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# Isolation of a Herpes Simplex Virus Type 1 Mutant Deleted for the Essential UL42 Gene and Characterization of Its Null Phenotype

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**We isolated a cell line, designated V9, stably transformed with the herpes simplex virus type 1 (HSV-1) UL42 gene, which is one of seven genes required in *trans* for the replication of plasmids containing an HSV origin of replication (C. A. Wu, N. J. Nelson, D. J. McGeoch, and M. D. Challberg, J. Virol. 62:435–443, 1988). V9 cells inducibly expressed the product of the UL42 gene, the 65-kDa DNA-binding protein ($65K_{DBP}$), and were used as a permissive host to construct a mutant virus deleted for this essential gene. The UL42 deletion mutant, designated CgalΔ42, displayed a tight early phenotype in nonpermissive Vero cells producing no infectious progeny, viral DNA, or late gene products but accumulated selected immediate-early and early transcripts with kinetics similar to those of wild-type virus. Wild-type levels of viral DNA and infectious progeny were produced in permissive V9 cells, despite the fact that V9 cells infected with CgalΔ42 accumulated less than 1% of the UL42 RNA and protein found in $Cgal^+$ virus-infected V9 or Vero cells. These results indicate that only small quantities of the $65K_{DBP}$ are required for the synthesis of HSV DNA and the production of infectious virus. Although we could find no evidence that the superinduction of the $65K_{DBP}$ in V9 cells infected with $Cgal^+$ repressed expression of HSV-1 genes as observed in cells expressing another DNA-binding protein, ICP8 (P. K. Orberg and P. A. Schaffer, J. Virol. 61:1136–1146, 1987), the induction of the $65K_{DBP}$ in V9 cells correlated with an approximately 2-h-earlier shift in the expression of genes from all three kinetic classes. The availability of the UL42 mutant should facilitate the construction of more subtle UL42 mutants which will be useful in the elucidation of the interrelationship between the $65K_{DBP}$ and other DNA replication proteins as well as in the characterization of additional important functional domains.**

Herpes simplex virus type 1 (HSV-1) encodes at least 70 different proteins (34), and 7 of these have been shown to be required in *trans* for the replication of plasmids containing an HSV-1 origin of replication (57). These seven genes encode a DNA polymerase (pol) (28, 44), an origin-specific DNA-binding protein (UL9) (37), a single-stranded DNA-binding protein (infected cell polypeptide 8 [ICP8]) (42, 56), a double-stranded DNA-binding protein which is the product of the UL42 gene (65-kDa DNA-binding protein [$65K_{DBP}$]) (33, 40), and three components of a helicase-primase complex composed of UL5, UL8, and UL52 (8). Evidence exists that each of these genes is essential for replication of the virus in vivo (3, 4, 19, 28, 31, 45, 56, 58).

One of the most effective means for studying the effects of defects in essential genes on virus replication is by construction of mutants in which the gene has been deleted. Such mutants are superior for characterizing the function of the gene inasmuch as they are free from the complications of leak and reversion common to temperature-sensitive (*ts*) mutants. However, propagation of deletion mutants for essential genes requires a permissive host cell, capable of providing the wild-type gene product in *trans*. This approach has been used to create mutants with deletions in the following genes required for HSV-1 DNA replication: ICP8 (9, 38), UL5 (58), UL8 (4), UL9 (3), UL52 (19), and pol (32). We were interested in constructing an HSV-1 mutant in which the UL42 ($65K_{DBP}$) gene was deleted to characterize the null phenotype as a means toward understanding more fully the function of this protein in virus replication.

The $65K_{DBP}$ is a strong double-stranded DNA-binding protein (33) with no apparent sequence specificity (13a). It has been shown to copurify with pol (7, 15, 51), and analysis of the apparent size of the pol-$65K_{DBP}$ protein complex by sedimentation through sucrose density gradients indicates that the complex is composed of a 1:1 ratio of the 140-kDa pol polypeptide and the $65K_{DBP}$ (7). Recently, the $65K_{DBP}$ has been shown to stimulate pol activity, indicating that it is likely to function as a pol accessory protein (14), although the 140-kDa pol protein has been shown to have intrinsic pol activity at least in vitro (11, 14, 22). Nevertheless, in vivo $65K_{DBP}$ is clearly essential as indicated by the lack of virus replication and the $DNA^-$ phenotype of a *ts* mutant (31) and of the UL42 deletion mutant we isolated and whose characterization is described herein.

In this report, we describe the construction of a transformed cell line which contains at least one functional UL42 gene capable of complementing $65K_{DBP}$ defects in *trans* and its use in constructing an HSV-1 mutant with a deletion in the UL42 gene. One of our long-term goals is also to construct a nonlytic HSV vector suitable for gene transfer, and mutants with defects in essential genes, such as the UL42 gene, might be suitable candidates for such a vector. To this end, we decided to construct the UL42 deletion mutant in the background of a virus containing the *Escherichia coli lacZ* reporter gene encoding β-galactosidase, which is easily detected by both enzymatic and histological staining methods, and which was under the control of a strong heterologous promoter from the human cytomegalovirus immediate-early (IE) regulatory region. Thus, in addition to providing a means for evaluating the function of the $65K_{DBP}$ in vivo, this virus construct will provide a means for

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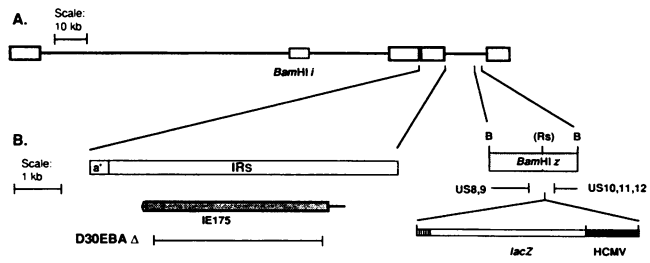


FIG. 1. Structures of the HSV-1 Cgal$^+$ and related genomes. (A) General structure of wild-type HSV-1 DNA is indicated and shows the location of the *Bam*HI i fragment (diagramed in detail in Fig. 2) containing UL42, the location of the IE3 gene (IE175) encoding the 175,000-Da IE protein (shown once here for the internal short repeat [IRs]), and the location of the *E. coli lacZ* gene insert within the *Bam*HI z fragment in the parental Cgal$^+$ strain. (B) Regions containing the IE3 and *lacZ* genes have been expanded. On the left, the extent of the deletion of the IE3 gene in D30EBA is shown, and on the right is the location and structure of the human cytomegalovirus-*lacZ* fusion gene inserted into the intergenic *Rsa*I site (Rs) in *Bam*HI (B) z, between the 3′ cotermini of genes US8,9 and US10,11,12. Viruses prefixed Cgal contain this insertion within *Bam*HI z.

