

The UL5 Gene of Herpes Simplex Virus Type 1: Isolation of a *lacZ* Insertion Mutant and Association of the UL5 Gene Product with Other Members of the Helicase-Primase Complex

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The UL5 gene product is required continuously during viral DNA synthesis (L. Zhu and S. K. Weller, *Virology* 166:366–378, 1988) and has been shown to be a component of a three protein helicase-primase complex encoded by herpes simplex virus type 1 (J. J. Crute, T. Tsurumi, L. Zhu, S. K. Weller, P. D. Olivo, M. D. Challberg, E. S. Mocarski, and I. R. Lehman, *Proc. Natl. Acad. Sci. USA* 86:2186–2189, 1989). The other members of the complex are viral proteins encoded by genes UL8 and UL52. In this study, we isolated a permissive cell line (L2-5) which contains the wild-type UL5 gene under the control of the strong and inducible promoter for the large subunit of herpes simplex virus type 1 ribonucleotide reductase (ICP6). An insertion mutant containing a mutation in the UL5 gene (*hr99*) was isolated by using the insertional mutagen ICP6::*lacZ*, in which the *Escherichia coli lacZ* gene is expressed under control of the viral ICP6 promoter. When grown on Vero cells, *hr99* does not form plaques or synthesize viral DNA, although both defects are complemented efficiently on the L2-5 cells. These results confirm that the UL5 gene product is essential for viral growth and DNA replication. Furthermore, since no detectable UL5 protein is synthesized in *hr99*-infected cells, these cells provide a valuable control not only for the detection of the UL5 protein itself but also for the detection of protein-protein interactions with UL8 and UL52 by coimmunoprecipitation. In addition, the *lacZ* insertion in *hr99* provides a convenient screening system for the introduction of site-specific mutations into the viral genome (L. Zhu and S. K. Weller, *J. Virol.* 66:469–479, 1992). Thus, *hr99* is a valuable tool in the structure-function analysis of the UL5 gene.

The 152-kb genome of herpes simplex virus type 1 (HSV-1) contains three *cis*-acting origins of DNA replication (45, 46, 54) and at least 72 open reading frames (31). Two complementary approaches have been used to identify *trans*-acting viral factors required for viral DNA synthesis. By using a genetic approach, HSV-1 mutants representing seven distinct complementation groups have been identified which fail to induce viral DNA synthesis under nonpermissive growth conditions (2–4, 19, 27, 30, 40, 51, 52, 57; reviewed in reference 50). These complementation groups represent seven viral genes which have been shown to be necessary and sufficient for origin-dependent plasmid amplification in a transient transfection assay (32, 55). Biochemical activities have now been assigned to the products of these genes: UL30 and UL42 encode the viral DNA polymerase and its accessory protein (4, 5, 17, 37, 39, 48); UL29 encodes the major DNA-binding protein, ICP8 (7, 52); UL9 encodes the HSV origin-specific DNA-binding protein (35); and UL5, UL8, and UL52 encode three members of a protein complex with helicase-primase activities (9).

The initial indication that UL5 is essential for DNA replication came through the physical mapping of the mutations in two temperature-sensitive (*ts*) mutants of HSV-1 (57). The existence of a consensus ATP binding site in the UL5 gene raised the possibility that the UL5 gene product may be an ATPase or a helicase (32, 57). We previously reported the expression of the UL5 gene product in *Escherichia coli* and the generation of specific anti-UL5 polyclonal antisera (α -UL5) (57); however, direct attempts to demonstrate that the UL5 protein expressed in *E. coli* has intrinsic

helicase activity were unsuccessful (60). Helicase activity was, however, detected in extracts of HSV-infected cells (8). In that report, a partially purified protein complex consisting of three major polypeptides with molecular weights of 120, 97, and 70 kDa was shown to exhibit helicase and DNA-dependent ATPase activities. Subsequently, α -UL5 was used to demonstrate that the UL5 protein is a component of this complex which exhibits not only helicase but also primase activity (9). The other two components of this complex were identified by specific antisera as the products of the UL8 and UL52 genes (9), both of which are essential for viral DNA replication (3, 19, 55). These three gene products coexpressed in insect cells form a functional complex exhibiting DNA-dependent ATPase, DNA helicase, and DNA primase activities (12). To date, UL5 expressed alone, either in *E. coli* (60) or in insect cells (12), fails to exhibit helicase activity; however, when UL5 is coexpressed with UL52, the two proteins together exhibit helicase activity (1). Subsequently, this two-protein complex was shown to exhibit both helicase and primase activities (13). The elucidation of the functional roles of each protein in helicase-primase activity and the significance of the interaction between the members of the complex await further biochemical and genetic characterization. This problem can be approached genetically through structure-function analysis, which will also facilitate the definition of protein domains that contribute to the enzymatic activities of helicase and primase and to the interactions between members of the complex.

The studies reported in this and the accompanying report (59) represent initial attempts to define domains required for UL5 function. *ts* mutants were useful in the initial identification of the UL5 gene as an essential DNA replication gene.

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In particular, temperature shift experiments have indicated that the UL5 gene product is required continuously during DNA replication (57). On the other hand, the analysis of *ts* mutants can be hindered by leaky *ts* mutations and possible interference caused by *ts* gene products. These problems can be circumvented by the isolation of host range mutations in DNA replication genes which can be propagated in cell lines capable of providing the wild-type gene products in *trans*. For the isolation of host range mutants, we previously reported an insertional mutagen consisting of the *lacZ* gene of *E. coli* expressed under the control of an inducible HSV promoter, permitting the screening of insertion mutants on the basis of plaque color (3, 19, 53). We report herein the establishment of a UL5 permissive cell line and the isolation and characterization of an ICP6::*lacZ* insertion mutant in the UL5 gene. The availability of this host range mutant, *hr99*, facilitates the analysis of functional domains of the UL5 protein in several ways. First, *hr99*-infected cells, in which no UL5 protein is expressed, provide a valuable control not only for the detection of the UL5 protein itself but also for the detection of protein-protein interactions by coimmunoprecipitation (this report). In addition, *hr99*-infected cells provide an ideal environment in which to test the function of cloned copies of UL5 genes containing site-specific mutations in the absence of potentially interfering mutant peptides (59). Finally, the availability of a mutant that produces blue plaques provides a convenient system for screening for the introduction of site-specific mutations into the viral genome (20, 59).

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero; American Type Culture Collection, Rockville, Md.) were propagated and maintained as described previously (52). HSV-1 strain KOS was used as the wild-type virus and the parental virus for the isolation of the UL5 host range mutant. Viruses were propagated and assayed as described previously (43). *ts* mutants *tsK13* and *tsM19* of strain KOS, isolated by nitrosoguanidine and UV radiation, respectively, were obtained from P. A. Schaffer (Dana-Farber Cancer Institute, Boston, Mass.) (42).

