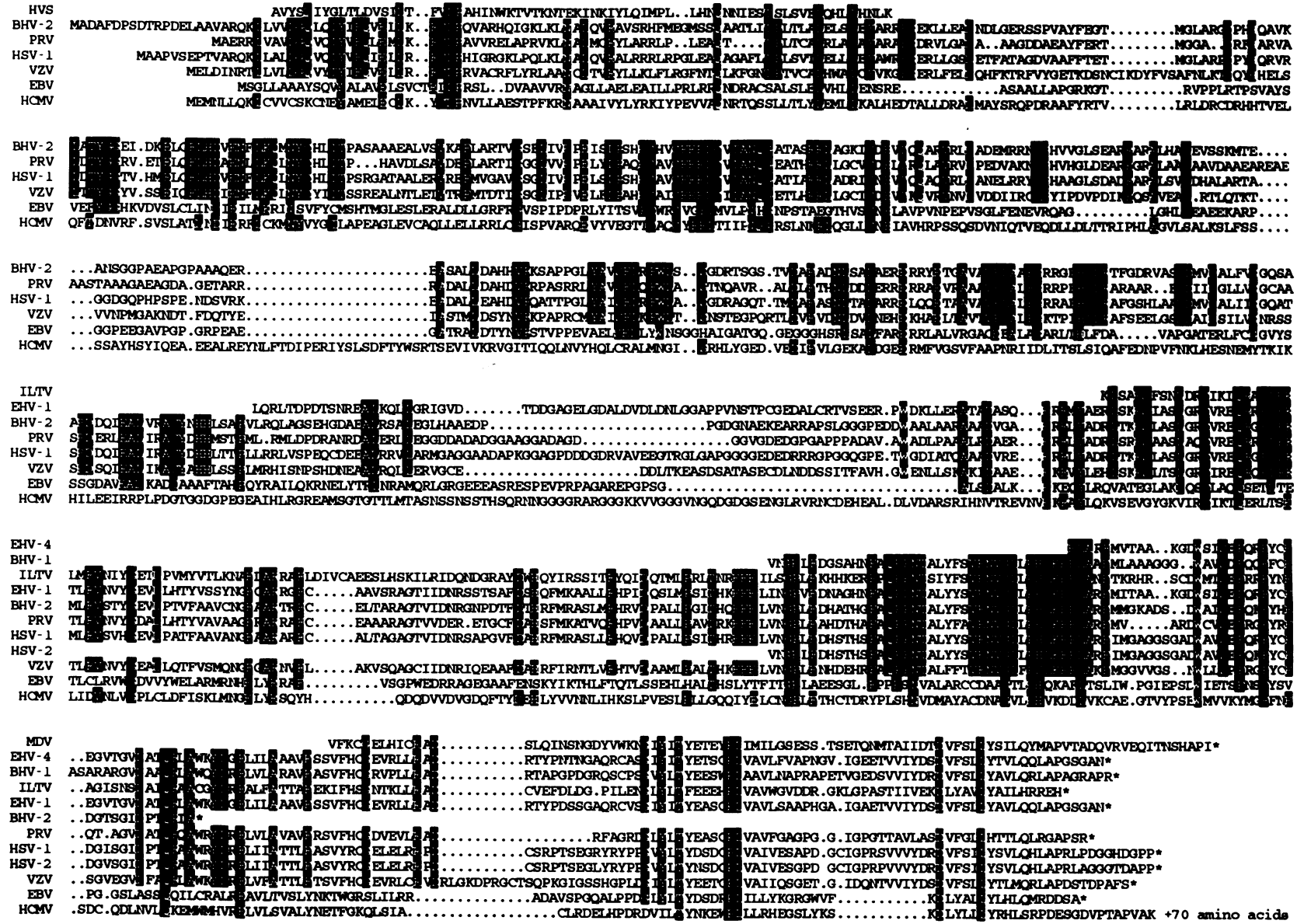

FIG. 11. ICP18.5 conservation among herpesviruses. Partial and complete predicted protein sequences from ICP18.5 genes were aligned with the assistance of the Genetics Computer Group BESTFIT and LINEUP programs (10). Amino acids on a black background are identical among all the alpha-herpesviruses shown. Amino acids from beta- and gamma-herpesviruses which also match are highlighted as well. Introduced gaps are marked with dots, and undetermined sequences are left blank. The following herpesviruses were used: bovine herpesvirus type 1 (BHV-1) (43), bovine herpesvirus type 2 (BHV-2) (15), Epstein-Barr virus (EBV) (3), equine herpesvirus type 1 (EHV-1) (41), equine herpesvirus type 4 (EHV-4) (30), human cytomegalovirus (HCMV; AD169) (7), HSV-1 Angelotti (20), HSV-2 (HG52) (4), herpesvirus saimiri (HVS) (13), infectious laryngotracheitis virus (ILTV) (14), Marek's disease virus (MDV) (35), PRV (Becker) (25), and varicella-zoster virus (VZV) (9).

The subcellular fractionation procedure demonstrated that ICP18.5 was synthesized in the cytoplasm before it associated with the nucleus. While ICP18.5 was detected immediately in cytoplasmic fractions, it was not found in nuclear fractions until at least 30 min later. The ICP18.5 fractionation results were distinctly different from those of the glycoprotein fractionation. Precursor glycoproteins gII and gIII were detected in nuclear fractions immediately after synthesis. Precursor forms were then detected in the cytoplasmic fractions as they left the nuclear fractions. The cytoplasmic precursor forms chased into mature glycoproteins with previously observed kinetics (36, 42). The results obtained by using the subcellular fractionation procedure described above compared well to those observed by others. Analysis of HSV-1 glycoprotein synthesis by electron microscopy showed that precursor glycoproteins appeared rapidly in the nuclear envelope before they were transported to the Golgi complex (28). Transiently expressed HSV-1 gB has also been identified in the nuclear envelope by indirect immunofluorescence (2).