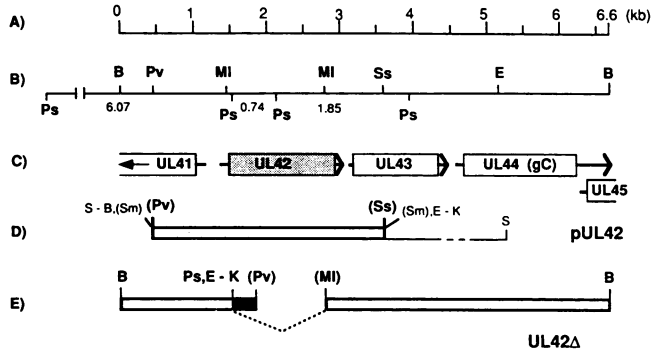


FIG. 2. Detail of the *Bam*HI i region showing the scale of the 6.6-kbp region (A) and a detail of relevant restriction sites in and around this fragment in the Cgal$^+$ parental genome (B). The sizes (in kilobase pairs) of genomic *Pst*I fragments are shown below the line. (C) Location of the UL41, UL42 (shaded), UL43, UL44 glycoprotein C (gC), and UL45 open reading frames. (D) Structure of the viral genomic sequences in plasmid pUL42, which contains a 3,275-bp *Pvu*II-*Sst*II subfragment of *Bam*HI-i (open box), including the entire UL42 coding region, inserted into pBluescript KSII (thin dotted line). (E) BamHI i fragment (open box) of pUL42Δ and CgalΔ42 which is deleted (dotted line) for 1,258 bp (*Pst*I-*Mlu*I) of the UL42 gene. The 280-bp *Pst*I-*Pvu*II fragment from pBluescript KSII (black box) containing all the polylinker restriction sites from *Pst*I to *Kpn*I marks the site of the deletion. Restriction sites: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; Ml, *Mlu*I; Ps, *Pst*I; Pv, *Pvu*II; S, *Sst*I; Ss, *Sst*II; Sm, *Sma*I.

characterizing the effects of the absence of the $65K_{DBP}$ on the ability for such a vector to express inserted genes.

## MATERIALS AND METHODS

**Growth of cells and virus.** African green monkey kidney (Vero) cells and a thymidine kinase (TK)-negative cell line of baby hamster kidney 21, clone 13 (BHK TK$^-$) cells were propagated in Dulbecco modified Eagle minimum essential medium (Flow Laboratories, Inc., McLean, Va.) containing 5 to 10% fetal bovine serum, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 0.075% $NaHCO_3$ for closed vessels or 0.225% $NaHCO_3$ for open vessels. V9 cells which contained both the transposon Tn*5* gene conferring neomycin resistance (Neo$^r$) and the HSV-1 UL42 gene were propagated similarly except that G418 (geneticin; GIBCO Laboratories, Grand Island, N.Y.) was added to culture medium at a concentration of 200 to 400 μg/ml at alternate passages. Vero and V9 cells were used for propagation and titration of all HSV-1 strains as appropriate, while BHK cells were used for most transfections. V9 cells to be used for the propagation of HSV-1 always were seeded in the absence of G418.

The HSV-1 strains KOS (46) and Cgal$^+$ were the parental strains used. The *ts* mutant *ts*701, which contains a lesion which maps in or close to the UL42 gene (31), was derived from the KOS strain (5) and was the kind gift of Priscilla Schaffer (Dana Farber Cancer Institute). The permissive temperature (pT) and nonpermissive temperature (npT) used for the propagation of *ts*701 were 34 and 39.7°C, respectively. Strain CgalΔ3 (Fig. 1) was constructed from D30EBA (gift of T. Paterson and R. D. Everett, Medical Research Council, Glasgow), which is an IE gene 3 deletion mutant of HSV-1 strain 17 syn$^+$ (41). The CgalΔ3 contains an insertion of a 4.6-kbp fragment (composed of 1.1 kbp of the human cytomegalovirus major IE regulatory region [1] upstream of the *E. coli lacZ* structural gene [3.5 kbp], obtained from the plasmid pON1 [49]) into the *Rsa*I site of a nonessential intergenic region in the unique short (Us) region of the genome. The IE3 deletion of CgalΔ3 was then repaired by marker rescue to generate the replication-competent virus Cgal$^+$. We have shown that Cgal$^+$ is indistinguishable from the parental strain 17 syn$^+$ in growth properties, production of viral DNA, and pattern of expression of viral genes (data not shown), and therefore, we refer to it as a wild-type strain for the purposes of this report. Stock preparations of the wild-type virus strains and *ts*701 were obtained by low-multiplicity passage in Vero cells as previously described (39). Stocks of the UL42 deletion mutant CgalΔ42 (described below) were similarly prepared in V9 cells.

**Plasmids.** The plasmid pBam i contains the HSV-1 *Bam*HI i fragment cloned into the *Bam*HI site of pUC19. A 3,275-bp *Pvu*II-*Sst*II fragment from pBam i containing the entire UL42 gene was inserted into the *Sma*I site of pBluescript KSII (Stratagene, La Jolla, Calif.) by blunt-end ligation to create the plasmid pUL42 (Fig. 2). The plasmid pUL42Δ was generated by a three-way ligation of (i) a 280-bp *Pst*I-*Pvu*II fragment from pBluescript KSII which includes polylinker restriction sites from *Pst*I to *Kpn*I; (ii) a 6,567-bp *Mlu*I-to-*Sst*I fragment from pBam i which includes UL43, UL44, and pUC19 sequences and in which the *Mlu*I site was filled in with DNA polymerase I large fragment (Klenow); and (iii) a 1,608-bp *Sst*I-to-*Pst*I fragment from pBam i which includes a portion of UL41. As shown schematically in Fig. 2, the resulting plasmid pUL42Δ contains a deletion of 1,258 bp within the coding region of UL42 and the deletion site is marked by a 280-bp insert of plasmid DNA which was used in subsequent screens for recombinant virus by plaque hybridizations.

Other plasmids used in this study were pSV2neo (48), used to confer resistance to geneticin; pGEM2-702, containing the HSV-1 pol and two late genes (11, 24) (kindly provided by Dave Dorsky, University of Connecticut); ptk109 (36), containing the HSV-1 TK gene; and plasmid pIE63, containing the IE2 (ICP27) gene.

**Generation of V9 cell line.** To generate a cell line permissive for the replication of HSV-1 containing lethal mutations

in the UL42 gene encoding $65K_{DBP}$, freshly trypsinized Vero cells previously grown to 50% confluence in a 100-mm culture dish were cotransfected in suspension with 5 μg of pUL42, 0.5 μg of pSV2neo, and 15 μg of herring sperm DNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by the $CaPO_4$ technique of Graham and van der Eb (21) essentially as described by DeLuca and Schaffer (10). After 16 days, 12 G418-resistant colonies were isolated and screened for their ability to complement the *ts*701 defect at the npT by direct plaque assay. One of these colonies, V9, was expanded for further study.

**One-step growth curve.** Equivalent numbers of Vero and V9 cells were seeded into 60-mm culture dishes, inoculated with $Cgal^+$ or CgalΔ42 at multiplicities of infection (MOIs) of 1 or 10 PFU per cell, and incubated at 37°C. At various times postadsorption (p.a.), cells were scraped into the medium and disrupted by sonication and total virus yield was determined by plaque assay in V9 cells at 34°C.

**Complementation analysis.** In the direct plaque assay, Vero and V9 cells were inoculated with dilutions of various virus isolates, adsorbed for 1 h at 37°C, overlaid with medium containing methylcellulose, and incubated at 34°C for 6 days, and the plaques were enumerated. In the yield of progeny assay, equal numbers of Vero and V9 cells in 25-$cm^2$ culture flasks were inoculated at an input MOI of 1 or 10 PFU per cell. Unadsorbed virus was removed 1 h later, the monolayers were washed, and the cells were grown for a single cycle (18 h) at 37°C. Cells scraped into the medium were disrupted by sonication, and total infectious virus was titrated by plaque assay at 34°C in Vero or V9 cells. The complementation index for CgalΔ42 was calculated by dividing the virus yield obtained from growth in V9 cells by that produced from growth in Vero cells.

