An ICP6::lacZ Insertional Mutagen Is Used To Demonstrate that the UL52 Gene of Herpes Simplex Virus Type 1 Is Required for Virus Growth and DNA Synthesis

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An insertional mutagen was developed which consists of the *lacZ* gene of *Escherichia coli* under the control of the regulatory elements of the large subunit of ribonucleotide reductase (ICP6). This ICP6::lacZ cassette was used to create a mutation in a gene designated UL52 (D. J. McGeoch, M. A. Dalrymple, A. Dolan, D. McNab, L. J. Perry, P. Taylor, and M. D. Challberg, *J. Virol.* 62:444-453, 1988), which is predicted to encode a 114,000-molecular-weight protein. To isolate and propagate this mutant, we generated a cell line, BL-1, by cotransfection of Vero cells with pSV2neo and a plasmid containing the herpes simplex virus type 1 KOS strain *Bam*HI L fragment (coordinates 0.708 to 0.745). An ICP6::lacZ insertion mutant, *hr114*, was capable of growing in BL-1 cells but not in normal Vero cells. In addition, *hr114* was defective in the synthesis of viral DNA and late proteins; however, this mutant appeared to exhibit normal early gene expression. Thus, the results presented in this report show that the UL52 gene product is required for viral DNA synthesis. Furthermore, our studies indicate that the ICP6::lacZ cassette will provide a useful tool for obtaining mutants of other herpes simplex virus genes.

The herpes simplex virus type 1 (HSV-1) genome is large (150 kilobases [kb]) and encodes many factors involved either directly in DNA chain growth or indirectly in DNA precursor biosynthesis, including DNA polymerase (26), the major DNA-binding protein (8, 17, 33), ribonucleotide reductase (7, 10), thymidine kinase (9), alkaline nuclease (13, 24), dUTPase (25), and uracil-DNA glycosylase (2). Of these, only the DNA polymerase and the major DNA-binding protein have been shown to be essential for viral DNA synthesis through the analysis of temperature-sensitive (*ts*) and other mutants (8, 26, 33). The analysis of several DNA-negative mutants has led to the identification of three additional genes whose products are also essential for viral DNA replication. These mutants, representing complementation groups 1-10 (32; L. Zhu and S. K. Weller, *Virology*, in press), 1-26 (E. P. Carmichael and S. K. Weller, unpublished data), and 1-36 (3, 20), have mutations which have been mapped to open reading frames designated UL5, UL8, and UL9, respectively (21).

An alternative approach to the identification of *trans*-acting functions required for viral replication has been developed by Challberg (5). Using a transient transfection assay, Challberg has identified seven viral genes that are required to amplify plasmids containing an HSV origin of replication (5, 35). Of these, only the DNA polymerase and major DNA-binding protein are factors whose functions have been previously assigned; the rest correspond to open reading frames UL5, UL8, UL9, UL42, and UL52 as revealed by the sequence data of McGeoch et al. (21). As described above, we have shown genetically that UL5, UL8, and UL9 are essential for viral DNA replication in infected cells. Thus, a good correspondence exists between genes required for plasmid amplification in a transient assay and

genes demonstrated by genetic means to be required for viral DNA replication in cells in culture.

To provide direct genetic evidence for the requirement of the UL52 gene product in viral DNA synthesis, we set out to isolate a null mutant carrying a potentially lethal defect in this gene. We recently described a strategy for insertional mutagenesis in HSV genes (14) by using an approach first used to construct vaccinia virus mutants (4, 23). The *Escherichia coli lacZ* gene was inserted in frame into the gene for the large subunit of the viral ribonucleotide reductase, ICP6, such that a fusion protein was synthesized under the control of the endogenous ICP6 regulatory signals (14). The advantage of using *lacZ* is that viral recombinants containing insertions can be easily detected in the presence of the chromogenic substrate for β -galactosidase (β -gal), 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-Gal). It was apparent from our studies with the ICP6 *lacZ* insertion mutant that substantial levels of ICP6:: β -gal fusion protein were synthesized under the control of the ICP6 regulatory signals. In the work described in this report, we exploited this phenomenon and constructed an ICP6::lacZ gene fusion which synthesizes β -gal under the control of the regulatory elements of the ICP6 gene. A cassette containing this gene fusion was then used as an insertional mutagen to isolate a mutant of the UL52 gene. Our results provide genetic evidence that the UL52 gene is essential for viral DNA replication in infected cells. In addition, our studies indicate that this insertional mutagen provides a useful tool for obtaining mutants of other HSV genes (Carmichael and Weller, unpublished).