Although we have demonstrated the association of ICP18.5 with the nucleus, the amino acid sequence of ICP18.5 does not contain a consensus nuclear localization signal (8, 11, 19, 22, 29). The most promising sequence of PRV ICP18.5 (461-RRRR-464) is not absolutely conserved in the ICP18.5 homologs among herpesviruses (Fig. 11). One possibility is that ICP18.5 is directed to the nucleus by the 70-kDa protein observed in a complex with ICP18.5 that is not antigenically related to ICP18.5 by Western blot analysis. Most ICP18.5 associates with this 70-kDa protein in infected cells, yet this interaction is noncovalent, as demonstrated by the sensitivity of the complex to SDS. The identity of this protein as a virally or cellularly encoded protein is not known. Since the 70-kDa protein was detected by pulse-labeling 5 h postinfection, it is synthesized at times when the majority of host protein synthesis is reduced, which suggests that it may be virally encoded. The sedimentation rate of the complex form is consistent with one 70-kDa monomer interacting with one ICP18.5 monomer.

Our findings do not readily explain how ICP18.5 functions in the model proposed by Pancake et al. (24). That model suggests that ICP18.5 may bind viral glycoproteins and either assist in their transport or mark them for transport to the cell surface and virions. We did not find ICP18.5 complexed to glycoproteins, but we saw it complexed to a 70-kDa protein. Another possibility might be that ICP18.5 would function in virus particles to coordinate glycoprotein distribution. We did not see ICP18.5 in virus particles (unpublished data); instead we found ICP18.5 in the nucleus in discrete, punctate regions. Significant progress toward our understanding can be expected once viruses deleted for ICP18.5 are established.

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ADDENDUM IN PROOF

Addison et al. (C. Addison, F. J. Rixon, and V. G. Preston, J. Gen. Virol. **71**:2377–2384, 1990) have suggested that ICP18.5 was involved in capsid maturation. Our results are consistent with this idea.

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