**Analysis of DNA synthesis.** Viral and cellular DNAs synthesized by Vero or V9 cells infected with various virus isolates (MOI of 1 PFU per cell) were measured by metabolic labeling of DNA with [$^3$H]thymidine (10 μCi/ml; specific activity, 50 Ci/mmol; ICN Biomedicals, Inc., Irvine, Calif.) from 4 through 18 h postinfection. Cells were harvested, lysed with Sarkosyl and sodium dodecyl sulfate, and digested with proteinase K (100 μg/ml) as described previously (39) to obtain high-molecular-weight DNA. Viral and cellular DNAs were separated by CsCl equilibrium density gradient centrifugation as previously described (39). The level of DNA synthesized was determined by integration of the area under the appropriate peak corresponding to incorporation of label into cellular (1.68 g/ml) or viral (1.72 g/ml) DNA.

**Hybridizations.** For Southern blot analysis (47), DNA subjected to restriction enzyme cleavage was separated by electrophoresis on 0.8% agarose gels and transferred to Nytran nylon membranes (Schleicher & Schuell, Inc., Keene, N.H.) by vacuum blotting with alkaline transfer buffer (0.4 M NaOH, 0.6 M NaCl). Total cytoplasmic RNA samples prepared as previously described (30) were separated on denaturing agarose gels containing 0.66 M formaldehyde and transferred to Nytran membranes according to the instructions of the manufacturer. The blots were analyzed with double-stranded DNA probes radiolabeled with [α-$^{32}$P]dATP (Amersham Corp., Arlington Heights, Ill.) by the method of Feinberg and Vogelstein (13) utilizing the hybridization and washing conditions suggested by the Nytran manufacturers (Schleicher & Schuell).

Plaque hybridizations were performed as described previously (25, 52). Briefly, we initially transfected BHK $TK^-$ cells with 2.5 μg each of intact and linearized plasmid pUL42Δ containing the UL42 deletion or with cleaved pUL42 (Fig. 2). At 16 h posttransfection, the cells were infected with the replication-competent parental strain $Cgal^+$ (2 PFU per cell) and harvested 24 to 48 h later. In this system, the replication of any recombinant mutant virus generated would have been complemented by the wild-type viral $65K_{DBP}$ also present in the infected cells. To separate recombinant from wild-type virus, the total virus yield from these cells was titrated on V9 cells in 100-mm dishes and overlaid with medium containing 1% electrophoresis-grade agarose. When plaques were evident, the agarose overlay was used as a template for transfer to 0.45-μm-pore-size Nytran membranes and the DNA on the membrane was denatured with 0.5 M NaOH and fixed by exposure to UV light. To detect plaques containing recombinant virus, we hybridized the filters with a probe composed of the 280-bp *Pvu*II-*Pst*I fragment from pBluescript KSII (Fig. 2) using the same conditions described above for Southern blot analysis. Plaques showing positive hybridization to the probe were picked from the agarose and purified an additional two times on V9 cells.

**Primer extension.** Primer extension reactions were performed with total cytoplasmic RNA essentially as described by Eisenberg and co-workers (12). The sequence of the 23-base synthetic oligonucleotide primer used to detect US11 RNA was d(CGATGTGCCACACCCAAGGATGC), which is complementary to nucleotides +103 to +81 (30). Nucleotide +1 of US11 corresponds to nucleotide 12859 of the published Us sequence (35).

**Quantitation of $65K_{DBP}$ induced by infection.** Vero and V9 cells in 100-mm culture dishes were mock infected or infected as described above at an MOI of 10 PFU per cell, harvested at 18 h postinfection, and washed with Tris-buffered saline. Cells were disrupted by sonication in 400 μl of extraction buffer containing 200 mM Tris (pH 7.5), 2 mM ATP, 2 mM $MgCl_2$, 3 mM dithiothreitol, and 20% glycerol; this was followed by low-speed centrifugation to remove debris. Serial twofold dilutions of each extract were made in Tris-buffered saline and applied to nitrocellulose by using a vacuum manifold system. The filter was probed with monoclonal antibody 6898, which specifically detects the $65K_{DBP}$ (40) and $^{125}$I-labeled protein A as previously described (15). The protein content of each sample was determined by the method of Bradford (2), using bovine serum albumin as a standard.

## RESULTS

**Derivation of cell line expressing $65K_{DBP}$.** The HSV-1 UL42 gene encodes the $65K_{DBP}$ (40), which is required for HSV origin-dependent replication (57) and viral replication in vivo (31). To propagate viral mutants deleted in the UL42 gene, it was first necessary to construct a transformed cell line which was capable of providing functional wild-type $65K_{DBP}$ in *trans*. To obtain such a cell line, we cotransfected Vero cells with the plasmids pUL42 (Fig. 2) and pSV2neo and selected for colonies resistant to G418 as described in Materials and Methods. Colonies were screened for their ability to support the replication at the npT of *ts*701, a *ts* mutant which contains a lesion which maps to UL42 (31). The cell line on which *ts*701 was best able to produce plaques at 39.7°C, designated V9, was chosen for further investigation. In both direct plaque assays and a standard yield of progeny assay, we determined quantitatively that V9 cells were able to compensate efficiently for the *ts*701 defect (data not shown).

**Detection of UL42 gene in V9 cells.** To confirm the presence

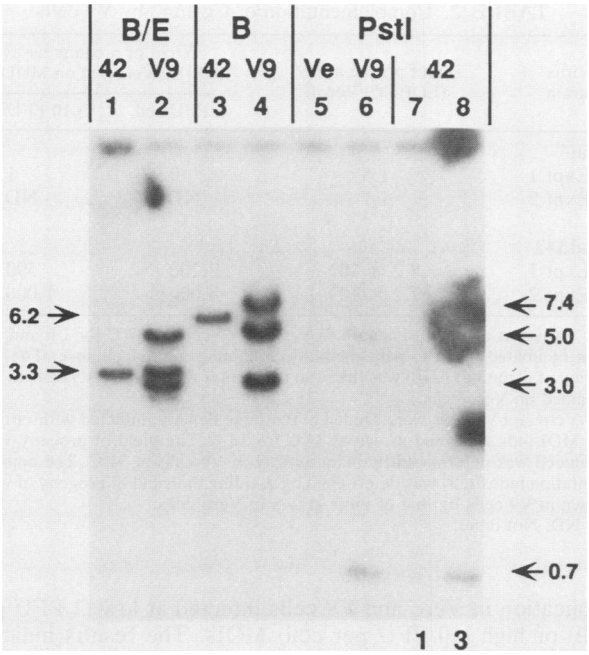


FIG. 3. Southern blot analysis of UL42 DNA in V9 cells. Electrophoretically separated *Bam*HI (B), *Bam*HI-*Eco*RI (B/E), and *Pst*I digests of V9 and Vero (Ve) cell DNA (5 μg) were transferred to a Nytran membrane and probed with the 738-bp *Pst*I fragment from pUL42. Digests of 10 pg of pUL42 (42) (lanes 1, 3, and 7) or 30 pg of pUL42 (lane 8) were included to estimate both the size and copy number of UL42 sequences in V9 cells. The amounts are equivalent to one and three copies, respectively, of the UL42 gene per $3 \times 10^9$ bp of cellular DNA. The numbers on the left correspond to the positions of full-length linearized pUL42 (6.2 kbp) and the 3.3-kbp viral insert, while those on the right correspond to the sizes (in kilobase pairs) of V9 DNA hybridizing to the probe and the 0.74-kbp *Pst*I fragment internal to the UL42 gene.

