VIROLOGY 237, 97–106 (1997)
ARTICLE NO. VY978763

Deletion Mutants of the Herpes Simplex Virus Type 1 UL8 Protein: Effect on DNA Synthesis

Eleanor C. Barnard,[1] Gaie Brown, and Nigel D. Stow[2]

MRC Virology Unit, Institute of Virology, Church Street, Glasgow, G11 5JR, United Kingdom

Received April 22, 1997; returned to author for revision June 25, 1997; accepted August 7, 1997

The herpes simplex virus type 1 (HSV-1) helicase-primase, an essential component of the viral DNA replication machinery, is a trimeric complex of the virus-coded UL5, UL8, and UL52 proteins. An assembly of the UL5 and UL52 subunits retains both enzymic activities, and the UL8 protein has been implicated in modulating these functions, facilitating efficient nuclear uptake of the complex and interacting with other viral DNA replication proteins. To further our understanding of UL8, we have constructed plasmids expressing mutant proteins, truncated at their N- or C-termini or lacking amino acids internally, under the control of the human cytomegalovirus major immediate-early promoter. Deletion of 23 amino acids from the N-terminus or 33 from the C-terminus abolished the ability of UL8 to support DNA replication in transient transfection assays. None of the UL8 mutants tested exhibited a strong dominant negative phenotype in the presence of the wild-type product, although some inhibition of replication was observed with mutants lacking 165 N-terminal or 497 C-terminal amino acids. The ability of the UL8 mutants to facilitate efficient nuclear localization of UL52 in the presence of coexpressed UL5 was examined by immunofluorescence. Selected mutants were also expressed by recombinant baculoviruses and tested for interaction with UL5 and UL52 in immunoprecipitation assays. The replicative ability of the mutants was found to correlate with their ability to localize UL52 to the nucleus, but not their interaction with UL5 and UL52. This property precluded the identification of any region of UL8 important for its presumed nuclear functions.

INTRODUCTION

A set of seven virus-coded proteins performs direct and essential roles in replicating the DNA genome of herpes simplex virus type 1 (HSV-1). UL30 (pol) and UL42 are the catalytic and accessory components of a dimeric DNA polymerase, UL9 functions as a sequence-specific origin-binding protein (OBP), UL29 (ICP8) as a single-stranded DNA binding protein, and the remaining three proteins (UL5, UL8, and UL52) constitute a tripartite complex which exhibits both DNA primase and DNA helicase activities (reviewed by Challberg, 1991).

In order to investigate the properties of the individual subunits of the helicase–primase complex the three proteins have been separately expressed in heterologous systems, most notably by recombinant baculoviruses in insect cells. Although the individual subunits are enzymically inactive, an *in vivo* subassembly of the UL5 and UL52 components produced in doubly infected cells was shown to possess DNA primase (assayed on homopolymeric templates) and DNA helicase activities essentially indistinguishable from the trimeric complex (Calder and Stow, 1990; Dodson and Lehman, 1991; Crute *et al.*, 1991). These observations and the subsequent finding that UL8 was unable to bind to single- or double-stranded DNA or to DNA/RNA hybrids (Parry *et al.,* 1993) led to puzzlement concerning its role within the complex and no obvious clues emerged from the analysis of its amino acid sequence. Nevertheless, several approaches have recently indicated important contributions that the UL8 protein may make. First, experiments using natural single-stranded DNAs as templates for the primase activity demonstrated that UL8 may serve a key role by stimulating either the synthesis of RNA primers (Tenney *et al.,* 1994, 1995) or the utilization of these primers by DNA polymerase (Sherman *et al.,* 1992). UL8 is also required for optimal helicase and primase activity on ICP8-coated templates (Tanguy Le Gac *et al.,* 1996). Second, immunofluorescence studies with HSV-1 temperature-sensitive mutants that, in addition to their immediate early genes, express additional copies of the UL5, UL8, or UL52 genes at the nonpermissive temperature revealed that UL8, although not translocated to the nucleus when expressed alone, was required for efficient nuclear uptake of the two other components of the helicase–primase complex (Calder *et al.,* 1992). A similar requirement for the presence of UL8 to allow intracellular localization of the UL52 subunit was noted using an HSV-1 mutant containing a suppressible nonsense mutation in the UL8 gene (Marsden *et al.,* 1996). Finally, in addition to directly interacting