Plasmids. Plasmid pDG-2 was constructed by inserting a 549-bp *NarI*-to-*BalI* fragment containing the HSV-1 strain KOS ICP6 gene upstream regulatory region into the vector Bluescribe (Stratagene, San Diego Calif.) which had been digested with *AccI* and *SmaI* (20a). pDG-2 contains 315 bp upstream from the ICP6 transcriptional start site and 228 bp of the ICP6 5' untranslated sequence (Fig. 1). pCW8 containing an *XbaI*-to-*KpnI* fragment from HSV-1 strain KOS (sequence coordinates 10636 to 16269) encompassing the entire UL5 gene (sequence coordinates 12487 to 15133; Fig. 2) inserted into the vector Bluescribe was provided by M. Challberg (National Institutes of Health, Bethesda, Md.). The expression clone p6UL5 was constructed by digesting pCW8 with *MluI*, releasing a 3,044-bp fragment (12127 to 15162) containing the entire UL5 coding region (Fig. 2). The *MluI* fragment was treated with Klenow fragment, ligated to *XhoI* linkers (New England Biolabs), and digested with *XhoI*. This fragment was inserted at the single *XhoI* site of pDG-2 such that the entire UL5 open reading frame is downstream from the ICP6 promoter and 5' untranslated region (Fig. 1). To create pUL5Lac, in which the UL5 gene is disrupted, the 4,276-bp *Bam*HI fragment from pD6p (19) containing the *lacZ* gene under the control of the ICP6 promoter (the ICP6::*lacZ* cassette) was inserted into the

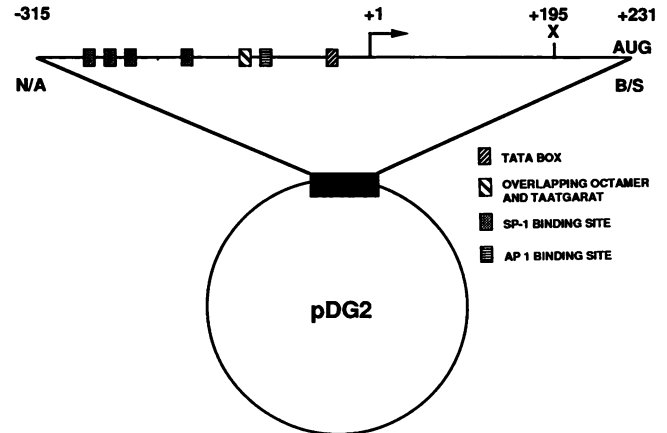


FIG. 1. Diagram of plasmid pDG-2 showing the ICP6 promoter region. A 549-bp *NarI*-to-*BalI* fragment from the ICP6 gene promoter region was inserted into the vector Bluescribe which had been digested with *AccI* and *SmaI*. The start site of the ICP6 transcript is marked by an arrow and numbered as +1 (33); the 315-bp region located upstream from +1 contains consensus sequence elements known to be responsive to various transcription factors, illustrated with boxes as shown. The ICP6 gene translation start codon is at position +231. Restriction enzyme recognition sites: X, *XhoI*; N, *NarI*; A, *AccI*; B, *BalI*; S, *SmaI*. For reference, the *XhoI* site marked is at position 86409 in the sequence of HSV-1 strain 17 (31).

BglII site in pCW8 (at position 14589, 544 bp downstream of the start codon of the UL5 gene; Fig. 2). pSV2neo containing the neomycin resistance gene under the control of the simian virus 40 promoter was described previously (44).

Generation of UL5 permissive cell lines. To generate UL5 permissive cell lines, Vero cells were cotransfected with plasmid pSV2neo and either pCW8 or p6UL5 as described previously (18). G418-resistant colonies were picked and screened for their ability to support the growth of *tsM19* at 39°C. Cell lines which supported the growth of *tsM19* at the nonpermissive temperature were analyzed for copy number of the UL5 gene by Southern blot analysis as described below.

Preparation and analysis of cellular and viral DNA. Intact infectious viral genomic DNA required in marker transfer experiments was prepared as described previously (57). Viral DNA used for Southern analysis was isolated as described for infectious DNA except that the DNA was deproteinized by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. Alternatively, viral DNA was isolated from infected cells as described by Coen et al. (6). Infected cells were scraped into medium and then subjected to three cycles of freezing and thawing. The sample was sonicated for 1 min and centrifuged at 2,500 rpm for 10 min to pellet the cell debris. The supernatant was transferred into a fresh tube and centrifuged at 13,000 × *g* for 1 h to pellet the crude virions. The virion pellet was resuspended in 400 μl of Tris-EDTA, deproteinized by extraction with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol. High-molecular-weight cellular DNA for Southern blot analysis was isolated as described previously (54). Southern blot analysis was performed on GeneScreen Plus membranes according to the manufacturer's instructions. DNA probes were prepared by random priming of gel-isolated restriction fragments with [α -³²P]dATP (14).

Marker transfer and marker rescue experiments. Marker

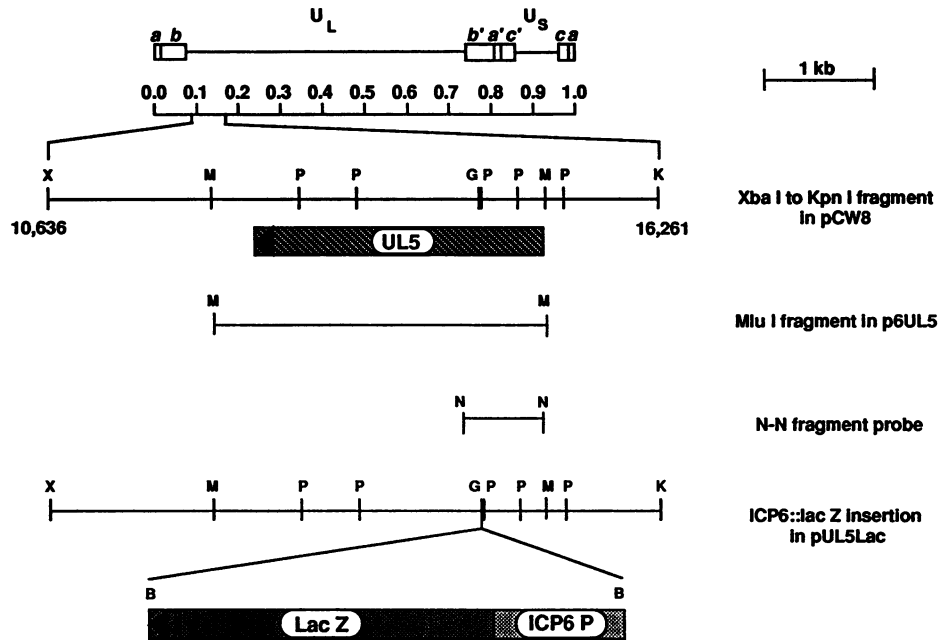


FIG. 2. Map location of the HSV-1 UL5 gene. The HSV-1 genome and map coordinates are shown in the first two lines. Below, the insert in pCW8, the *Xba*I-to-*Kpn*I fragment (sequence positions 10636 to 16261) spanning the UL5 open reading frame, has been expanded to show internal cleavage sites: X, *Xba*I; M, *Mlu*I; P, *Pst*I; G, *Bgl*II; K, *Kpn*I. The next three lines correspond to the *Mlu*I fragment in p6UL5, an *Nae*I fragment used as a probe for Southern blot hybridization, and a diagram of the ICP6::lacZ insert in pUL5Lac. N refers to *Nae*I sites and B refers to *Bam*HI sites.

transfer and marker rescue experiments were performed as described previously (20).