of the UL42 gene in the V9 cells and to determine its apparent organization and copy number in the cell clone, we analyzed high-molecular-weight DNA isolated from Vero and V9 cells by Southern blot hybridization to a UL42 gene-specific probe (Fig. 3). The DNA samples were digested with *Bam*HI or with *Bam*HI and *Eco*RI, subjected to electrophoresis, and probed with the UL42 internal *Pst*I fragment to ascertain the presence of integrated full-length copies of pUL42. Three bands were readily detected in each of these restriction digestions of V9 cell DNA. The presence in V9 cell DNA of a *Bam*HI fragment of 7.4 kbp, which is greater in size than the 6.2-kbp linearized full-length pUL42 used for transfections, and its conversion after *Eco*RI digestion to a 3.3-kbp fragment corresponding in size to the viral sequence insert in pUL42 indicate that there is at least one full-length copy of the insert integrated into the V9 genome. Likewise, the presence of a 3.0-kbp *Bam*HI fragment, which is smaller than the insert in pUL42, and the presence of a 5.0-kbp *Bam*HI fragment lacking the *Eco*RI site at the end of the insert are indicative of at least two additional integrations of less-than-full-length copies of the pUL42 viral gene insert. Thus, there are at least one full-length and probably two partial copies of the pUL42 insert present in V9 cells. Analysis of V9 cell DNA cleaved with *Pst*I and probed with the *Pst*I fragment located entirely within the UL42 open reading frame compared with copy number plasmid controls

TABLE 1. Marker rescue of CgalΔ42 in BHK cells transfected with pUL42

| Sample | Transfection[a] | Progeny titer (PFU/ml) on[b]: | | EOP (Vero/V9)[c] |
|---|---|---|---|---|
| | | Vero | V9 | |
| 1 | pUL42 | $9.4 \times 10^5$ | $6.3 \times 10^6$ | 0.15 |
| 2 | pUL42 | $3.3 \times 10^5$ | $2.1 \times 10^6$ | 0.15 |
| 3 | | NP[d] | $8.5 \times 10^2$ | |

[a] BHK TK$^-$ cells ($2 \times 10^5$) were transfected with 10 μg of cleaved pUL42 (*Bam*HI and *Hin*dIII) (sample 1), 5 μg each of cleaved and uncleaved pUL42 (sample 2) by the standard $CaPO_4$ technique as described in Materials and Methods, or were not transfected (sample 3). The efficiency of transfection of the cell line was typically >10%. After 16 h, the cells were washed with medium and superinfected with $10^6$ PFU of CgalΔ42.

[b] Progeny virus was harvested 3 days after superinfection by freeze-thawing and titrated on Vero and V9 cells. Three well-isolated plaques arising on Vero cells at a high dilution were picked for further analysis.

[c] Efficiency of plaquing (EOP) in Vero cells compared with V9 cells. The data indicate the proportion of the progeny virus that was host cell independent for lytic growth.

[d] NP, No plaques detected with undiluted inoculum.

cleaved with *Pst*I confirmed that there are approximately three copies of the UL42 gene present in V9 cells. As expected, no hybridization of the probe to Vero cell DNA was observed.

**Construction of UL42 deletion mutant CgalΔ42.** It was reasonable to expect that V9 cells which contained UL42 sequences and complemented *ts*701 at the npT would also be able to complement a UL42 deletion mutant. The availability of such a deletion mutant and subsequent characterization of the null phenotype would facilitate the analysis of the functional role of the $65K_{DBP}$ in virus replication as well as the construction of additional directed mutations in the UL42 gene within the virus. To this end, we constructed such a mutant by allowing the homologous recombination of replicating viral DNA with a plasmid insert containing a viral sequence in which most of the UL42 open reading frame had been deleted and replaced by a 280-bp indicator plasmid sequence (Fig. 2). Recombinant viruses containing the modified UL42 gene were generated as detailed in Materials and Methods. A stock of one such isolate, designated CgalΔ42, was prepared in V9 cells and further analyzed.

**CgalΔ42 contains a deletion in UL42.** To confirm that the isolate obtained did, in fact, possess the expected deletion, we tested for the ability of a transfected intact UL42 gene to rescue the CgalΔ42 mutation (Table 1). Because the transfections and subsequent infections were done in BHK TK$^-$ cells which were nonpermissive for the replication of the CgalΔ42, very little virus was recovered from controls that did not receive pUL42 DNA. That which was recovered was capable of replicating only in V9 cells, indicating that it most likely represented surviving input virus. By contrast, approximately 15% of the abundant progeny virus produced in transfections containing pUL42 was host cell independent for lytic growth.

Three plaques from the marker rescue experiment described above which appeared on Vero cells were picked, and the organization of DNA from rescued virus amplified by replication in Vero cells was compared with that of Cgal$^+$ and CgalΔ42 grown in V9 cells by Southern blot hybridization with $^{32}$P-labeled pUL42 as a probe (Fig. 4). When cleaved with *Pst*I, three fragments of the parental Cgal$^+$ genomic DNA hybridized to the probe, corresponding to the sizes predicted from Fig. 2 of 6.07, 1.85, and 0.74 kbp. By

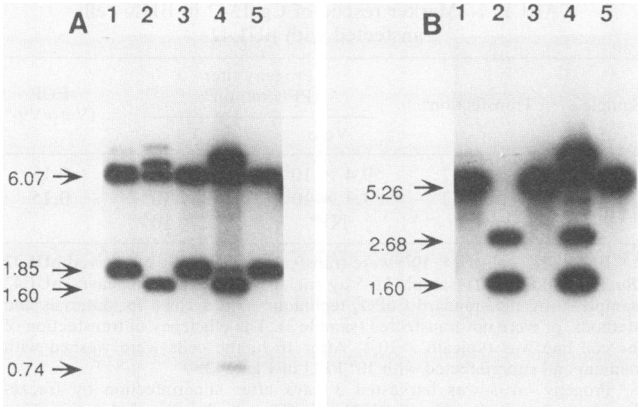


FIG. 4. Southern blot analysis of UL42-specific sequences in virus isolates. DNA samples were digested with *Pst*I (A) or *Bam*HI and *Eco*RI (B), subjected to electrophoresis on a 0.8% agarose gel, and probed with $^{32}$P-labeled pUL42. Lanes contain viral DNA as follows: 1, Cgal$^+$; 2, CgalΔ42; 3 to 5, three marker-rescued isolates of CgalΔ42 (described in Table 1). Specific bands denote sizes of fragments in kilobase pairs and are referred to in the text.

contrast, the CgalΔ42 DNA contained a 1.6-kbp fragment in place of the 1.85- and 0.74-kbp fragments, corresponding to the size predicted if the entire deletion (with 280-bp plasmid insertion) mutation had been transferred. The CgalΔ42 band migrating above the 6.07-kbp band (Fig. 4) was a partial digestion product and was not present in other blots of the same DNA (data not shown). All three of the rescued isolates contained the parental 1.85- and 0.74-kbp *Pst*I fragments, although one isolate (Fig. 4A, lane 4) was obviously from a mixed plaque and additionally contained a predominant 1.60-kbp mutant fragment. In a double digestion with *Bam*HI and *Eco*RI, the expected 5.26-kbp fragment of Cgal$^+$ was replaced by two bands of 2.68 and 1.60 kbp in CgalΔ42, consistent with the insertion of a new *Eco*RI site within the 280-bp plasmid sequences contained in pUL42Δ together with the loss of UL42 sequences. Again, the hybridization patterns of two of the rescued isolates were identical to the parental Cgal$^+$, while the third (Fig. 4B, lane 4) had a mixed pattern. Taken together, these results confirm that CgalΔ42 contains the same deletion in UL42 as the plasmid pUL42Δ (Fig. 2).

**Growth properties of CgalΔ42.** Although the marker rescue experiment described above indicated that the UL42 deletion mutant was incapable of growing in cells in which the $65K_{DBP}$ was not provided in *trans*, we were interested in more accurately quantifying the ability of permissive (V9) and nonpermissive (Vero) host cells to complement the replication of CgalΔ42. At low dilutions of CgalΔ42 in Vero cells, general cytolysis was observed. However, the ability of CgalΔ42 to form plaques on V9 cells was at least 6 orders of magnitude greater than that on Vero cells (Table 2). Stocks of the parental Cgal$^+$ produced plaques nearly as efficiently on Vero cells as on V9 cells (Table 2), although we consistently observed larger plaques on V9 cells despite the fact that V9 and Vero cells were at the same density at the time of infection.