[1] Present address: Department of Biochemistry, University of Edinburgh, George Square, Edinburgh, EH8 9XD, UK.
[2] To whom correspondence and reprint requests should be addressed. Fax: +44 141 337 2236. E-mail: n.stow@vir.gla.ac.uk.

0042-6822/97 $25.00

with both the UL5 and UL52 components, UL8 has also been shown to bind specifically to two other DNA replication proteins, UL9 (McLean *et al.,* 1994) and UL30 (Marsden *et al.,* 1997). The physical interactions of UL8 with other DNA replication proteins are likely to be important in the assembly of replication complexes in HSV-1-infected cells (Liptak *et al.,* 1996; Lukonis and Weller, 1996) and possibly contribute to loading of the helicase–primase complex at the replication origins and coordination of leading and lagging strand DNA synthesis with replication fork movement.

UL8 is one of the six genes encoding HSV-1 DNA replication proteins (UL5, UL8, UL29, UL30, UL42, and UL52) that are conserved in the alpha-, beta-, and gamma-herpesvirus subfamilies (reviewed by Davison, 1993). The UL8 homologues of both Epstein–Barr virus (EBV, a gammaherpesvirus) and human cytomegalovirus (HCMV, a betaherpesvirus) have been shown to be required for origin-dependent DNA synthesis in transient assays (Fixman *et al.,* 1992; Pari and Anders, 1993; Smith and Pari, 1995; Sarisky and Hayward, 1996), and are assumed to contribute to a tripartite helicase–primase complex along with homologues of the UL5 and UL52 proteins. The roles of UL8 in modulating the enzymatic activities of the helicase–primase complex, facilitating its nuclear localization, and interacting with the UL30 protein are therefore likely to be conserved in the different subfamilies. In contrast, the interaction between HSV-1 UL8 and UL9 appears to represent a largely alphaherpesvirus-specific function, since, with the exceptions of human herpesviruses 6 and 7 (Inoue *et al.,* 1994; Lawrence *et al.,* 1995; Nicholas, 1996), the beta- and gammaherpesviruses do not encode UL9 homologues.

In order to investigate further the structure/function relationships of the UL8 protein we have isolated and characterized UL8 mutants. This paper describes the generation of N- and C-terminally truncated derivatives of UL8 and assessment of their properties including ability to support HSV-1 origin-dependent DNA synthesis and facilitate efficient nuclear uptake of the helicase–primase complex.

MATERIALS AND METHODS

Cells and viruses

Baby hamster kidney 21 clone 13 (BHK) cells were grown in Eagle's medium (Glasgow MEM) supplemented with 10% tryptose phosphate broth, 10% newborn calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Vero cells were grown in the same medium supplemented with 10% fetal calf serum and antibiotics. Spodoptera frugiperda (*Sf*) cells (strain IPLB-SF-21; Kitts *et al.,* 1990) were maintained in TC100 medium containing 5% fetal calf serum and supplemented with the same antibiotics. Recombinant baculoviruses AcUL5, AcUL8, and AcUL52 have been described previously (Calder and Stow, 1990). The UL8-containing fragments from plasmids pCΔ033, pCΔ280, pNΔ165, and p8ΔN1 (see below) were inserted in the appropriate orientation into the *Bam*HI site of the transfer vector pAcYM1 (Matsuura *et al.,* 1987) and recombinant viruses (AcUL8Δ1, AcUL8Δ2, AcUL8Δ3, and AcUL8Δ4, respectively) isolated essentially as described by Kitts *et al.* (1990), except that the parental viral DNA was AcPAK6 DNA cleaved with *Bsu*36I (Bishop, 1992).