Analysis of viral DNA synthesis. Viral DNA synthesis was determined according to a method described by Gadler (15). Specifically, cells in 35-mm plates were infected with virus at a multiplicity of infection of 2 to 5 for 16 h at 37°C. The plates were washed with phosphate-buffered saline (PBS) twice, and the cells were scraped into 15-ml tubes. The cells were precipitated by centrifugation at 4°C for 10 min at 2,000 rpm, and the pellets were resuspended in 100 μ l of fresh PBS. A series of fivefold dilutions was spotted onto a GeneScreen Plus membrane, using a Microsample Filtration Manifold (Schleicher & Schuell). The membrane was treated twice with 0.4 N NaOH and twice with 1 M Tris-HCl (pH 7.5). The membrane was dried at room temperature and probed with a 32 P-labeled HSV-specific probe (*Eco*RI F fragment from plasmid pSG18; map coordinates 0.315 to 0.422) as described above.

Detection of UL5 proteins in viral infection and transient transfection by immunoprecipitation and immunoblotting. Cells in 100-mm plates were infected with virus stocks at a multiplicity of infection of 20 PFU per cell at 34°C. L- 35 S]methionine was added at 5 h; cells were harvested at 17 h postinfection and processed for immunoprecipitation as described below. To detect UL5 protein in cells transiently transfected with the expression clones pCW8 and p6UL5, 3×10^6 Vero cells were transfected with 12 μ g of pCW8 or p6UL5 as described previously (20) and plated in a 100-mm tissue culture dish. Induction of expression was achieved by superinfection at 30 h posttransfection with hr99. L- 35 S]methionine was added at 32 h posttransfection, and cells were harvested at 48 h. Prior to labeling, cells were washed with prewarmed methionine-free minimal essential medium (GIBCO) and incubated in this medium for 30 min. The medium was removed, L- 35 S]methionine (716 Ci/mmol;

NEN Research Products, Wilmington, Del.) was added to a concentration of 400 μ Ci/ml in 0.5 ml of medium, and the plates were rocked constantly at 34°C. At the end of the labeling period, each plate was washed three times with cold PBS and cells were lysed on the plate by the addition of 1 ml of RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% deoxycholate, 1.0% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 μ g of aprotinin per ml, 200 μ M tolylsulfonyl phenylalanyl chloromethyl ketone, (TPCK), and 200 μ M tolylsulfonyl leucylalanyl chloromethyl ketone (TLCK) at 4°C for 30 min with occasional rocking. The lysate was scraped into 1.5-ml microcentrifuge tubes, sonicated for 1 min, and cleared by a 10-min centrifugation in a microcentrifuge at 4°C. The resulting lysate was precleared twice by the addition of nonimmune rabbit serum (50 μ l) at 4°C for 30 min. Then 40 μ l of a 50% solution of protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) in RIPA buffer plus 2% bovine serum albumin (BSA) was added, and the incubation was continued for another 30 min with rocking. The immune complexes were centrifuged for 2 min in the microcentrifuge. The supernatant was transferred to a fresh tube to which 2.5 to 10 μ l (depending on the experiment) of α -UL5 (57) was added; the mixture was incubated for 1 h in ice, and the immune complexes were pelleted after the addition of 40 μ l of protein A beads as described above. The beads were washed once with RIPA buffer plus 2% BSA, once with RIPA buffer plus 1 M NaCl, twice with RIPA buffer, once with RIPA buffer minus SDS, once with RIPA buffer plus 0.2 M LiCl and 1 M urea, once with RIPA buffer minus SDS, and once with RIPA buffer. The pellet containing the immune complexes was boiled for 5 min in protein SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (0.0625 M Tris [pH 8.6], 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue, 10% glyc-

TABLE 1. Plaquing efficiencies of HSV *ts* mutants on Vero, E22, and L2-5 cells

Cell	Copy no. ^a	Promoter ^b	Titer (PFU/ml)					
			KOS		<i>ts</i> K13		<i>ts</i> M19	
			34°C	39°C	34°C	39°C	34°C	39°C
Vero	0		8×10^8	5×10^8	3×10^8	$<1 \times 10^3$	2×10^8	$<1 \times 10^3$
E22	40	UL5	3×10^7	6×10^6	1×10^7	$<1 \times 10^3$	6×10^7	7×10^6
L2-5	66	ICP6	5×10^8	2.2×10^8	4×10^7	6×10^6	3×10^8	1×10^8

^a See Fig. 3 for determination of UL5 copy number in L2-5 cells.

^b Refers to promoter used for UL5 expression.

erol) (25). The proteins were separated by SDS-PAGE (9% acrylamide). The gel was fixed in H₂O-methanol-acetic acid (5:5:1) for 1 h, rinsed in water for 15 min, and soaked in Autofluor for 2 h. The dried gel was fluorographed at -70°C . ¹⁴C-labeled rainbow protein molecular weight markers were purchased from Amersham (Arlington Heights, Ill.).

In some experiments, a combination of immunoprecipitation and immunoblotting was used to detect the UL5 protein. The infected cells were not labeled, and the immune complexes were washed only three times with RIPA buffer. Immune complexes were subjected to SDS-PAGE as described above, and proteins were electrophoretically transferred to filters essentially as described by Towbin et al. (47). Immobilon membrane filters (Millipore, Bedford, Mass.) were used as recommended by the supplier. Filters were incubated with a 1:500 dilution of serum, and the immunoreactive proteins were detected by reaction with a goat anti-rabbit immunoglobulin G (IgG) (Fc) antibody conjugated to alkaline phosphatase (Promega, Madison, Wis.). Alkaline phosphatase color development was carried out as described previously (57).

Detection of protein complexes by coimmunoprecipitation. Coimmunoprecipitation was performed in a manner similar to immunoprecipitation except that cells were lysed in TNE buffer (50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA) and the immune complexes were washed three times with TNE buffer. Following SDS-PAGE, individual proteins in the complex were detected by immunoblotting with their corresponding antibodies as described above. α -UL8 was raised in a rabbit against a UL8 protein expressed in *E. coli* (3a); α -UL52 was provided by M. Challberg (36).

RESULTS

Isolation of UL5 permissive cell lines. To isolate and propagate a UL5 insertion mutant, a cell line that can provide a wild-type copy of the UL5 gene product in *trans* was necessary. Initially, Vero cells were cotransfected with plasmid pCW8 containing the UL5 gene under its endogenous promoter and pSV2neo. Stably transfected G418-resistant cell lines were isolated and tested for their ability to support the growth of UL5 *ts* mutant, *ts*M19. One cell line, E22, which could support *ts*M19 at the nonpermissive temperature, was chosen for further study. Surprisingly, however, this cell line was unable to support *ts*K13 at the nonpermissive temperature (Table 1). This allele-specific difference was also observed in another cell line containing pCW8. Although this phenomenon is not understood, the simplest explanation is that the endogenous UL5 gene promoter is not very active and that levels of the UL5 gene product are insufficient to complement certain *ts* mutants. This explanation is consistent with the finding that the UL5

protein is not abundant in infected cells (36, 57). To express higher levels of the UL5 protein in infected cells, we decided to express the UL5 gene under the control of the promoter for ICP6, the large subunit of ribonucleotide reductase. The reason for choosing the ICP6 promoter is threefold: (i) the ICP6 protein is an abundant protein in HSV-infected cells; (ii) the ICP6 promoter is inducible by viral infection (18, 58); and (iii) in previous experiments, a reporter gene (the *lacZ* gene) was expressed successfully at relatively high levels under the control of the ICP6 regulatory elements (18, 19). Plasmid p6UL5, in which the UL5 gene is under control of the ICP6 regulatory region (Fig. 1), was used to transform Vero cells as described in Materials and Methods. G418-resistant clones were tested for their ability to support *ts*M19 and *ts*K13 at the nonpermissive temperature. Of 16 clones which were able to support *ts*M19, 13 were also able to support the growth of *ts*K13 at the nonpermissive temperature (data not shown). One of the 13 cell lines, L2-5, was chosen for further study, its ability to support the *ts* mutants is shown in Table 1.