MATERIALS AND METHODS

Cells and viruses. Procedures for the growth and maintenance of African green monkey kidney cells (Vero; American Type Culture Collection, Rockville, Md.) have been previously described (33). BL-1 cells (see below) were maintained in the same way as Vero cells, except that the

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FIG. 1. Map location of the UL52 gene. The figure shows an expanded version of the *Bam*HI L fragment from the HSV-1 KOS genome and the relative position of the open reading frame designated UL52 by McGeoch et al. (21). Plasmid pKB-L contains the entire *Bam*HI L fragment and was used with pSV2neo to construct the cell line BL-1. Da, Daltons.

medium contained 250 μ g of G418 (Geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml.

The KOS strain of HSV-1 was used as the wild-type virus. Mutants *tsA24* (complementation group 1-1), *tsJ12* (complementation group 1-9), and *tsM19* (complementation group 1-10) were obtained from P. Schaffer and have been previously described (27). *ts1206*, derived from HSV-1 strain 17, was provided by V. Preston and has been previously described (20).

Plasmids and bacteria. Plasmid pKB-L contains the *Bam*HI L fragment of HSV-1 KOS cloned into the *Bam*HI site of vector Bluescribe (Vector Laboratories, San Diego, Calif.) and was generously provided by M. Challberg, National Institute of Allergy and Infectious Diseases, Bethesda, Md. (35) (Fig. 1). pSV2neo contains the bacterial gene for neomycin resistance under the control of the simian virus 40 early promoter (30). Plasmid pD6p was constructed as depicted in Fig. 2. A 1.2-kb *Sma*I fragment (coordinates 0.557 to 0.565 of strain KOS from plasmid pKHF [14]) containing upstream regulatory regions and 180 base pairs of the coding region of the ICP6 gene (22) was blunt-end ligated into the unique *Sma*I site of plasmid pDP503 (generously provided by D. Panicali [23]). This construct generates an in-frame gene fusion between the 5' end of the coding region of the ICP6 gene and the structural gene for *E. coli* β -gal. The *Bam*HI fragment from pD6p, containing the ICP6::*lacZ* gene fusion, can be used as an insertional mutagen and will be designated as the ICP6::*lacZ* cassette in this publication. Plasmid pBL-D6p was constructed by ligating the ICP6::*lacZ* cassette into the unique *Bcl*I site of plasmid pKB-L (Fig. 1). All recombinant plasmids were propagated in either

E. coli DH5 or the *dam* strain, 1275 (for propagation of pKB-L), by standard procedures (19).

Transformation of Vero cells and isolation of cell line BL-1. Vero cells were cotransfected with plasmids pKB-L and pSV2neo as described previously (14). G418-resistant colonies were picked and screened for the presence of the *Bam*HI L fragment as described in Results.

Marker rescue analysis. Marker rescue was performed by transfecting cells in suspension with intact infectious mutant DNA and individual linearized clones containing regions of wild-type DNA. The transfection procedure was based on the method developed by Graham and Van der Eb (15). A total of 10^6 cells were added to each tube and pelleted by low-speed centrifugation. To each cell pellet was added 0.6 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline solution containing 200 to 1,000 PFU of viral DNA (ca. 1 μ g), a 10-fold molar excess of a cloned HSV DNA fragment (ca. 1 to 2 μ g), carrier salmon sperm DNA to bring the final DNA concentration to 20 mg/ml, and CaCl_2 at a final concentration of 125 mM. The DNA was allowed to precipitate for 20 to 25 min at room temperature before being added to the cell pellet. After a 30-min incubation in a shaking incubator or with agitation at 37°C, 5×10^5 fresh cells were added to each tube in a total of 5 ml of growth medium. The suspension was mixed well, used to seed duplicate 35-mm petri dishes, and incubated at 37°C. At 4 and 16 h after seeding, the medium was removed and fresh medium was added. When maximal cytopathic effect was observed, duplicate cultures were scraped into media, pooled, and frozen at -70°C . The infectious virus was