Because the ability of virus to form plaques is related, among other things, to its ability to replicate at low input MOIs, the amount of virus produced, and its ability to pass from one infected cell to another, we measured the amount of infectious progeny virus produced in a single cycle of

TABLE 2. Complementation of CgalΔ42 by V9 cells

| Virus strain | Direct plaque assay[a] (EOP [V9/Vero]) | Yield of progeny assay (CI [V9/Vero]) at an MOI of[b]: | |
|---|---|---|---|
| | | 1 PFU/cell | 10 PFU/cell |
| Cgal$^+$ | | | |
| Expt 1 | 1.5 | 0.97 | 1.6 |
| Expt 2 | 1.1 | ND[c] | ND |
| CgalΔ42 | | | |
| Expt 1 | $8.2 \times 10^6$ | 2,200 | 590 |
| Expt 2 | $7.2 \times 10^6$ | 4,000 | 1,000 |

[a] Virus was allowed to adsorb to Vero or V9 cells at 37°C for 1 h and was then incubated at 34°C with medium containing methylcellulose. The efficiency of plaquing (EOP) was the ratio of the titer obtained on V9 cells to that obtained on Vero cells.

[b] Vero and V9 cells were seeded at the same density, infected with virus at the MOI indicated, and grown at 34°C for 18 h. The yield of progeny virus produced was determined by plaque assay in V9 cells at 34°C. The complementation index (CI) was determined by dividing the yield of progeny of virus grown in V9 cells by that of virus grown in Vero cells.

[c] ND, Not done.

replication in Vero and V9 cells infected at low (1 PFU per cell) or high (10 PFU per cell) MOIs. The results indicate that infectious CgalΔ42 yield (titrated in the V9 permissive host) was on the order of 1,000 times greater in V9 cells than in Vero cells and that low MOIs consistently produced a greater complementation index than high MOIs (Table 2). By contrast, no evidence of significant positive complementation of Cgal$^+$ by V9 cells was observed at either MOI.

Based on the above results, it appeared that CgalΔ42 could not replicate in Vero cells, although these data did not rule out the possibility of a prolonged replication cycle in Vero cells. To address this possibility as well as to compare the rate of replication of the deletion mutant in permissive V9

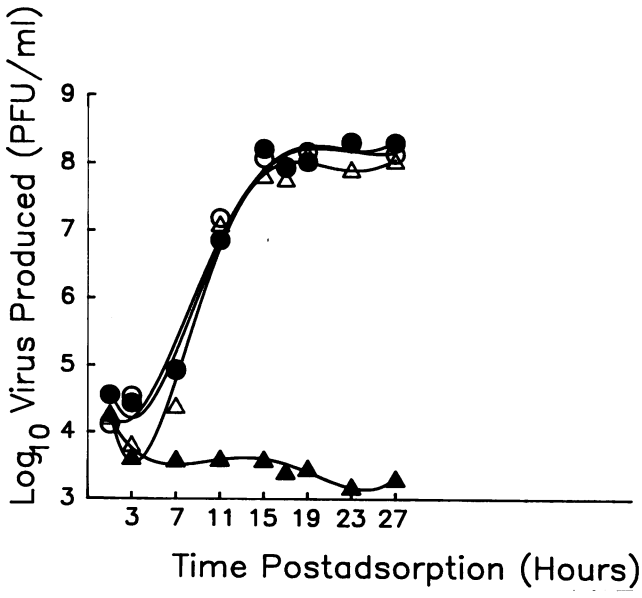


FIG. 5. One-step growth curves of Cgal$^+$ and CgalΔ42 in Vero and V9 cells. Cells were infected at an MOI of 1 PFU per cell, and the total virus yield (PFU per milliliter) from each sample at the times indicated was determined by plaque assay in V9 cells. Symbols: ●, Cgal$^+$ in Vero cells; ○, Cgal$^+$ in V9 cells; ▲, CgalΔ42 in Vero cells; △, CgalΔ42 in V9 cells.

TABLE 3. Levels of cellular and viral DNA synthesized in HSV-1-infected Vero and V9 cells

| Strain | Vero (34°C) | | V9 (34°C) | | Vero (39.7°C) | | V9 (39.7°C) | |
|---|---|---|---|---|---|---|---|---|
| | Cellular[a] | Viral[b] | Cellular | Viral | Cellular | Viral | Cellular | Viral |
| KOS | 100 | 100 | 110 | 89 | 99 | 76 | 77 | 42 |
| Cgal$^+$ | 18 | 51 | 71 | 79 | ND[c] | ND | ND | ND |
| *ts*701 | 130 | 45 | 160 | 77 | 220 | <10 | 200 | 33 |
| CgalΔ42 | 160 | <10 | 94 | 92 | ND | ND | ND | ND |

[a] Levels of cellular DNA synthesis were determined by integration of the area under the peak corresponding to a buoyant density of approximately 1.68 g/ml on CsCl equilibrium density gradients. Gradients were fractionated as described in the legend to Fig. 6. Values are expressed as the percentage of cellular DNA synthesized by Vero cells infected with the wild-type strain KOS at 34°C.

[b] Levels of viral DNA synthesis were determined by integration of the area under a detectable peak or shoulder corresponding to a buoyant density of approximately 1.72 g/ml on CsCl equilibrium density gradients. Values are expressed as the percentage of viral DNA synthesized by Vero cells infected with the wild-type strain KOS at 34°C.

[c] ND, Not done.

cells with that of the parental Cgal$^+$ strain, we determined the one-step growth curve of each virus in Vero and V9 cells infected at low and high MOIs. The growth curves produced by infection at an MOI of 1 PFU per cell are shown in Fig. 5, and similar results (not shown) were obtained at MOIs of 10 PFU per cell. The lack of replication of CgalΔ42 in Vero cells is striking when compared with its kinetics of replication in V9 cells, with consistently lower amounts of recoverable CgalΔ42 from Vero cells through at least 27 h p.a. compared with that present within 1 h p.a. The results further attest to the ability of V9 cells to fully complement the replication of CgalΔ42. In fact, the overall kinetics of production of infectious progeny CgalΔ42 in V9 cells were virtually indistinguishable from those of the parental virus grown in either Vero or V9 cells. We performed a similar analysis of growth kinetics of one of the rescued variants and found the kinetics to be the same as those of Cgal$^+$ in both Vero and V9 cells (data not shown).

Although we consistently observed a lower titer of CgalΔ42 than of Cgal$^+$ at 3 h p.a., replication of Cgal$^+$ and CgalΔ42 was completed at approximately the same time with similar yields of virus. If we define the latent period as that time required to attain a yield of virus greater than that present immediately following adsorption, the latent period was the same (7 h) for CgalΔ42 in V9 cells as for Cgal$^+$ in either cell type.

To rule out the possibility that a recombinant generated by rescue of the deletion in CgalΔ42 by the endogenous UL42 sequences in V9 cells was responsible for the wild-type growth kinetics, we also titrated progeny from the growth curve experiment in Vero cells. No plaques were observed, but because of cytolysis at low dilutions, we estimate that the frequency of recombination with the endogenous genome sequence must be less than one event per $10^6$ infectious virions (data not shown).