Antibodies

Rabbit antiserum raised against a synthetic decapeptide from the C-terminus of the UL52 protein (Olivo *et al.,* 1989) was a kind gift of Dr M. D. Challberg. Rabbit antiserum 094 (Parry *et al.,* 1993) and mouse monoclonal antibodies (MAbs) 811, 814, and 817 (McLean *et al.,* 1994) were generated using purified UL8 protein as immunogen. MAbs 811, 814, and 817 recognize epitopes located in the regions of UL8 comprising amino acids 166–253, 470–554, and 718–750, respectively (Marsden *et al.,* 1997).

Plasmids

Plasmids pE5, pE8, pE9, pE29, pE30, pE42, and pE52 contain the UL5, UL8, UL9, UL29, UL30, UL42, and UL52 DNA replication genes under the control of the human cytomegalovirus major immediate early promoter in the vector pCMV10 (Stow *et al.,* 1993). Plasmid pM2, a similar construct containing the *Escherichia coli* β-galactosidase gene, was used as a control for comparing transfection efficiencies in transient DNA replication assays.

Plasmids expressing C-terminally truncated UL8 proteins, designated pCΔx, where x corresponds to the number of amino acids deleted were constructed as follows. Plasmid pE8, which contains the UL8 gene cloned as a *Bam*HI fragment, was linearized at the *Xba*I site immediately downstream of the gene and digested for varying times with nuclease Bal31. DNA was ligated to synthetic 14-bp *Xba*I linkers containing termination codons in all three reading frames (New England Biolabs), digested with *Bam*HI plus *Xba*I, and ligated to plasmid pCMV10X [a modified version of pCMV10 (Stow *et al.,* 1993) in which the original *Xba*I site had been replaced by the 14-bp *Xba*I termination codon linker] cut with the same enzymes. *E. coli* DH5 cells were transformed and the resulting plasmids were analyzed by a combination of restriction endonuclease digestion and DNA sequencing. Plasmids expressing N-terminally truncated UL8 proteins (pNΔx) were similarly constructed following Bal31 digestion of pE8 DNA linearized at the *Eco*RI site upstream of the UL8 gene. A linker containing an initiating ATG codon and an *Eco*RI cohesive end (5′ AATTCGCCACCATGGGG plus 3′ GCGGTGGTACCCC) was ligated, the DNA digested with *Bam*HI plus *Eco*RI, and ligated to pCMV10 cleaved with the same enzymes. Plas-

mids with the UL8 gene sequences in frame with the ATG codon were identified by transfecting BHK cells and sceening for expression of UL8-related products by Western blot analysis of lysed cells using polyclonal antiserum (094). The deletion end points were determined by DNA sequencing. Plasmid pNΔ005 was generated following ligation of the same initiation codon linker, in frame, at the unique *Eco*RV site near the 5′ end of the UL8 gene. Plasmid p8ΔN1 was constucted by an in-frame deletion between the two internal *Not*I sites in pE8 and specifies a protein lacking amino acids 78–339.

Expression of mutant UL8 proteins

Sparse monolayers of BHK cells (approximately 1.5×10^5 cells per 35-mm plate) were transfected with 2 μg plasmid DNAs using liposomes as described by Rose *et al.* (1991). Twenty-four hours posttransfection the cell monolayer was washed with Tris-buffered saline, the cells were scraped from the plate, and pelleted. The pelleted cells were washed once with SE buffer (100 m*M* NaCl, 20 m*M* Tris–HCl, pH 8.0, 10% glycerol) and lysed in 100 μl SE buffer containing 1% Nonidet-P40 for 15 min on ice. Soluble extracts were defined as the supernatant fraction obtained after centrifugation of the lysed cells at 100,000 *g* for 30 min (Beckman TLA 100.2 rotor). Samples of whole cell and soluble extracts were analyzed by polyacrylamide gel electrophoresis and Western blotting using the indicated antibodies as described previously (McLean *et al.,* 1994).