FIG. 2. Strategy for the construction and use of the ICP6::lacZ cassette. Plasmid pKHF contains the *Hpa*I F fragment of HSV-1 KOS (coordinates 0.535 to 0.620) which encodes the large (ICP6) and small (38-kilodalton [38K]) subunits of the viral ribonucleotide reductase. Shown are relative positions of the reductase open reading frames. A 1.2-kb *Sma*I fragment, containing the upstream regulatory elements of the ICP6 gene and 180 base pairs of the ICP6 coding region, was cloned into a unique *Sma*I site upstream of the structural gene of *lacZ* of plasmid pDP503 such that β -gal could be synthesized under the control elements of the ICP6 gene. The resulting plasmid, pD6p, contains an ICP6::lacZ cassette which can be excised by digestion with *Bam*HI and used as an insertional mutagen. Plasmid pBL-D6p was constructed by cloning the isolated ICP6::lacZ cassette into the compatible *Bcl*II site of plasmid pKB-L, which disrupts the UL52 gene at this site. A stop codon exists at the end of the *lacZ* gene, thereby preventing the synthesis of the remaining 90% of the UL52 gene product. Plasmid pBL-D6p was used in a marker transfer experiment for the construction of mutant *hr114*.

processed as described previously (33), and titers in Vero and BL-1 cells were determined.

Complementation tests. Complementation tests were carried out as previously described (28). Complementation indices (CIs) were calculated from the formula $CI = (A + B)_{np} / (A_{np} + B_{np})$, where *A* and *B* refer to the titers of two mutants being tested and *np* refers to the nonpermissive growth condition. Virus yields from infections of Vero cells at 39.5°C (nonpermissive condition) were assayed for plaque formation in BL-1 cells at 34°C (permissive condition).

Marker transfer and isolation of *hr114*. Marker transfer experiments were performed essentially as described above for marker rescue. Briefly, 200 to 1,000 PFU (ca. 1 μ g) of wild-type viral DNA were cotransfected with a 10-fold molar excess of the recombinant fragment. For isolation of mutant *hr114*, the 9.2-kb insert of plasmid pBL-D6p was excised by digestion with *Bam*HI and used with infectious KOS DNA to cotransfect BL-1 cells. When maximal cytopathic effects were observed, progeny were harvested and titers were determined on the permissive BL-1 cells. Recombinant viruses were detected with X-Gal and neutral red as described by Goldstein and Weller (14). Blue plaques were picked and purified four times in BL-1 cells before stocks were made.

Viral and cellular DNA isolation. *hr114* and KOS DNAs were prepared from partially purified virions as follows. BL-1 or Vero cells were infected with KOS or mutant virus at a multiplicity of infection of 0.005 PFU per cell and

incubated at 37°C until maximal cytopathic effect was observed. Crude HSV virions were isolated as described previously (6), except that Vero cells were used instead of RK cells and the sucrose gradient was omitted. HSV DNA was isolated by using a procedure based on the method of Longmire et al. (18). Virus pellets were lysed by the addition of sodium dodecyl sulfate and proteinase K to final concentrations of 0.1% and 100 μ g/ml, respectively. Tubes were inverted twice and incubated at 37°C overnight. Samples were dialyzed and concentrated by using an autoclaved Schleicher & Schuell model UH020/2A microdialysis apparatus at room temperature against four 60-min changes of sterile 20% (wt/vol) polyethylene glycol 8000 (Sigma Chemical Co., St. Louis, Mo.) in TE. Sample volumes decreased approximately 10-fold during polyethylene glycol dialysis. Desalting was accomplished by dialysis against two or three 30-min changes of sterile TE at room temperature. DNA samples were carefully transferred (to avoid shearing) to sterile tubes and stored at -70°C. Cellular DNA was isolated as described by Weller et al. (34).

Analysis of cellular and viral DNA. Total cellular or viral DNA was digested with the restriction endonuclease *Bam*HI, separated by agarose gel electrophoresis in a Tris-borate buffer, and transferred by the method of Southern (29) to GeneScreen Plus filters (New England Nuclear Corp., Boston, Mass.) as recommended by the supplier. DNAs used as hybridization probes were labeled by the method of Feinberg and Vogelstein (12).