**UL42 gene is absolutely required for viral DNA synthesis.** The *ts*701 mutant was previously reported to have a DNA$^-$ phenotype at the npT (31), and we confirmed that *ts*701 did not synthesize any viral DNA at 39.7°C in Vero cells (Table 3). However, in V9 cells which complement the replication of *ts*701 at the npT, viral DNA was synthesized at 39.7°C at 33% of the level made by the wild-type parental KOS strain. The availability of CgalΔ42 allowed us to determine whether or not viral DNA could be synthesized in nonpermissive Vero cells at the less restrictive temperature of 34°C. The profiles of DNA synthesized in Vero and V9 cells infected with CgalΔ42 indicated that no labeled viral DNA could be detected in Vero cells, while large quantities were obtained in V9 cells (Fig. 6). Significantly, the amount of labeled viral DNA observed in V9 cells infected with CgalΔ42 was approximately the same as that synthesized in V9 or Vero cells infected with the wild-type strain KOS or V9 cells infected with the parental Cgal$^+$ (Table 3). The lower amount of viral DNA synthesized by *ts*701 compared with CgalΔ42 in V9 cells most likely reflects temperature effects on DNA synthesis since even wild-type viral DNA synthesis is reduced at 39.7°C compared with that at 34°C (Table 3).

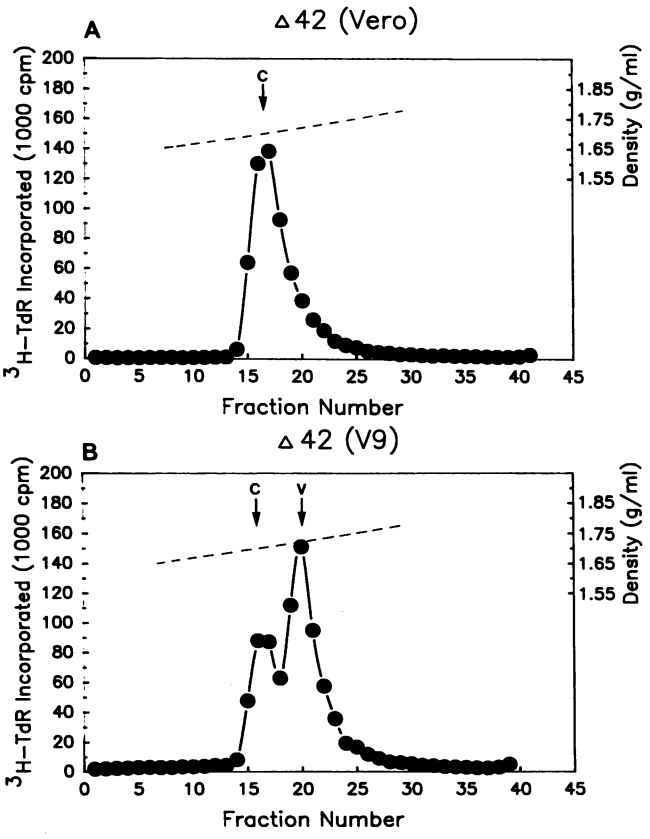


FIG. 6. DNA profiles of Vero (A) and V9 (B) cells infected with CgalΔ42 at an MOI of 1 PFU per cell. Cellular (C) and viral (V) DNAs were separated by centrifugation through CsCl equilibrium density gradients, and the amount of [$^3$H]thymidine incorporated into DNA (●) was determined as described in Materials and Methods. The density of fractions (– – – –) was determined from the refractive index, and the gradient was linear in the range indicated.

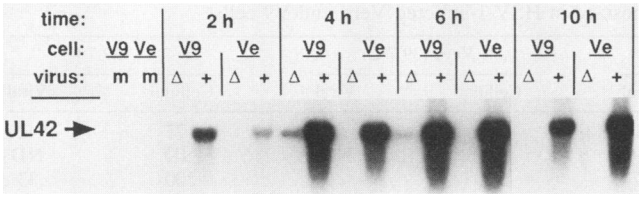


FIG. 7. Northern blot analysis of UL42 RNA accumulation during infection. V9 and Vero (Ve) cells were mock infected (m) or infected with CgalΔ42 (Δ) or Cgal$^+$ (+) at an MOI of 5 PFU per cell, and total cytoplasmic RNA was prepared at the various times indicated. RNA separated by electrophoresis was transferred to Nytran and probed with the $^{32}$P-labeled internal *Mlu*I fragment of gene UL42 isolated from pUL42. An autoradiogram of the resulting blot is shown.

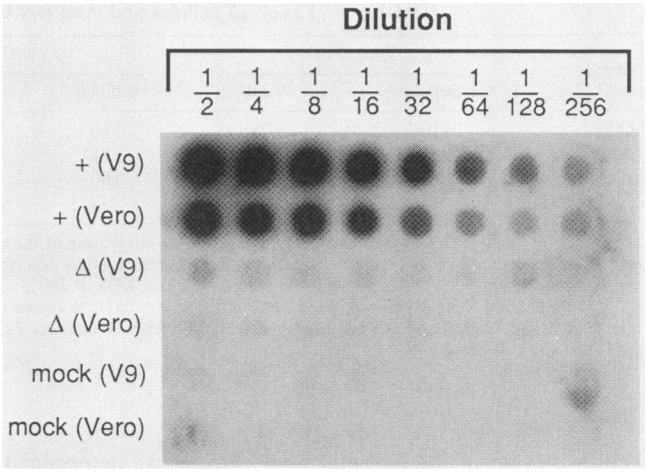


FIG. 8. Quantitative immunoblotting of 65K$_{DBP}$. Vero and V9 cells were infected with Cgal$^+$ (+) or CgalΔ42 (Δ) or mock infected as indicated and harvested at 18 h p.i., and the proteins were extracted. Dilutions as indicated were applied to nitrocellulose, and the blot was probed with 65K$_{DBP}$-specific antibody. The most concentrated spot represents one-fourth of each sample. Protein concentrations (milligrams per milliliter) of undiluted samples were as follows: Cgal$^+$ (V9), 2.9; Cgal$^+$ (Vero), 2.7; CgalΔ42 (V9), 2.7; CgalΔ42 (Vero), 2.7; mock (V9), 2.4; mock (Vero), 2.7.

Thus, the amount of 65K$_{DBP}$ provided in *trans* by V9 cells infected with CgalΔ42 is sufficient to allow the production of wild-type levels of viral DNA.

**Induction of UL42 gene expression in V9 cells.** The ability of the V9 cells to replicate the UL42 deletion mutant to levels similar to those of the parental virus and to complement fully the defect in viral DNA synthesis suggested that the V9 cells produce close to wild-type levels of the 65K$_{DBP}$, at least following infection. We therefore studied the expression of the UL42 gene by Northern (RNA) analysis of RNA isolated from Vero and V9 cells at various times following infection with Cgal$^+$ or the UL42 deletion mutant (Fig. 7). No UL42 transcripts could be detected in uninfected V9 cells, indicating little, if any, constitutive expression of the gene in the transformed cell line. At 2 h p.a., there was substantial UL42 RNA in V9 cells infected with Cgal$^+$ but barely detectable levels in CgalΔ42 virus-infected V9 cells at the same time. A marked increase in the level of UL42 RNA was apparent in Cgal$^+$ virus-infected Vero and V9 cells at 4 and 6 h p.a., but the level declined thereafter, consistent with the previously reported kinetics of accumulation of this transcript by the wild-type strain KOS (20). Surprisingly, little UL42 RNA accumulated in V9 cells infected with the deletion mutant, with maximum amounts present at 4 h p.a. and declining thereafter. The amount of UL42 RNA present in V9 cells undergoing permissive replication of the deletion mutant was less than 1% of that present at 4 or 6 h p.a. in V9 cells infected with Cgal$^+$. Interestingly, significantly more UL42 RNA was observed in Cgal$^+$-infected V9 cells than in infected Vero cells at early times, while the subsequent decline in the abundance of UL42 RNA was more rapid in V9 than in Vero cells. These results demonstrate that the endogenous UL42 gene in V9 cells is inducibly expressed following infection, although substantially less of the transcript accumulates in V9 cells infected with the deletion mutant than in wild-type infections.