To confirm the presence of the UL5 construct and to determine its copy number in the L2-5 cell genome, Southern blot analysis of high-molecular-weight cellular DNA was performed (Fig. 3). Cellular DNA and p6UL5 DNA were digested, subjected to electrophoresis, transferred to a nylon membrane, and probed with a ³²P-labeled *Mlu*I-*Mlu*I fragment covering the UL5 gene (Fig. 2). Various dilutions of p6UL5 provided standards for the determination of copy number (Fig. 3, lanes 1 to 5). Digestion of p6UL5 and cellular DNA with *Bgl*III and *Eco*RI generates three internal fragments, 523 bp, 2.0 kb, and 4.3 kb. The differing intensities of each band are due to the position of each fragment relative to the probe. Cell line L2-5 DNA exhibits the same pattern as p6UL5 (Fig. 3, lane 7), confirming the presence of p6UL5 in the cell genome. By comparing the intensity of the internal 2.0-kb fragment in lane 7 with the standards, it appears that the L2-5 genome contains about 60 copies of p6UL5 DNA per haploid genome. L2-5 DNA was also digested with *Hind*III, generating two expected fragments of 3.0 and 3.8 kb (Fig. 3, lane 9). Vero cell DNA, when digested and probed in parallel, exhibited no specific hybridization to the probe (Fig. 3, lanes 6 and 8). The E22 cell line and 15 other L-cell lines were subjected to Southern blot analysis in parallel (data not shown). The E22 cell line contains about 40 copies of the UL5 gene per haploid genome. The UL5 gene copy numbers of those L-cell lines that supported both *ts*K13 and *ts*M19 ranged from as little as 2 copies to about 60 copies per haploid genome. The three L-cell lines that supported only *ts*M19 contain approximately one copy of the UL5 gene per haploid genome. These results support the hypothesis that the allele-specific differences in ability to complement mutants *ts*K13 and *ts*M19 are due to levels of

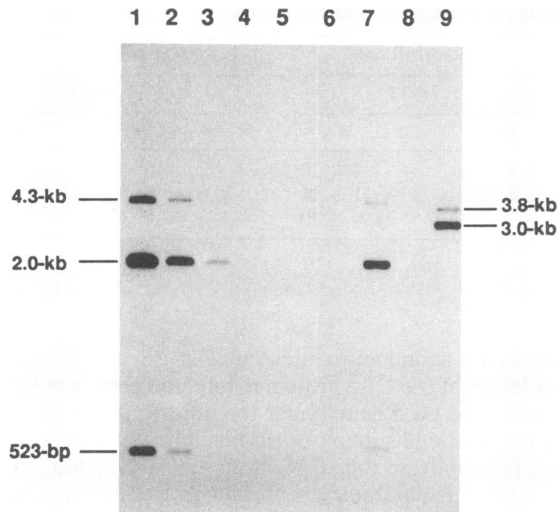


FIG. 3. Southern analysis of cell line L2-5 DNA. DNA from plasmid p6UL5, Vero cells, or L2-5 cells was digested with the appropriate restriction enzyme, subjected to electrophoresis, and transferred to GeneScreen Plus membranes. In lanes 1 to 7, DNAs were digested with *Bgl*II and *Eco*RI; in lanes 8 and 9, DNAs were digested with *Hind*III. Lanes 1 to 5 contain various amounts of digested p6UL5 DNA: lane 1, 3.125 ng; lane 2, 0.625 ng; lane 3, 0.125 ng; lane 4, 0.025 ng; lane 5, 0.005 ng. Lanes 6 to 9 contain 7 µg of cellular DNA from Vero (lanes 6 and 8) or L2-5 (lanes 7 and 9). The blot was probed with the ³²P-labeled *Mlu*I fragment containing the entire UL5 gene (see Fig. 2). Fragment sizes are indicated.

expression of the wild-type version of the UL5 gene in the cell lines. This hypothesis was tested directly by looking at the ability of the E- and L-cell lines to express detectable UL5 protein (see below).

Isolation of a UL5 insertion mutant. It was anticipated that the availability of a null mutant in the UL5 gene would facilitate the analysis of functional domains of the UL5 protein and extend our understanding of its role in viral DNA replication. We previously demonstrated that the *E. coli lacZ* gene can be introduced into the HSV genome by marker transfer, permitting the screening of insertion mutants on the basis of plaque color (3, 19, 53). We initially attempted to construct a UL5 disruption mutant with an in-frame insertion of the structural gene of *E. coli* β-galactosidase at the *Bgl*II site such that a UL5-*lacZ* fusion would be synthesized under the control of the endogenous UL5 regulatory signals. It was expected that following marker transfer, recombinant viruses would easily be detected by screening for β-galactosidase activity in the presence of 5-bromo-4-choro-3-indolyl-β-D-galactopyranoside (X-Gal); however, no blue plaques were detected in several separate marker transfer experiments. Our inability to detect blue plaque formation led us to postulate that β-galactosidase synthesis from these recombinants was below the limits of detection, consistent with our belief that the UL5 promoter is weak. We previously reported the construction and use of a plasmid, pD6p, bearing an insertional mutagen consisting of the *lacZ* gene expressed under the control of the upstream regulatory signals of the ICP6 gene (3, 19, 53). Restriction digestion of pD6p with *Bam*HI results in the excision of a 4.2-kb ICP6::*lacZ* cassette. This cassette was inserted into a UL5 clone at a *Bgl*II site located at a position 544 bp downstream from the 5' end of the initiation AUG of the UL5 gene (Fig. 2). The resulting construct, pUL5lacZ,

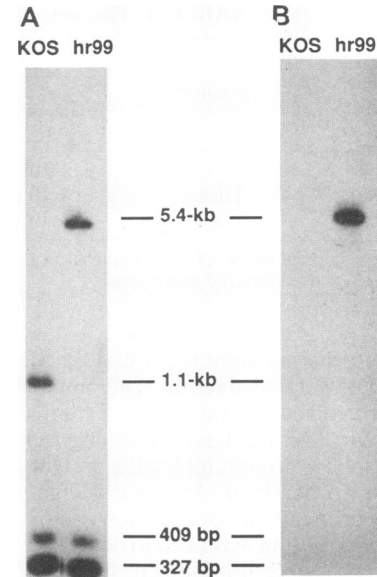


FIG. 4. Southern blot analysis of viral DNA. Viral DNAs from wild-type KOS and *hr99* were digested with *Pst*I, subjected to electrophoresis, and transferred to a GeneScreen Plus membrane. Membranes were probed either with the HSV-1 *Nae*I fragment (see Fig. 2) (A) or *lacZ* DNA (B). Fragment sizes are indicated.

would be expected to encode an N-terminally truncated UL5 peptide containing the first 181 amino acid residues of UL5 which would terminate within the upstream regulatory sequences of the ICP6 gene to generate a protein of approximately 20 kDa.