Synthesis of viral DNA in infected cells. Viral DNA synthesis was analyzed as described by Aron et al. (1), except that 150 μ g of proteinase K per ml was used instead of pronase. Levels of [3 H]thymidine incorporation into viral DNA were measured in either Vero or BL-1 cells.

Analysis of viral proteins. Vero or BL-1 cells were infected with KOS or *hr114* at a multiplicity of infection of 10 PFU per cell. Cells were labeled at 5 h with [35 S]methionine as previously described (32) and were harvested at 18 h post-infection. Extracts were prepared as described previously (14) and analyzed by polyacrylamide gel electrophoresis with 9% polyacrylamide (acrylamide-bisacrylamide, 30:0.8) containing 0.1% sodium dodecyl sulfate. Gels were fixed in methanol-water-acetic acid (5:5:1, vol/vol/vol), treated with Autofluor (National Diagnostics Inc., Manville, N.J.), dried, and exposed at -70°C to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

The UL52 gene is contained within the *Bam*HI L fragment of the HSV-1 genome (coordinates 0.708 to 0.745) and is predicted to encode a 114-kilodalton protein (21) (Fig. 1). To provide genetic evidence that the UL52 gene product is essential in viral DNA replication, we set out to isolate a mutant with a null mutation in this gene. Null mutations are especially useful because they express no residual activity under nonpermissive growth conditions (i.e., they are not leaky). To isolate and propagate potentially lethal null mutations in essential genes, cell lines that can express the wild-type version of the gene must be isolated.

Permissive hosts for UL52 mutants. Permissive cell lines containing the UL52 gene were constructed as previously described (14) by using the plasmid pKB-L (Fig. 1) and a plasmid containing the gene conferring neomycin resistance (pSV2neo). Following transfections, G418-resistant colonies were isolated and screened for the presence of DNA from the *Bam*HI L region by Southern blot analysis. Total cellular DNA was isolated, digested with *Bam*HI, subjected to electrophoresis, transferred to a filter, and probed with the gel-purified and labeled *Bam*HI L fragment. Although several cell lines were shown to contain the entire *Bam*HI L fragment, lines containing between 5 and 10 copies per haploid genome (BL-1 and BL-9, data not shown) were used in subsequent experiments.

Isolation of an ICP6::lacZ insertion mutant with a mutation in the UL52 gene. We initially attempted to construct a mutant which would disrupt the UL52 gene with an in-frame insertion of the structural gene of *E. coli* β -gal at the *Bcl*I site (coordinate 0.719) shown in Fig. 1. This construct was designed such that a protein containing the first 181 amino acids of the UL52 gene product fused to β -gal would be synthesized under the control of the endogenous UL52 regulatory signals. Recombinant viruses should easily be detected by screening for β -gal activity in the presence of the chromogenic substrate X-Gal. The transformed cell lines BL-1 and BL-9 were transfected with a plasmid containing the desired mutation and infectious KOS DNA in a marker transfer experiment. However, upon screening for viral recombinants in the presence of X-Gal, no blue plaques were detected in two separate marker transfer experiments. We consider it likely that the UL52 regulatory signals generate insufficient β -gal expression to detect blue plaques (see Discussion).

As mentioned in the Introduction, our previous studies indicated that the ICP6 *lacZ* insertion mutant, *hrR3*, gener-

FIG. 3. Southern blot analysis of KOS and *hr114* DNA. KOS and *hr114* DNAs were digested with *Bam*HI and subjected to electrophoresis in parallel with *Bam*HI-digested pKB-L and pBL-D6p. DNAs were separated on a 0.8% agarose gel, blotted onto GeneScreen Plus, and hybridized to either the 32 P-labeled *Bam*HI L fragment (HSV probe; left-hand blot) or the *lacZ* fragment (right-hand blot). The 3.2-kb fragment present in the right-hand blot represents linearized Bluescribe vector which contains *lacZ* sequences that hybridize to the *lacZ* probe.