To determine whether the low level of UL42 RNA accumulation corresponded to low levels of 65K$_{DBP}$ induced in CgalΔ42-infected V9 cells, we performed quantitative immunoblot analysis using the 65K$_{DBP}$-specific monoclonal antibody 6898 (Fig. 8). The results demonstrate that the amount of 65K$_{DBP}$ induced by infection of V9 cells with the UL42 deletion mutant was less than 1% of that induced by the wild-type virus in either V9 or Vero cells. The results further demonstrate that the superinduction of the UL42 RNA observed in V9 cells infected by Cgal$^+$ also was evident at the protein level.

**Characterization of cascade of viral gene expression induced by CgalΔ42.** The pattern of viral gene expression during productive replication of HSV-1 has been well characterized (6, 23, 26, 27, 30, 50, 53, 54) and is composed of three general phases: IE (or α), early or delayed early (β), and late (L or γ). We were interested to determine how the absence of the 65K$_{DBP}$ in CgalΔ42-infected Vero cells or its superinduction in Cgal$^+$-infected V9 cells would affect the expression of viral genes from each temporal class. Some effects, particularly on late gene expression, were expected as an indirect result of the critical role of the 65K$_{DBP}$ in DNA replication. However, inasmuch as 65K$_{DBP}$ is a double-stranded DNA-binding protein, we also considered the possibility that the 65K$_{DBP}$ has the ability to directly affect viral gene expression. For instance, defects in the major DNA-binding protein ICP8 have been shown to lead to overexpression of genes from all temporal classes, distinct from their effects on DNA replication (16–18). Therefore, the Northern blot described above was stripped and reprobed with DNA sequences which would detect the accumulation of IE gene 2 (IE2) encoding ICP27, the early gene encoding TK, and a late gene which maps to UL31 (Fig. 9).

**IE and early gene expression.** The defect in UL42 had little effect on the accumulation of IE2 RNA except for a slightly lower early rate of accumulation in Vero cells infected with CgalΔ42 (Fig. 9A). Analysis of Northern blots with a TK-specific gene probe used to detect the early 1.5-kb TK RNA (43) indicated a substantial accumulation of TK transcript for both the mutant and wild-type viruses in both cell types between 2 and 4 h p.a. (Fig. 9B). Clearly, the absence of the 65K$_{DBP}$ did not lead to a significant over- or underproduction of the model IE or early gene transcripts. However, the superinduction of the 65K$_{DBP}$ by the wild-type virus in V9 cells does appear to correlate with a more rapid rise as well as decline in the accumulation of these transcripts.

**Late gene expression.** Using a probe corresponding to the 1.4-kb transcript of the putative late gene encompassing the UL31 open reading frame (24, 34), we found that expression

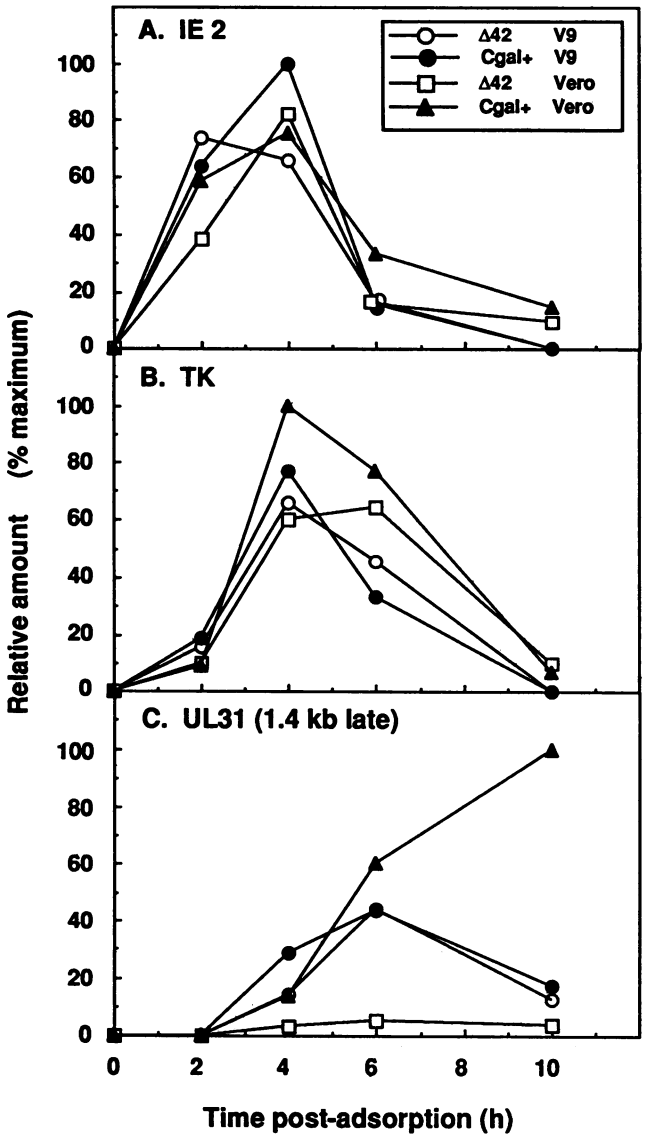


FIG. 9. Blot shown in Fig. 7 was stripped and reprobed to detect IE2 (A), TK (B), and 1.4-kb late UL31 (C) transcripts. The IE2 RNA was detected with the *Rsr*II-*Sal*I fragment located within the ICP27 open reading frame, and TK RNA was detected by an internal *Pst*I fragment probe. The UL31 transcript was detected with a probe from pGEM2-702 (11) which simultaneously resolved the 4.4-kb pol transcript, the 3.0-kb late UL32 transcript, and the abundant 1.4-kb UL31 transcript. The intensities of bands appearing on autoradiograms were determined by densitometry and were normalized to the maximum intensity band (100%) on each blot.

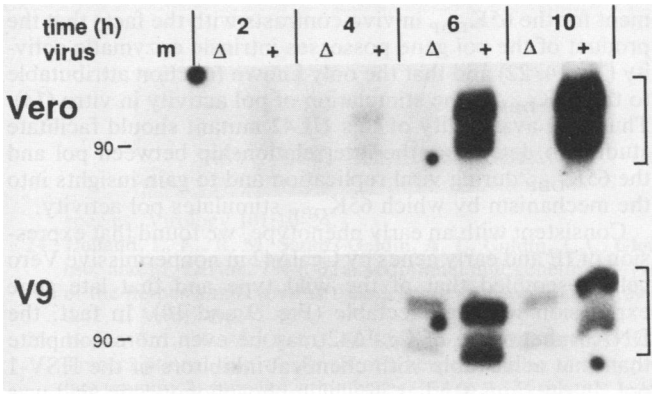


FIG. 10. Primer extension analysis of US11 RNA accumulation. RNA samples from the experiment described in the legends to Fig. 7 and 9 were hybridized to a $^{32}$P-labeled synthetic primer complementary to US11 (30) as described in Materials and Methods. Extension products were analyzed by electrophoresis on a denaturing gel. The positions of US11 transcription are in agreement with previously published data (30). The position of the 90-base *Hpa*II-cut pBR322 size marker is indicated on the left.

was undetectable in Vero cells infected with the UL42 deletion mutant (Fig. 9C). These results are consistent with a strong dependence on DNA replication for the expression of true late genes. We are uncertain of the cause of the underproduction of UL31 by both viruses in V9 cells compared with that of the wild-type virus in Vero cells, but the results may reflect a shift in the kinetics of expression leading to an early decline in accumulation.