An HSV mutant containing a *lacZ* insertion was recovered by cotransfecting L2-5 cells with infectious wild-type DNA and pUL5LacZ. Homologous recombination between KOS genome DNA and pUL5LacZ resulted in the generation of recombinants which formed dark blue plaques in the presence of X-Gal. Mutant viruses were picked, purified, and propagated in L2-5 cells. One recombinant virus, *hr99*, was used in further studies. To confirm that *hr99* contains the ICP6::*lacZ* cassette at the appropriate position, DNAs from KOS and *hr99* were purified, digested with *Pst*I, and subjected to gel electrophoresis and Southern blot hybridization in duplicate (Fig. 4). Filters were probed with a ³²P-labeled *Nae*I fragment from inside the UL5 gene (see Fig. 2 for position of probe) or with a ³²P-labeled LacZ fragment probe. KOS contained a 1.1-kb fragment as expected, and *hr99* contained the 5.4-kb version expected if the ICP6::*lacZ* fragment were inserted (Fig. 4A); furthermore, the 5.4-kb fragment from *hr99* was also recognized by the LacZ probe as anticipated (Fig. 4B). The 409- and 327-bp fragments seen in KOS and *hr99* DNA probed with the *Nae*I fragment correspond to *Pst*I fragments from within the UL5 gene (Fig. 2).

Phenotypic properties of *hr99*. The mutant *hr99* was tested for its ability to form plaques in untransformed Vero cells. A stock of *hr99* was propagated on L2-5 cells, and the titers on L2-5 and on Vero cells were determined. Table 2 shows that *hr99* fails to form plaques on Vero cells and that this growth defect can be complemented by the L2-5 cell line. These results confirm that the UL5 gene is essential for virus growth. The ability of *hr99* to induce DNA synthesis in L2-5 and Vero cells was assessed by dot blot hybridization. Previously we measured viral DNA synthesis by the incor-

TABLE 2. Plaquing efficiencies of KOS and *hr99* on Vero and L2-5 cells

Virus	Cell line	Titer (PFU/ml)
KOS	Vero	6×10^8
	L2-5	6×10^8
<i>hr99</i>	Vero	$<1 \times 10^3$
	L2-5	1.6×10^9

poration of [*methyl*-³H]thymidine into viral DNA in mutant and wild-type virus-infected cells; by this method, we demonstrated that *tsK13* and *tsM19* were unable to synthesize viral DNA at the nonpermissive temperature (39.6°C) (57). In this study, we measured viral DNA synthesis by dot hybridization of total DNA from infected cells to a virus-specific probe (see Materials and Methods). The results for *tsK13*- and *tsM19*-infected cells are entirely consistent with our previous results (data not shown), thereby establishing the validity of this method. The results in Fig. 5 clearly demonstrate that *hr99* was not able to synthesize detectable viral DNA in Vero cells. The figure shown was exposed to film for 5 h, but no viral DNA in *hr99*-infected Vero cells was detected even after 1 week of exposure (data not shown). In contrast, *hr99* was able to synthesize wild-type levels of viral DNA in L2-5 cells (Fig. 5). These results clearly demonstrate that the UL5 gene is essential for HSV DNA synthesis, consistent with results obtained with the UL5 *ts* mutants.

The ability of *hr99* to form plaques in L2-5 cells and not in Vero cells clearly demonstrates that the lesion in *hr99* is in the UL5 gene. This was further verified by marker rescue experiments: transfections which included *hr99* DNA and p6UL5 generated approximately 4,000-fold more recombinants that grew on Vero cells than did transfections with *hr99* DNA alone (data not shown). These recombinants form white plaques in the presence of X-Gal, since wild-type UL5 sequences have replaced the UL5 gene containing a *lacZ* insertion. The ability to pick white plaques from a background of blue plaques forms the basis for the selection of additional UL5 mutations introduced into the viral genome (59).

Detection of UL5 protein in KOS- and *hr99*-infected Vero and L2-5 cells. In a previous report, polyclonal antibodies (α -UL5) raised against an *E. coli*-expressed UL5 protein were described (57). Immunoblot analysis of infected cell extracts with α -UL5 demonstrated that a protein of 99 kDa

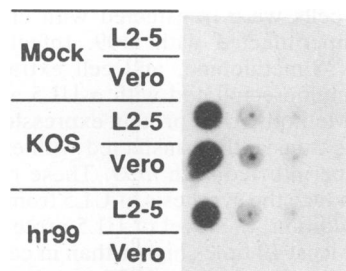


FIG. 5. Analysis of viral DNA synthesis. Vero cells or L2-5 cells (as marked) were infected with the indicated virus at 37°C for 18 h. For each sample, a series of fivefold dilutions of a cell suspension was spotted onto GeneScreen Plus membranes, and the cells were lysed on the membrane. ³²P-labeled *EcoRI* F fragment (coordinates 0.315 to 0.422 on the HSV genome) was used as a hybridization probe.

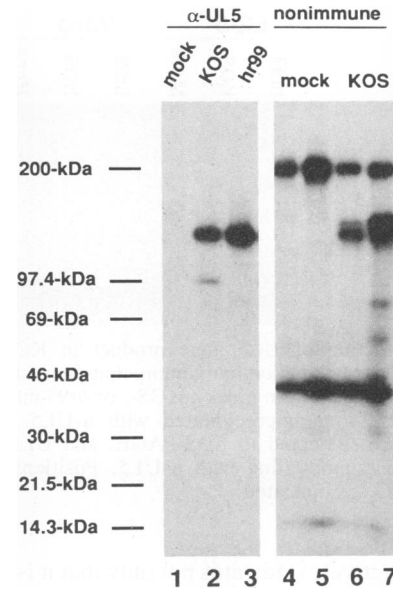


FIG. 6. Detection of UL5 protein in KOS- and *hr99*-infected cells by immunoprecipitation. Vero cells were mock infected or infected with KOS or *hr99* and labeled with [³⁵S]methionine as described in Materials and Methods. Cell extracts were prepared in RIPA buffer and precleared twice by precipitation with nonimmune rabbit serum and subsequently with α -UL5 (lanes 1 to 3). Lanes 4 and 6 are samples from the first preclearing step; lanes 5 and 7 are samples from the second preclearing step. Only preclearing steps of mock- and KOS-infected cell extracts are shown, although extracts from *hr99*-infected cells were treated identically prior to precipitation with α -UL5. Positions of molecular weight markers are indicated.