ates levels of β -gal synthesis which are easily detected in the presence of X-Gal in infected cells. To take advantage of the control elements of the ICP6 gene, the upstream regulatory region, including 60 amino acids of the N terminus of ICP6, was fused in frame to the structural gene of β -gal from clone pDP503 to generate plasmid pD6p (Fig. 2; see Materials and Methods). Restriction digestion of plasmid pD6p with *Bam*HI results in the excision of an ICP6::lacZ cassette which can be used as an insertional mutagen. Plasmid pBL-D6p was constructed by inserting the ICP6::lacZ cassette into the *Bcl*I site at coordinate 0.719 of plasmid pKB-L and was used to isolate a UL52 mutant as described below. Introduction of these sequences into the viral genome at the *Bcl*I site should result in the disruption of the UL52 gene product and synthesis of the ICP6::lacZ fusion protein driven by the control elements of the ICP6 gene (Fig. 2).

To construct a null mutant in the UL52 gene, we used the plasmid pBL-D6p (Fig. 2) and infectious KOS DNA to cotransfect BL-1 and BL-9 cells in a marker transfer experiment as described in Materials and Methods. Recombinant viruses were detected by staining progeny from the marker transfer experiment with X-Gal. Blue plaques appeared at a frequency of about 1% when the cell line BL-1 was used, whereas no blue plaques appeared when BL-9 cells were used. These results indicated that BL-9 may be incapable of supporting a lethal UL52 mutant and was therefore not used in further experiments. Blue plaque recombinant viruses were picked, purified, and propagated in BL-1 cells. One recombinant virus, *hr114*, was used in further studies.

Restriction analysis of *hr114*. To confirm that *hr114* contains the ICP6::lacZ cassette at the appropriate position, DNAs from KOS and *hr114* were purified, digested with *Bam*HI, and subjected to gel electrophoresis and Southern blot hybridization in duplicate as described in Materials and Methods (Fig. 3). Plasmids pKB-L and pBL-D6p (containing the wild-type and mutant versions of the *Bam*HI L region,

TABLE 1. Plaquing efficiencies of *hr114*, KOS, two marker rescue isolates of *hr114*, and *ts1206* on Vero and BL-1 cells^a

Virus	Cell	Temp (°C)	Titer (PFU/ml)
KOS	Vero	34	1.3×10^9
KOS	BL-1	34	1.0×10^9
<i>hr114</i>	Vero	34	1.2×10^{3b}
<i>hr114</i>	BL-1	34	5.5×10^7
<i>hr114</i> ⁺ -1 ^c	Vero	34	2.1×10^7
<i>hr114</i> ⁺ -1	BL-1	34	8.4×10^6
<i>hr114</i> ⁺ -2 ^c	Vero	34	8.0×10^6
<i>hr114</i> ⁺ -2	BL-1	34	4.1×10^6
<i>ts1206</i>	Vero	34	2.4×10^7
<i>ts1206</i>	Vero	39.5	3.5×10^3
<i>ts1206</i>	BL-1	34	8.4×10^7
<i>ts1206</i>	BL-1	39.5	1.7×10^6

^a Plaquing efficiencies were measured by determining the titers of virus stocks on monolayers of Vero or BL-1 cells as indicated.

^b All plaques present failed to stain blue in X-Gal and most probably represent wild-type contaminants generated during preparation of stocks (see Results).

^c *hr114*⁺-1 and *hr114*⁺-2 are two independent isolates of *hr114* rescued with the *Bam*HI L fragment of strain KOS (coordinates 0.708 to 0.745).

respectively; Fig. 2) were also digested with *Bam*HI and subjected to electrophoresis in parallel. Duplicate filters were hybridized to either the ³²P-labeled *Bam*HI L fragment or the ³²P-labeled *lacZ* fragment from pDP503. The left half of Fig. 3 (HSV-1 probe) shows that pKB-L and KOS DNA contain the wild-type, 5.2-kb form of *Bam*HI fragment L, whereas pBL-D6p and *hr114* contain the 9.4-kb version expected if the ICP6::*lacZ* cassette were present. When the duplicate filter was hybridized with a *lacZ* probe (right-hand portion of Fig. 3), only the 9.4-kb fragment of pBL-D6p and *hr114* was detected. This shows that the ICP6::*lacZ* fragment of pBL-D6p was inserted into the expected position of the genome of *hr114*.