Transient assay for origin-dependent DNA synthesis

Assays were performed as previously described (Stow *et al.,* 1993). BHK cell monolayers in 35-mm petri dishes were transfected by the calcium phosphate procedure followed by treatment with DMSO after 4 hr. Duplicate monolayers received 0.4 ml of precipitate containing 0.5 μg of each of the plasmids encoding DNA replication genes (pE5, pE9, pE29, pE30, pE42, pE52, plus test plasmid encoding wt or mutated UL8), 0.4 μg plasmid pS1 (containing a functional HSV-1 ori_S replication origin; Stow and McMonagle, 1983), 0.1–0.5 μg pM2, and 6 μg calf thymus DNA. Total cellular DNA was prepared 30 hr posttransfection from one of the plates and analyzed for pS1 replication. DNA samples from 2×10^5 cells were cleaved with *Eco*RI plus *Dpn*I and subjected to agarose gel electrophoresis and Southern blotting. Replicated molecules (*Dpn*I-resistant) were detected by hybridization to a probe containing plasmid vector sequences. Quantification was performed on a PhosphorImager (Molecular Dynamics). To confirm that there were no significant variations in transfection efficiency when different plasmids were used, the other plate was stained for β-galactosidase expression and stained cells in representative fields were counted. Replication assays investigating possible inhibitory effects of the UL8 mutants were performed as above except that monolayers received 0.5 μg each of pE5, pE8, pE9, pE29, pE30, pE42, and pE52 supplemented with an additional 0.5 μg of the plasmid under test.

Immunofluorescence

Monolayers of Vero cells (4×10^5cells) in 35-mm plastic petri dishes were cotransfected with plasmids using the calcium phosphate method followed by treatment at 4 hr posttransfection with 25% DMSO (Stow and Wilkie, 1976). Each monolayer received 1 ml of precipitate containing 18 μg calf thymus carrier DNA and 1 μg of each of the indicated plasmids, and, following transfection, the cells were maintained in Eagle's medium containing 5% fetal calf serum plus antibiotics. Twenty-four hours posttransfection the plates were washed and fixed with a 3:1 methanol/acetone mixture. The cells were treated with antibody against the UL52 protein as previously described (Calder *et al.,* 1992) and the stained cells viewed under a Nikon Microphot-SA fluorescence microscope. Photographs were taken on Kodak TMAX 3,200 film with a 40× objective lens.

Immunoprecipitation

All procedures were performed essentially as described by McLean *et al.* (1994). Sf cells were infected with recombinant baculoviruses and L-[^{35}S]methionine-labeled extracts were incubated with MAbs 811 or 817. The bound proteins were collected with protein A–Sepharose beads and analyzed on 8.5% polyacrylamide gels.

RESULTS

Expression of mutated UL8 polypeptides

Plasmids containing deleted copies of the UL8 gene located immediately downstream of the HCMV major immediate-early promoter were generated as described under Materials and Methods. The precise end-points of the deletions were determined by DNA sequencing and the predicted UL8 products are described in Table 1.

The ability of plasmids used in subsequent experiments to express UL8-related proteins was confirmed by Western blot analysis of lysates of transfected BHK cells (Fig. 1). Examination of the total protein samples (lanes T) shows that each plasmid expressed a product detectable by an antiserum against UL8 protein. The apparent sizes of several of these deleted proteins determined from blots which included molecular weight markers are shown in Table 1 and show a good correlation with their predicted molecular weights. The amounts of truncated polypeptide accumulating in transfected cells were similar to the amount of full-length UL8 produced in cells transfected with the parental plasmid pE8. The presence of the UL8 mutants in soluble extracts of the transfected cells was also examined (lanes S). Full-length UL8 and

TABLE 1

UL8 Mutants Used in These Studies[a]