was barely visible but that a majority of the proteins recognized by the antibody migrated at positions corresponding to proteins of approximately 45 to 50 kDa (57). These proteins were not recognized by preimmune rabbit serum. The significance of these faster-migrating species was not known at the time. To improve the sensitivity of detection of the UL5 protein in infected cells and to determine whether the 45- to 50-kDa proteins correspond to an alternate form of the UL5 protein, infected cells were labeled with [³⁵S]methionine and subjected to immunoprecipitation as described in Materials and Methods. In the experiment shown in Fig. 6, extracts from mock-, *hr99*-, or KOS-infected cells were precleared twice by precipitation with nonimmune rabbit serum and then specifically precipitated with α -UL5 (lanes 1 to 3). In KOS-infected cell extracts, a band of 99 kDa can readily be detected after immunoprecipitation with specific antisera, although no such band was observed in *hr99*-infected cells (Fig. 6, lanes 2 and 3, respectively). The sensitivity of detection was greatly improved over that of the initial immunoblots, and the 45- to 50-kDa bands are not observed, indicating that these smaller bands were probably nonspecific reaction products. A protein of approximately 155 kDa was also precipitated by α -UL5 from KOS- and *hr99*-infected cells. This protein was not seen in immune complexes from mock-infected cell extracts (Fig. 6, lane 1), indicating that it was induced by HSV infection. To determine whether this protein reacted specifically with α -UL5, mock- or KOS-infected cell extracts were precipitated with nonimmune rabbit serum and subjected to electrophoresis. The presence of the 155-kDa protein in both the first and second preclearing steps in KOS-infected cells (Fig. 6, lanes

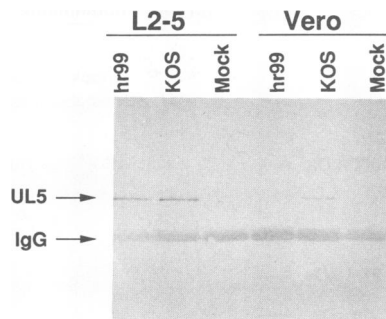


FIG. 7. Detection of UL5 gene product in KOS- and *hr99*-infected cells by combination immunoprecipitation and immunoblot analysis. Cell extracts from mock-, KOS-, or *hr99*-infected Vero or L2-5 cells were immunoprecipitated with α -UL5. The immune complexes were subjected to SDS-PAGE, and UL5 protein was detected by immunoblotting with α -UL5. Positions of the UL5 protein and IgG are indicated.

6 and 7, respectively) indicates not only that it is precipitated nonspecifically but also that it is a fairly abundant protein, since preclearing twice failed to deplete it. The absence of the 155-kDa band in the first and second preclearing steps from mock-infected cells (Fig. 6, lanes 4 and 5, respectively) confirms that it is virally induced.

To confirm the results obtained by immunoprecipitation and to further characterize UL5 expression in Vero and L2-5 cells, an alternative method for detecting immunoreactive material in infected cells was employed. Unlabeled extracts from mock-, *hr99*-, and KOS-infected cells were immunoprecipitated with α -UL5 and immune complexes were subjected to SDS-PAGE and immunoblot analysis with α -UL5 (Fig. 7). The only bands detected were the 99-kDa UL5 protein and a band corresponding to immunoglobulin protein present in the immune complexes (marked IgG). *hr99* is predicted to encode a peptide of approximately 20 kDa; however, a band of this size was not observed either by immunoprecipitation or by the combined immunoprecipitation-immunoblot method (Fig. 6, lane 3; Fig. 7), indicating that this truncated protein may not be stable. The inability of *hr99* to induce the expression of either full-length or a 20-kDa truncated version of the UL5 protein indicates that it can be considered to be a null mutant. The ability of the cell line L2-5 to synthesize the UL5 protein was investigated. The results of a combined immunoprecipitation and immunoblot analysis are presented in Fig. 7. In mock-infected L2-5 cells, no UL5 protein could be detected, indicating that UL5 expressed from p6UL5 is not constitutively expressed in these cells. On the other hand, in *hr99*-infected L2-5 cells, significant amounts of the UL5 gene product were readily detected (Fig. 7). These results demonstrate that although *hr99* is incapable of synthesizing full-length UL5 protein in infected Vero cells, it is capable of providing the necessary factors in *trans* for the induction of the ICP6 regulatory element, allowing expression of the UL5 gene in infected L2-5 cells (see Discussion). In addition, this experiment confirms that the ability of L2-5 cells to support viral DNA replication and growth of *hr99* is due to its ability to provide the missing UL5 protein.

Detection of UL5 in transient and stable transfections. To demonstrate directly that the ICP6 regulatory region is significantly more efficient than the UL5 endogenous promoter region in directing the synthesis of UL5 protein, levels of UL5 protein expression level from the two promoters

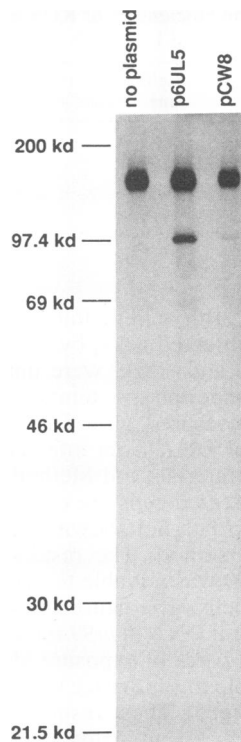


FIG. 8. Detection of UL5 protein expressed in Vero cells transiently transfected with plasmid pCW8 or p6UL5. Vero cells were transfected with 12 μ g of pCW8 or p6UL5 as indicated. In the leftmost lane, Vero cells were transfected with 12 μ g of salmon sperm DNA. After transfection, cells were superinfected with *hr99* and labeled with [35 S]methionine, and extracts were immunoprecipitated with α -UL5 as described in Materials and Methods. [14 C]-labeled rainbow protein molecular weight markers (Amersham) were electrophoresed in parallel. Positions of molecular weight markers are indicated.

were compared in a transient transfection experiment. As shown above, *hr99* is unable to synthesize UL5 protein but can *trans* activate the ICP6 promoter in L2-5 cells, leading to the expression of the UL5 protein; therefore, it seemed logical that *hr99* would also be able to induce p6UL5 in transient transfection assays. In pCW8, the UL5 gene is under control of its own promoter and is also expected to be induced by superinfection with *hr99*, since the expression of immediate-early genes is predicted to be normal in this mutant. Vero cells were transfected with either pCW8 or p6UL5 and superinfected with *hr99*. Infected cells were labeled with [35 S]methionine, and cell extracts were prepared and immunoprecipitated with α -UL5 as described in Materials and Methods. UL5 protein expression (Fig. 8) was induced in cells transiently transfected with either pCW8 or p6UL5 and superinfected with *hr99*. These results confirm that *hr99* can induce the synthesis of UL5 from either p6UL5 or pCW8. In addition, the level of UL5 protein synthesized by p6UL5 is at least 10 times higher than in cells transfected with pCW8, indicating that the ICP6 promoter is considerably stronger than the endogenous UL5 gene promoter. The relative strength of the ICP6 promoter is also indicated by analysis of cell lines transfected with pCW8 (E-cell lines) or with p6UL5 (L-cell lines). As mentioned above, several such cell lines were isolated, some capable of supporting the growth of both *tsM19* and *tsK13* and others capable of