Growth properties of *hr114*. The mutant *hr114* was tested for its ability to grow in untransformed Vero cells. Table 1 shows the titers of mutant and KOS grown in either Vero or BL-1 cells. These results show that *hr114* produces plaques very inefficiently in Vero cells and that this growth defect can be complemented by the BL-1 cell line. However, *hr114* forms small plaques in BL-1 cells compared with the wild type, suggesting that BL-1 cannot fully compensate for the defect in UL52. In addition, the stock of *hr114* contains a very low level of wild-type contaminants (Table 1; frequency of 2 in 10⁵). Since the mutant was plaque purified extensively, these contaminating particles are most probably due to recombination with the wild-type version of the UL52 gene in the BL-1 cell line during preparation of stocks. The low levels of wild-type virus did not appear to affect the phenotypic analysis described below.

Viral DNA synthesis of *hr114*. The ability of *hr114* to synthesize viral DNA was analyzed by measuring the incorporation of [*methyl*-³H]thymidine into viral DNA in mutant-infected Vero or BL-1 cells. Wild-type- and mutant-infected cells were labeled, total DNA was isolated (1), and cellular and viral DNAs were resolved by equilibrium centrifugation in cesium chloride. Figure 4 shows the fractionation and density distribution of [³H]thymidine incorporation into viral and cellular DNA by the wild-type and mutant viruses. Table 2 summarizes these results and shows that the mutant was

incapable of synthesizing detectable viral DNA when grown in Vero cells. This defect, however, could be complemented to 85% of wild-type levels by the BL-1 cell line. Wild-type virus was capable of synthesizing substantial levels of DNA in either cell line. These results indicate that the lesion in mutant *hr114* is in a gene whose product is required for viral DNA synthesis.

Ability of *hr114* to induce viral polypeptides. Mutants with a block in viral DNA synthesis are defective in the induction of late viral (γ₁ and γ₂) polypeptides. To determine the ability of *hr114* to express viral proteins, we analyzed extracts of ³⁵S-labeled mutant- and KOS-infected Vero and BL-1 cells by polyacrylamide gel electrophoresis (Fig. 5). The pattern of protein synthesis of BL-1 cells infected with *hr114* resembled that of KOS-infected Vero or BL-1 cells (compare lane 6 with lanes 2 and 3). In contrast, *hr114*-infected Vero cells showed no detectable synthesis of true late (γ₂) polypeptides such as ICP1/2, ICP15, ICP19/20, ICP33, and ICP43/44 and reduced levels of early-late (γ₁) polypeptide such as ICP5 and ICP25 (lane 5). There was, however, no defect in the ability of the mutant to synthesize early polypeptides, as indicated by the presence of normal levels of proteins such as ICP8 and ICP36. The profile of *hr114*-infected Vero cells closely resembles the pattern of protein synthesis of KOS-infected Vero cells in the presence of the replication inhibitor, phosphonoacetic acid (lane 4). These features are consistent with a defect in the ability of *hr114* to synthesize viral DNA.

Complementation of *hr114* and other known HSV-1 mutants. Complementation tests were carried out to determine the relationship between mutant *hr114* and other HSV *ts* mutants. *ts1206*, a spontaneous mutant derived from strain 17, was included since its lesion has been mapped to within or near the region of the UL52 gene (between coordinates 0.708 and 0.720 [20]). The mutation in *hr114* was efficiently complemented by several other mutants including *tsM19*, *tsJ12*, and *tsA24* (Table 3). *hr114*, however, failed to complement *ts1206*, indicating that both mutations are in the same gene. The *ts1206* mutant forms small plaques in BL-1 cells at the nonpermissive temperature (39.5°C; Table 1), indicating that its growth can be complemented by BL-1 cells, albeit with low efficiency.