Plasmid	Amino acid sequence	Predicted M_r	Apparent size (kDa)[b]
pE8	Wild type (amino acids 1–750)	79924	86
pCΔ004	1–746	79446	—[c]
pCΔ033	1–717 LV	76484	82
pCΔ041	1–709 SLD	75532	—
pCΔ079	1–672	71147	76
pCΔ280	1–470 SLD	49952	56
pCΔ359	1–391	41598	46
pCΔ497	1–253 PSLD	27549	29
pNΔ005	MG 6–750	79579	—
pNΔ023	MG 24–750	77519	—
pNΔ043	MG 44–750	75156	—
pNΔ097	MG 98–750	69642	75
pNΔ165	MG 166–750	62453	68
p8ΔN1	1–77 and 340–750	52109	—

[a] Plasmids were generated as described under Materials and Methods and the encoded products (shown relative to full length UL8) predicted from their DNA sequences. The pCΔ and pNΔ series lack the indicated number of amino acids from their C or N terminus, respectively, and additional amino acids resulting from the presence of the linker oligonucleotides are shown in single letter code.

[b] Determined from blots performed in the presence of molecular weight markers.

[c] Not determined.

the N-terminal truncations were efficiently recovered. The p8ΔN1 protein and C-terminal truncations lacking 4, 33, 41, or 71 amino acids appeared slightly less soluble than the full-length protein but were nevertheless readily detected in the high speed supernatants. In contrast, the three mutants with the largest C-terminal deletions (CΔ280, CΔ359, and CΔ497) were poorly recovered in the soluble fraction. This suggests that at least when expressed alone these three truncated proteins form aggregates.

Ability of truncated UL8 proteins to support HSV origin-dependent DNA synthesis

The ability of the mutant UL8 proteins to participate in DNA synthesis was tested in transient transfection assays. Plasmids encoding wild-type or mutated UL8 proteins were cotransfected into BHK cells together with plasmids specifying the other six HSV-1 DNA replication proteins and a functional copy of HSV-1 ori_S (plasmid pS1). Total cellular DNA was analyzed 30 hr after tranfection. Figure 2 shows that efficient replication of the origin-containing plasmid occurred in the presence of all seven wt DNA replication proteins. Substitution of plasmid pE8 with either pCΔ004 or pNΔ005 resulted in a reduced level of replication. The replicative abilities of pCΔ004 and pNΔ005 were quantified by PhosphorImager analysis of hybridized blots or scanning of autoradiographs. In three independent experiments pNΔ005 exhibited 89, 73, and 56% of the activity of pE8 with corresponding values of 23, 10, and 18% for pCΔ004. None of the other plasmids supported detectable origin-dependent DNA synthesis in any experiment (Fig. 2 and data not shown). Removal of 23 N-terminal or 33 C-terminal amino acids is thus sufficient to inactivate the replicative function of UL8.

Ability of truncated UL8 proteins to interfere with HSV origin-dependent DNA synthesis

Several of the UL8 products were tested for their ability to interfere with HSV-1 origin-dependent DNA synthesis in transfected BHK cells. Plasmids encoding the NΔ005, NΔ023, NΔ165, CΔ004, CΔ033, CΔ497, and 8ΔN1 pro-