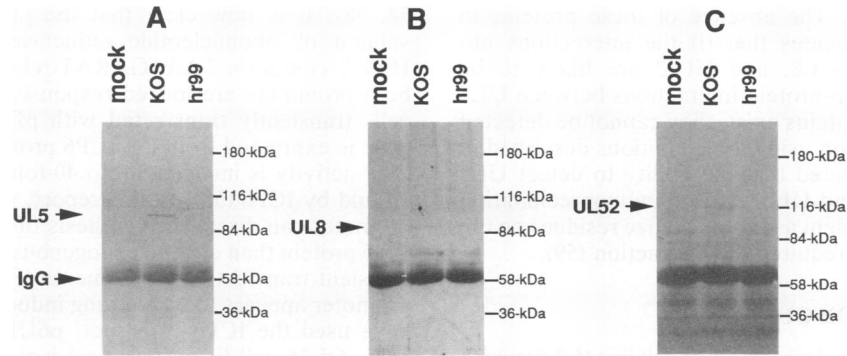


FIG. 9. Detection of UL5 protein interaction with UL8 and UL52 proteins by coimmunoprecipitation. Extracts from mock-, KOS-, or *hr99*-infected Vero cells were immunoprecipitated with α -UL5. Immune complexes were divided into three aliquots and subjected to electrophoresis in parallel. Each blot was probed with either α -UL5 (A), α -UL8 (B), or α -UL52 (C). Positions of the UL5 and IgG proteins are marked in panel A; positions of the UL8 and UL52 proteins are marked in panels B and C, respectively. Positions of molecular weight markers are indicated.

supporting the growth of *tsM19* only. We suggested that the ability to support both *ts* mutants was due to the levels of expression of the UL5 gene. This hypothesis was confirmed by immunoprecipitation of [35 S]methionine-labeled extracts from representative cell lines. When E4 cells, containing approximately 50 copies of pCW8, were infected with *hr99*, no detectable UL5 protein was observed; however, in *hr99*-infected L-cell lines, containing between 1 and 60 copies of p6UL5, the UL5 protein could be detected (data not shown). In these cells, the amount of UL5 protein correlates well with the copy number of the UL5 gene. Barely detectable UL5 protein was observed in one cell line (L1-18) which contains approximately one copy of p6UL5 and is not supportive of *tsK13*; on the other hand, considerably more protein was seen in two permissive cell lines, L2-13 and L2-5, containing 2.5 and 60 copies of the plasmid, respectively (data not shown). The fact that E4 and L2-5 cells which contain approximately the same numbers of copies of the UL5 gene vary so greatly in levels of UL5 synthesis confirms that the ICP6 promoter is much stronger than the endogenous UL5 promoter. In addition, the observation that the cell lines which support both *ts* mutants exhibit higher levels of UL5 protein than do cell lines which do not support *tsK13* confirms our hypothesis that the ability to support both *ts* mutants is due to levels of UL5 expression.

Detection of associations among UL5, UL8, and UL52.

UL5, UL8, and UL52 proteins were copurified as an active helicase-primase complex, indicating that physical associations among the proteins exist (9). We wished to provide additional evidence for the specific interaction between these three proteins. We reasoned that since UL5 could be immunoprecipitated with α -UL5, it might be possible to coimmunoprecipitate UL8 and UL52 with the same sera. Immunoprecipitation experiments described above (Fig. 6) in which [35 S]methionine-labeled KOS-infected cell extracts were precipitated with α -UL5 did not reveal any other proteins specific to KOS-infected cells; however, in those experiments, cells were lysed in RIPA buffer containing deoxycholate, Nonidet P-40, and SDS and the immune complexes were washed very stringently to decrease non-specific background. To detect interactions between proteins, the conditions were modified as described in Materials and Methods. In addition, instead of looking for UL8 and UL52 directly in immune complexes after SDS-PAGE, the presence of UL8 and/or UL52 in the α -UL5 immune complexes was determined by immunoblot analysis using α -UL8

or α -UL52 (see Materials and Methods). To establish the specificity of coimmunoprecipitation in KOS-infected cells, *hr99*, which is unable to synthesize UL5 protein, was used to infect cells in parallel. In the experiment shown in Fig. 9, mock-, *hr99*- or KOS-infected Vero cell extracts were immunoprecipitated with α -UL5. The immune complexes were washed three times with TNE buffer and resolved by SDS-PAGE. Three identical protein gels were blotted onto Immobilon membranes and probed with α -UL5, α -UL8, or α -UL52 to detect the presence of each protein in each blot. α -UL52 raised against a C-terminal oligopeptide was previously reported to immunoprecipitate a 115-kDa protein from KOS-infected cell extracts (36). α -UL8 reacts specifically with a protein of approximately 85 kDa from KOS-infected cells which is absent in cells infected with a UL8 null mutant (3, 43a). In the gel system that we used, UL8 consistently migrates at approximately 85 kDa whether it is expressed in KOS-infected cells or in cells transiently transfected with three different UL8 expression clones, slightly higher than the previously reported size of 80 kDa or smaller (9, 36). Figure 9A demonstrates that as shown above, if KOS-infected cell extracts are initially precipitated with α -UL5, UL5 can be detected by immunoblot analysis and no UL5 protein is detected in *hr99*- or mock-infected cells. In Fig. 9B and C, UL8 and UL52 (marked by arrows) are detected in immune complexes from KOS-infected cells precipitated with α -UL5. The bands marked UL8 and UL52 comigrated with UL8 and UL52 proteins detected by the combined immunoprecipitation-immunoblot method using antisera to UL8 and UL52, respectively (data not shown). That these interactions specifically require UL5 is suggested by the fact that they are not observed in *hr99*- or mock-infected cells (Fig. 9, lanes *hr99*). In Fig. 9B, a protein just above the IgG band is also visible. The appearance of this band was not reproducible, and it was not observed in extracts immunoprecipitated and immunoblotted with α -UL8. Therefore, we believe that it is nonspecific. The results presented in Fig. 9 indicate that we can detect specific physical interactions between members of the helicase-primase complex. These results are consistent with the fact that these three proteins can be copurified as an helicase-primase complex from infected cells (8, 9). To further demonstrate the specificity of this interaction, the UL5 immune complexes were assayed for the presence of several other viral proteins by immunoblot. We tested and were unable to detect the presence of the UL9, DNA polymerase, alkaline nuclease, and ICP8 pro-

teins (data not shown). The absence of these proteins in immune complexes indicates that (i) the interactions observed between UL5, UL8, and UL52 are likely to be specific and (ii) if protein-protein interactions between UL5 and other replication proteins exist, they cannot be detected by coimmunoprecipitation using the conditions described in this report. It is anticipated that the ability to detect UL5 association with UL8 and UL52 by coimmunoprecipitation should provide a convenient assay to localize residues on the UL5 protein which are required for interaction (59).

DISCUSSION

In this study, we isolated a permissive cell line (L2-5) which contains the wild-type UL5 gene and is capable of supporting the growth of *ts* mutants in the gene at the nonpermissive temperature. A mutant with a *lacZ* insertion in the UL5 gene, *hr99*, was isolated and propagated in L2-5 cells. The expression of UL5 protein in mutant- and KOS-infected Vero and L2-5 cells was monitored by immunoprecipitation and by immunoblotting with a UL5-specific antiserum. In addition, coimmunoprecipitation was used to detect the specific interaction between UL5, UL8, and UL52.