Marker rescue of *hr114*. To confirm that the growth defects of *hr114* were due exclusively to the insertion in the UL52 gene, we performed a marker rescue experiment in which BL-1 cells were transfected with infectious *hr114* DNA either alone or with plasmid pKB-L (coordinates 0.708 to 0.745). The titer of progeny virus in Vero cells was then determined. Since *hr114* plaques are blue in the presence of X-Gal, wild-type recombinants were easily detected by screening for white plaques. Plasmid pKB-L efficiently rescued the mutation in *hr114*, since we observed at least 100-fold more white plaques than when pKB-L was excluded from marker rescue transfections. Two white plaques were picked, purified, and analyzed for their ability to produce wild-type-sized plaques in Vero cells. Both had restored wild-type plaque size and produced high titers in Vero cells (Table 1), indicating that the insertion into the UL52 gene is most probably responsible for the defects observed in the growth properties of *hr114*. It should be noted that it is not possible to distinguish between white plaques obtained from recombination between input plasmid and mutant DNA and from recombination between the resident UL52 genes in the BL-1 cells and mutant DNA. Nevertheless, the fact that we obtained a significantly higher efficiency of marker rescue compared with the frequency of appearance of white plaques

FIG. 4. Separation of viral and cellular DNAs by CsCl equilibrium centrifugation. Vero or BL-1 cells, infected with either mutant or wild type at a multiplicity of 5 PFU per cell, were incubated at 34°C and were exposed to 10 μ Ci of [3 H]thymidine per ml from 6 to 24 h postinfection. Lysates of these cells were then subjected to equilibrium centrifugation in neutral CsCl gradients as described by Aron et al. (1). Cellular DNA corresponds to a peak with a buoyant density of 1.690, and viral DNA peaks at a buoyant density of 1.725.

in the no-fragment control suggests that it is likely that the plaques picked from the marker rescue experiment were indeed due to recombinant with input plasmid.

DISCUSSION

A gene corresponding to an open reading frame designated UL52, situated within the right-hand end of the HSV-1 genome, was shown by Wu et al. in a transient transfection

TABLE 2. Viral DNA synthesis of wild-type and *hr114* viruses in Vero and BL-1 cells^a

Virus	Cell	DNA synthesis (% of wild-type HSV-1 synthesis)
KOS	Vero	100
KOS	BL-1	100
<i>hr114</i>	Vero	0
<i>hr114</i>	BL-1	85

^a Values under peaks from Fig. 4 were determined, and results are expressed as the percentage of viral DNA in wild-type virus-infected cultures.

assay to be required for amplification of HSV origin-containing plasmids (35). We have isolated a mutant with a null mutation in this gene and demonstrated that it is essential for DNA replication in infected cells in culture. The gene for UL52 was disrupted by the insertion of a cassette containing the *lacZ* gene under the control of ICP6 regulatory elements. *hr114*, a recombinant virus capable of forming blue plaques, was isolated and propagated in cells transformed with the wild-type UL52 gene. This mutant failed to produce plaques or synthesize viral DNA when grown in Vero cells. Consistent with a defect in viral DNA synthesis, *hr114* was defective in the synthesis of late polypeptides. The lesion in a DNA-negative *ts* mutant of HSV-1 strain 17, *ts1206*, was previously mapped to the right-hand end of U_L between coordinates 0.708 and 0.720. *ts1206* failed to complement *hr114*, indicating that these two mutants have defects in the same gene and define a new complementation group, designated 1-37, according to the nomenclature of Schaffer et al. (28). These data demonstrate that UL52 is essential for viral DNA replication in infected cells.

FIG. 5. Synthesis of viral polypeptides in wild-type- and mutant-infected cells. Monolayers of Vero and BL-1 cells were infected with KOS or *hr114* at a multiplicity of 10 PFU per cell, labeled from 5 to 16 h postinfection, and harvested at 16 h postinfection. Samples were electrophoretically separated on a denaturing 9% polyacrylamide gel. Positions of viral polypeptides are indicated to the right of the gel. Lane 4 represents KOS-infected Vero cells in the presence of 150 µg of phosphonoacetic acid (PAA) per ml.

Thus, mutants representing four complementation groups (1-10 [32; Zhu and Weller, in press], 1-26 [Carmichael and Weller, unpublished] 1-36 [3], and 1-37 [this paper]) have now been reported whose mutations lie within genes required for viral DNA synthesis; these genes correspond to open reading frames UL5, UL8, UL9, and UL52, respectively, as predicted by the nucleotide sequence of UL (21). These four uncharacterized genes, and a fifth (UL42), have been shown by Wu et al. in a transient transfection assay to be required for origin-containing plasmid amplification (35).