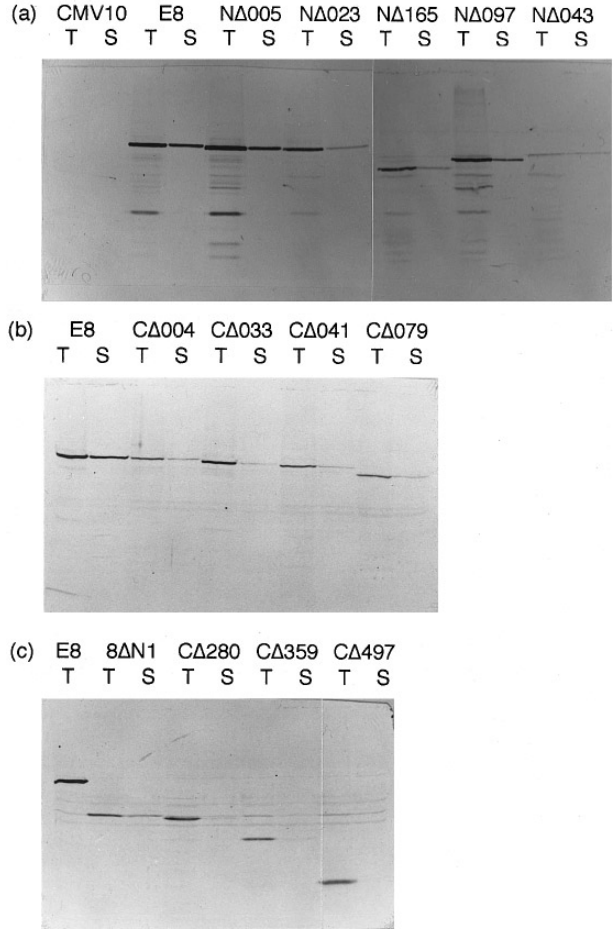

FIG. 1. Expression and solubility of mutated UL8 proteins. Whole cell lysates (T) from BHK cells transfected with the indicated plasmids were compared with soluble extracts (S) obtained following centrifugation at 100,000 *g* for 30 min. Samples recovered from equivalent numbers of cells (approximately 2×10^4) were resolved by electrophoresis through 9% polyacrylamide gels and transferred to nitrocellulose. The blots were incubated with MAb 817 (a), MAb 814 (b), or polyclonal serum 094 (c), washed, and bound antibody was detected with anti-mouse (a and b) or anti-rabbit (c) IgG alkaline phosphatase-conjugated antibody in conjunction with the Promega Protoblot system.

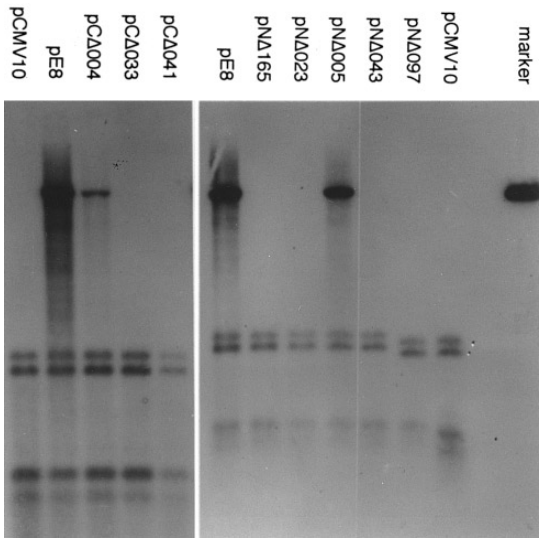

FIG. 2. Ability of mutated UL8 proteins to support HSV-1 origin-dependent DNA synthesis. BHK cells were cotransfected with the origin-containing plasmid pS1, plasmids pE5, pE9, pE29, PE30, pE42, and pE52 and a UL8 containing plasmid or the vector pCMV10 as indicated. 30 hr posttransfection DNA was prepared and analyzed as described under Materials and Methods. The marker lane contains linearized pS1 DNA, and bands detected in the lower half of the gel represent the *Dpn*I cleavage products of unreplicated input plasmid molecules.

teins were individually cotransfected with pS1 and the seven plasmids encoding the wt HSV-1 DNA replication proteins. The level of pS1 replication was assessed in independent experiments by Phosphorimager analysis (Table 2). The addition of vector pCMV10 DNA or of an additional 0.5 μg pE8 DNA did not significantly affect the amount of replicated pS1 DNA detected. Similarly, with the possible exception of plasmids pNΔ165 and pCΔ497, none of the plasmids encoding mutated UL8 proteins had an inhibitory effect. In the presence of the pNΔ165 and pCΔ497 products, DNA synthesis was reduced to 50 and 38% of that seen in their absence (averages of two experiments). This level of inhibition is much less than that observed with a powerful dominant negative inhibitor such as the C-terminal domain of the UL9 protein (Perry *et al.,* 1993; Baradaran *et al.,* 1996), which inhibits synthesis approximately 30-fold in similar experiments (Stow *et al.,* 1993).