Isolation of a complementing cell line. In our first attempt to isolate a permissive cell line for UL5 mutants, Vero cells were cotransfected with pSV2neo and pCW8, a plasmid containing the UL5 gene under the control of its endogenous promoter. The resulting cell lines (the E series) were able to support the growth of a mutant with a *ts* mutation in the UL5 gene, *tsM19*; however, these cell lines were not able to support another UL5 *ts* mutant, *tsK13*. We hypothesized that the level of UL5 expression from its endogenous promoter in the E-series cells is too low to allow efficient complementation of certain mutants. That the UL5 promoter itself is weak is suggested by two observations: (i) the UL5 protein is not abundant in the infected cells (36, 57) and (ii) initial attempts to isolate an in-frame *lacZ* insertion mutant in which the *lacZ* gene is expressed from the endogenous UL5 promoter failed, presumably because β -galactosidase levels were insufficient for blue color formation.

To isolate a cell line capable of increased levels of UL5 protein expression, we wished to use a promoter which is both strong and inducible, since it was possible that overexpression of UL5 in a constitutive manner might be toxic to cells. The ICP6 promoter was chosen for many reasons: ICP6 is an abundant protein in infected cells, and its unusual regulation has generated considerable interest. Although in infected cells the kinetics of induction of ribonucleotide reductase activity is similar to that of other HSV early proteins such as thymidine kinase (26), the large subunit, ICP6, is regulated in some respects like an immediate-early gene. The ICP6 transcript and protein are expressed within 2 h postinfection (21–23), and small amounts of the transcript are synthesized in the presence of cycloheximide (49). Furthermore, the ICP6 transcript and protein are expressed in ICP4 mutants under nonpermissive growth conditions (10, 11, 28, 33). We have previously reported the isolation of cell lines (D14) containing the entire ICP6 gene (18). In D14 cells, ICP6 is not constitutively expressed and its synthesis is induced by ICP0 but not ICP4, unlike typical early (β) promoters. The existence of an immediate-early-type *cis* response element (TAATGARAT) in the promoter for ICP6 (Fig. 1) provides a partial explanation for its unusual regulation. This sequence found upstream from several immediate-early genes is the target for the viral transcriptional activator VP16 (also known as Vmw65 and α -TIF) (16, 24,

34, 38). It is now clear that the promoters for the large subunit of ribonucleotide reductase in both HSV-1 and HSV-2 contain a TAATGARAT-related sequence and that both promoters are indeed responsive to VP16 (56, 58). In cells transiently transfected with pD6p in which the *lacZ* gene is expressed from the ICP6 promoter (19), β -galactosidase activity is induced 30- to 40-fold by VP16 and at least 10-fold by ICP0 (58). In this report, we show that the ICP6 promoter can direct the synthesis of at least 10 times more UL5 protein than can the endogenous UL5 gene promoter in transient transfection experiments (Fig. 8). Thus, the ICP6 promoter appears to be a strong inducible promoter, and we have used the ICP6 construct, p6UL5, to transform Vero cells. Of 16 cell lines (L series) isolated, 13 were found to support the growth of both *tsM19* and *tsK13*. We speculate that this is due to increased expression of UL5 in these L-cell lines. The three L-cell lines which were unable to support the growth of *tsK13* contained only one copy of the UL5 gene per haploid genome, and one such cell line synthesized barely detectable levels of UL5 when induced by infection with *hr99*. The 13 complementing cell lines, on the other hand, contained between 2 and 60 copies per haploid genome, and two such cell lines synthesized easily detectable UL5 following *hr99* infection. These results support our hypothesis that ability to complement *tsK13* correlates with levels of UL5 expression. Taken together, these results indicate that the ICP6 promoter is both strong and inducible and has general application in the overexpression of poorly expressed HSV proteins.

Isolation of an ICP6::*lacZ* insertion mutant. To isolate a mutant which would be incapable of expressing functional UL5, the gene was disrupted by the insertion of an ICP6::*lacZ* mutagenic cassette at the unique *Bgl*III site within the gene. *hr99*, a recombinant virus capable of forming blue plaques, was isolated and propagated in L2-5 cells. This mutant failed to produce plaques or synthesize viral DNA when grown in Vero cells, consistent with previous results with *ts* mutants indicating that UL5 is essential for viral DNA replication (57). This insertion mutant has several advantages over previously identified *ts* mutants in the UL5 gene for the analysis of functional aspects of the UL5 protein. Since no UL5 gene product was detected in *hr99*-infected cells, it will now be possible to characterize functional activities of mutant UL5 proteins in complementation assays without interference from *ts* gene products (59). The fact that *hr99* does not synthesize detectable UL5 protein also makes it ideal as a control in coimmunoprecipitation experiments designed to study UL5 interaction with other HSV proteins (see below). In addition, *hr99* is able to provide the necessary *trans*-acting factors for the induction of the ICP6 promoter in p6UL5 not only in stable cell lines but also in transiently transfected cells. Furthermore, the availability of the blue plaque mutant, *hr99*, provides a convenient system for screening for the introduction of site-specific mutations into the viral genome (59).

UL5 interaction with other HSV proteins. The specific interaction of UL5 with other HSV replication proteins was originally suggested by the fact that partially purified preparations of the HSV-1 helicase contained three polypeptides (8). By using specific antisera, the three proteins were subsequently identified as the products of the UL5, UL8, and UL52 genes (9). In this report, we provide additional evidence for the specific interaction between these three proteins. We show that the UL52 and UL8 gene products are specifically coimmunoprecipitated with UL5 protein by α -UL5 (Fig. 9). This interaction was observed by immunoprecipitation of

infected cell extracts with α -UL5 followed by SDS-PAGE of the immunocomplexes; UL8 and UL52 were detected by immunoblot with appropriate antibodies. This method was developed to circumvent high levels of background observed in direct immunoprecipitation of [35 S]methionine-labeled proteins. The specificity of this method was demonstrated by including *hr99* as a control; no UL8 or UL52 is precipitated by α -UL5 in *hr99*-infected cells (Fig. 9). This assay will provide a system to map functional domains required for specific protein-protein interactions. In the accompanying report (59), three mutants which abolish the replication ability of the UL5 protein are shown to maintain their ability to form specific interactions with UL8 and UL52. This result strongly suggests that the sites for interaction are separable from regions of the UL5 protein required for replication activity. Further site-directed mutagenesis will be required to extend these initial findings and demonstrate whether these interactions are essential for enzymatic function.

Function of UL5 in helicase-primase activity. UL5, UL8, and UL52 can be coexpressed in insect cells to form a functional complex exhibiting helicase and primase activities (12). A subcomplex made up of only UL5 and UL52 was shown to exhibit helicase (1) and subsequently helicase and primase (13) activities. The question of which activities are associated with each protein has not been addressed directly because neither UL5 nor UL52 appears to be functional when expressed alone (1, 12, 13); however, the presence of six conserved helicase motifs in the UL5 gene (59) strongly suggests that the UL5 protein will turn out to be associated with helicase activity. It is possible however, that correct association between the UL5 and UL52 is required for both helicase and primase activity. The UL5-UL52 complex may be analogous to the primeosome of T4 bacteriophage, which is believed to consist of two proteins, a helicase (gene 41) and a primase (gene 61). The T4 primase-helicase requires both the 41 and 61 proteins for optimal primase and helicase activities (29, 41). In the HSV system, the question of which proteins have intrinsic helicase and primase activities awaits further genetic and biochemical characterization. It is anticipated that structure-function analysis of the relevant genes will not only aid in the assignment of function to the individual members of the complex but also define protein domains that contribute to the enzymatic activities of helicase and primase (59).

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