TABLE 3. Complementation among five mutants of HSV-1

Mutant	Complementation index ^a				
	<i>hr114</i>	<i>tsM19</i>	<i>tsJ12</i>	<i>tsA24</i>	<i>ts1206</i>
<i>hr114</i>		153.7	35.2	433.1	1.28 ^b
<i>tsM19</i>			14.6	36.4	114.0
<i>tsJ12</i>				6.1	78.2
<i>tsA24</i>					8.9
<i>ts1206</i>					

^a Complementation indices were calculated as described in Materials and Methods.

^b This number is considered negative for complementation.

The functions of these five gene products are currently unknown; however, by analogy to other replication systems, we might expect HSV-1 to encode, in addition to DNA-binding protein and DNA polymerase, an origin-recognition protein(s), a primase, a helicase, polymerase accessory proteins, a topoisomerase, and a ligase (16). Biochemical studies (11) have led to the identification of an HSV-induced protein that binds specifically to one HSV origin of replication. The possibility exists that this protein is encoded by one of the genes described above. In efforts to elucidate the functions of these uncharacterized gene products, we are currently overexpressing them in *E. coli* (Carmichael and Weller, unpublished).

These studies describe a useful genetic approach to the creation of mutations in target genes. A similar approach was recently described for insertion and deletion mutagenesis of regions of the cytomegalovirus genome which may be dispensable for growth in culture (31). We have combined the use of the *lacZ* gene as a genetic marker (insertional mutagen) and transformed, permissive host cell lines containing the wild-type versions of genes to be mutated, thus providing us with a means to create insertion and deletion mutations in genes which are essential for viral growth. Our first target for mutagenesis was the large subunit (ICP6) gene of the HSV-1-encoded ribonucleotide reductase (14). An in-frame insertion of the structural gene of β -gal led to the isolation of a mutant (*hrR3*) which is incapable of inducing viral ribonucleotide reductase activity in infected cells. This mutant synthesizes substantial levels of β -gal from the regulatory elements of the ICP6 gene which can easily be detected in the presence of X-Gal.

As mentioned in the Results, we attempted to create an analogous, in-frame *lacZ* insertion into the UL52 gene. Our inability to detect blue plaque formation with this construct led us to postulate that β -gal synthesis from these viral recombinants was below the limits of detection. This would imply that the UL52 regulatory signals result in low levels of expression of this gene. In fact, attempts to use in-frame insertions of the *lacZ* gene into two other viral genes believed to be essential for viral DNA replication, UL5 and UL8, also resulted in failure to detect blue-plaque formation. Although many explanations exist for these results, we favor the explanation that replication genes UL5, UL8, and UL52 may be expressed at low levels in infected cells. Further experiments are required to elucidate the nature of the regulatory elements controlling the expression of this class of replication proteins.

To circumvent this problem, we have constructed an ICP6::*lacZ* gene fusion which can be introduced into the viral genome for identification of viral recombinants containing *lacZ* insertions in specific genes. By taking advantage of the promoter and upstream regulatory elements of the ICP6 gene, the detection of recombinant viruses is therefore not dependent on the undefined control elements of uncharacterized genes. This method has led to the isolation of a mutant with an insertion mutation in the UL52 gene (described in this report) and a mutant with an insertion-deletion mutation in the UL8 gene (Carmichael and Weller, unpublished). These studies demonstrate the usefulness of this approach for mutating genes thought to play roles in viral DNA synthesis and provide a general strategy for mutating any HSV gene.

Blue-plaque mutants can be exploited for the construction of additional mutations in specific genes. By screening for recombinant viruses that have lost the *lacZ* gene (white plaques), deletions, other insertions, or point mutations can easily be introduced into the viral genome. For instance, we

recently showed that a deletion can be created in the ICP6 gene by isolating white plaques following cotransfection of a plasmid containing the desired deletion and infectious DNA from the blue-plaque, *lacZ* insertion mutant *hrR3* (D. J. Goldstein and S. K. Weller, *Virology*, in press). Once the functions of genes altered by *lacZ* insertional mutagenesis have been identified, several new mutations can therefore be easily created in specific sites. Isolation and characterization of white-plaque viruses will then allow for molecular probing of functional domains of these proteins.

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