Effect of UL8 mutations on the intracellular localizationof the UL52 protein

We previously established, using HSV-1 recombinants, that coexpression of UL5, UL8, and UL52 was necessary for the efficient localization of any one of these proteins to the cell nucleus (Calder *et al.,* 1992). Since the recombinant viruses also expressed HSV-1 immediate early proteins, it remained possible that the helicase–primase complex alone was not capable of localizing to the nucleus. To address this possibility we therefore carried out similar immunofluorescence experiments on Vero cells transfected with plasmids expressing the components of the helicase–primase complex under the control of the constitutive major immediate early promoter of HCMV.

Since UL5 and UL52 together form a stable subassembly, we initially investigated the ability of UL8 to influence the cellular localization of this subassembly by staining the transfected cells with antibody against the UL52 protein (Fig. 3). When UL5 and UL52 were coexpressed, UL52 was found almost exclusively in the cytoplasm (Fig. 3A). In contrast, when wt UL8 was also present the staining was predominantly nuclear (Fig. 3B). In agreement with previous results (Calder *et al.,* 1992), UL52 remained cytoplasmic when expressed alone or with only UL8 (data not shown). Essentially similar results were obtained when the localization of UL5 was examined.

Nuclear localization of UL52 was observed in 100% of the expressing cells cotransfected with plasmids pE5, pE8, and pE52, indicating that all three proteins were coexpressed in the population of competent cells (usually 1–5%). Some heterogeneity was, however, observed in the pattern of cytoplasmic staining of UL52 in the presence of UL5 alone. While fairly diffuse staining typified by Fig. 3A was seen in approximately half the positive cells, the remainder exhibited a more granular appearance (resembling the pattern seen in Figs. 3I and 3J). Similar heterogeneity was also noted with the UL8 mutants which failed to facilitate nuclear localization of UL52 (see below) and may be a consequence of differences in the levels of protein expressed from the transfected plasmids in individual cells.

UL8 expressed alone was detected as bright cytoplasmic fluorescence with additional weaker staining of the nuclei in approximately 25% of cells. Coexpression

TABLE 2

Inhibition of HSV-1 Origin-Dependent DNA Synthesis[a]

Plasmids	Relative amount of replicated pS1 DNA			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
7 wt	100	100	100	100
7 wt + pCMV10	97	92	107	135
7 wt + pE8	112	109	140	nd
7 wt + pNΔ005	nd	nd	73	177
7 wt + pNΔ023	nd	nd	nd	101
7 wt + pNΔ165	55	44	nd	nd
7 wt + pCΔ497	44	32	nd	nd
7 wt + pCΔ033	nd	nd	87	nd
7 wt + pCΔ004	nd	nd	153	131
7 wt + p8ΔN1	nd	nd	149	121

[a] Transient replication assays were carried out using plasmids encoding the seven wt HSV-1 DNA replication genes plus the indicated additional plasmids. The relative amounts of replicated pS1 DNA were determined using a Phosphorimager. Data for four separate experiments are shown, with the amount of replicated DNA in cells receiving just the 7 wt plasmids assigned a value of 100 in each case. nd signifies not done.