As a more sensitive means for detecting late gene expression, we performed primer extension analysis on the previously well-characterized true late gene US11 (29, 30). RNA samples from the same time course described above were incubated with an oligonucleotide primer complementary to a 5′ region of the US11 gene, and the extension products produced in the presence of reverse transcriptase were analyzed (Fig. 10). In productively infected cells, we detected extension products of US11 corresponding to the previously documented transcription start sites (30), although in V9 cells infected with CgalΔ42, US11 transcripts accumulated to a reduced level compared with that of the parental virus. The nonproductive infection of Vero cells by the UL42 deletion mutant clearly failed to produce detectable US11 transcripts. This result is consistent with the DNA$^-$ phenotype of CgalΔ42 and the dependence of late gene expression on DNA replication. The primer extension studies also demonstrate an earlier decline in the abundance of the US11 RNA produced by the wild-type virus in V9 compared with Vero cells, consistent with the pattern observed for the other true late RNA examined, UL31 (Fig. 9C).

## DISCUSSION

We derived a Vero host cell line, designated V9, which contains a functional UL42 gene and is permissive for replication of HSV-1 mutants with defects in the UL42 gene product, the 65K$_{DBP}$. This cell line enabled us to construct and characterize an HSV-1 UL42 deletion mutant, CgalΔ42, as described in this report.

**Characterization of null phenotype.** In Vero cells, CgalΔ42 failed to replicate, with no progeny virus detected up to 28 h after infection (Fig. 5). In addition, we could detect no viral DNA synthesis in these cells, indicative of an early block in replication (Fig. 6A). This phenotype is essentially the same as that described at the npT for *ts*701, a *ts* mutant with a lesion which maps to UL42 (31), and confirms that the 65K$_{DBP}$ is absolutely essential for the synthesis of viral DNA and the production of infectious virus. Taken together with the results of Wu and co-workers (57), who demonstrated that the product of UL42 was required in *trans* for the amplification of plasmids containing an HSV-1 origin of replication, it is likely that the 65K$_{DBP}$ participates directly in the synthesis of viral DNA in vivo. The absolute require-

ment for the $65K_{DBP}$ in vivo contrasts with the facts that the product of the pol gene possesses intrinsic enzymatic activity (11, 14, 22) and that the only known function attributable to the $65K_{DBP}$ is the stimulation of pol activity in vitro (14). Thus, the availability of this UL42 mutant should facilitate studies to determine the interrelationship between pol and the $65K_{DBP}$ during viral replication and to gain insights into the mechanism by which $65K_{DBP}$ stimulates pol activity.

Consistent with an early phenotype, we found that expression of IE and early genes by CgalΔ42 in nonpermissive Vero cells resembled that of the wild type and that late gene expression was undetectable (Fig. 9 and 10). In fact, the $DNA^-$ phenotype of CgalΔ42 may be even more complete than that achievable with chemical inhibitors of the HSV-1 pol. In previous studies in which phosphonoacetic acid was used to block viral DNA synthesis, vastly reduced, yet detectable, transcription of the late genes US11 and gC was shown (18, 30, 55).

The relationship between DNA replication and late gene expression is not well understood. An intriguing possibility is that differences in the nature of the block to DNA replication may account for whether or not any late gene expression can be detected. For example, in the presence of phosphonoacetic acid, formation of a replication complex may occur allowing limited initiation of late gene transcription, which may differ from the type of complex that is formed in the complete absence of the $65K_{DBP}$. Therefore, it will be interesting to study the formation of DNA replication complexes in nonpermissive cells infected with the UL42 deletion mutant and with mutants containing more subtle defects in the $65K_{DBP}$. This in turn may lead to insights into the regulation of late gene expression. In any case, since viral DNA replication has an absolute requirement for the UL42 gene, the $65K_{DBP}$ may prove to be a suitable target for the development of novel antiviral chemotherapeutic agents.

**Effect of $65K_{DBP}$ induction on kinetics of HSV-1 replication.** We could detect no constitutive expression of the endogenous UL42 gene(s) in V9 cells at the RNA or protein level (Fig. 7 and 8). Expression was induced by 4 h after infection with CgalΔ42. Nevertheless, RNA and protein accumulation resulting from transcription and translation of the endogenous UL42 gene in V9 cells infected with CgalΔ42 was less than 1% of the level observed in wild-type virus-infected cells. Therefore, we were surprised to find wild-type levels of viral DNA synthesis and infectious virions in V9 cells infected with the UL42 deletion mutant. These results suggest that only small quantities of $65K_{DBP}$ are required for efficient HSV-1 replication.

Accumulation of UL42 transcripts in $Cgal^+$-infected V9 cells preceded that in infected Vero cells by approximately 2 h (Fig. 7). In addition, when the kinetics of expression of IE, early, and late genes in $Cgal^+$-infected Vero and V9 cells were compared, we consistently observed in V9 cells a shift in the pattern of expression by approximately 2 h, with an earlier increase as well as an earlier decrease in the accumulation of transcripts representing each temporal class (Fig. 9 and 10). The shift in the kinetics of gene expression in V9 cells implies that the earlier presence of threshold levels of the $65K_{DBP}$ in $Cgal^+$-infected V9 cells may facilitate or speed up early stages in the viral replicative cycle.

**Effect of MOI on replication of CgalΔ42.** Mutants containing deletions in the gene encoding ICP8 have been shown to be impaired for replication in host cells which inducibly expressed ICP8, and this impairment was greater at high compared with low input MOIs (38). Previous studies also have indicated that abundant functional ICP8 may downregulate the expression of certain IE, early, and late genes (16–18), presumably in its capacity as a DNA-binding protein. Because the product of the UL42 gene is also a strong DNA-binding protein (15, 33), we were interested to determine whether we could observe a similar repression in replication of CgalΔ42 at high input MOI. However, we found replication of the mutant to be as efficient at an MOI of 10 PFU per cell as at 1 PFU per cell, although we did find that the V9 cells complemented both *ts*701 (data not shown) and CgalΔ42 somewhat less efficiently at high compared with low MOIs (Table 1). Together with the studies comparing gene expression between CgalΔ42 and $Cgal^+$, these data provide no evidence that the $65K_{DBP}$ significantly represses transcription of HSV-1 genes. We cannot, however, exclude the possibility that the absence of multiplicity effects is related to a relatively low level of induction of the $65K_{DBP}$ in V9 cells (Fig. 7 and 8) compared with the induction of ICP8 in stable ICP8-gene transformed cells (38).

There is much interest in the development of HSV-1 vectors for the purpose of transferring transcriptionally active genes to postmitotic cells such as neurons. Since the choice of mutant is likely to be crucial to both the outcome of infection and the expression of inserted foreign genes, it is essential that an understanding of these factors be gained. The construction of the UL42 deletion in the background of a virus containing the *lacZ* reporter gene driven by a heterologous promoter will enable us to assess the effect of this lesion on heterologous gene expression and its potential utility as a gene transfer vector.

In summary, we constructed an HSV-1 mutant deleted for gene UL42, which will provide a useful tool in analyzing the role of the $65K_{DBP}$ in the viral replicative cycle. This virus has a tight early phenotype with no viral DNA replication or late gene expression occurring in nonpermissive cells. In the future, the availability of this deletion mutant should facilitate the construction of other UL42 mutants containing targeted lesions in the gene. Such mutants will be invaluable in the elucidation of the important functional domains of the $65K_{DBP}$.

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