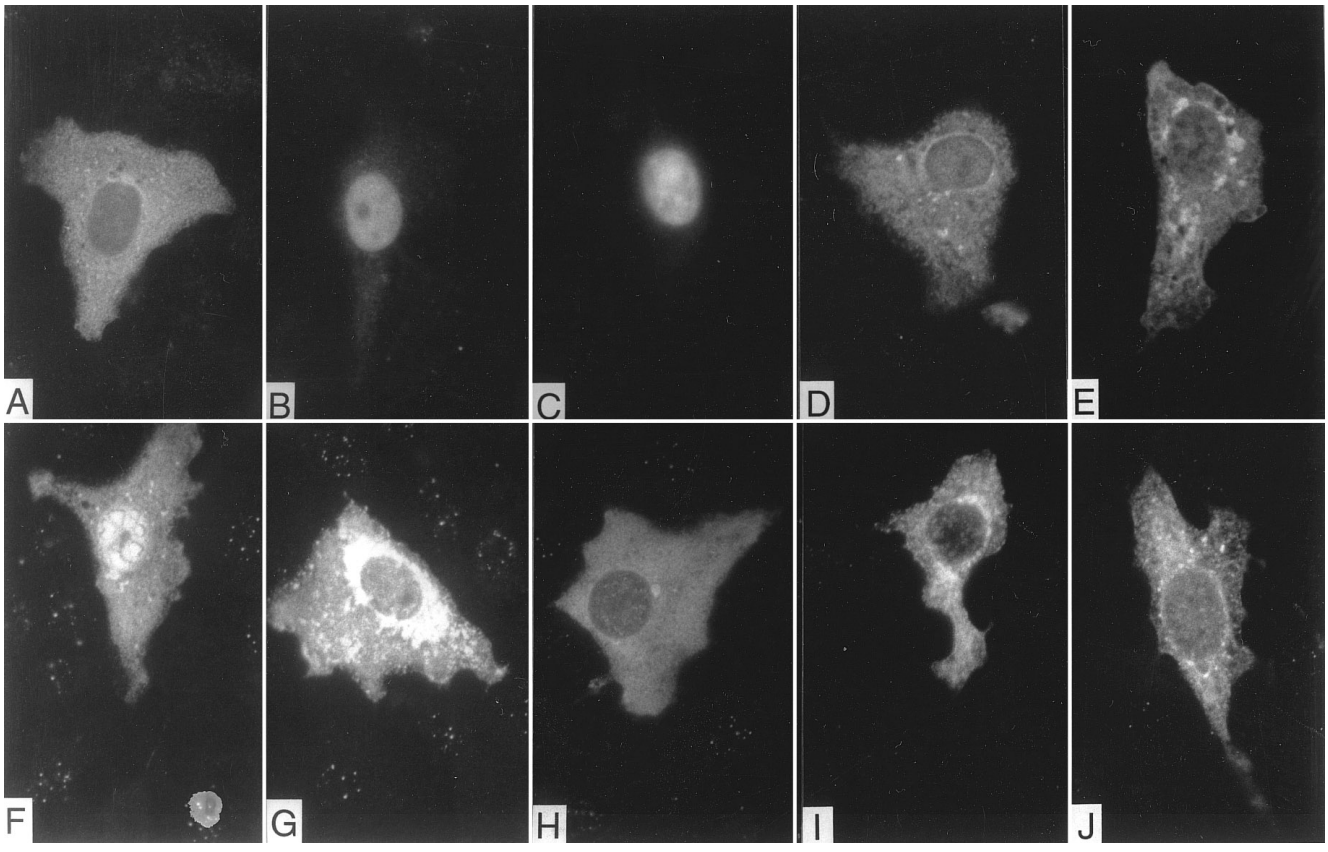

FIG. 3. Cellular localization of the UL52 protein in the presence of UL5 and wt or mutated UL8 proteins. Vero cells were transfected with plasmids pE5 and pE52 in the presence of the following additional plasmids: A, none; B, pE8; C, pNΔ005; D, pNΔ023; E, pNΔ043; F, pCΔ004; G, pCΔ004; H, pCΔ033; I, pCΔ041; J, p8ΔN1. 24 hr posttransfection the cells were fixed and stained for the presence of UL52 protein. Photographs of representative cells expressing UL52 are shown.

of UL8 with UL5 and UL52 resulted in combined cytoplasmic and nuclear staining for UL8 in all expressing cells. Double-labeling experiments on these triply transfected cells showed that UL8 colocalized with UL5 and UL52 in the nucleus but not the cytoplasm of expressing cells (data not shown). These results suggest that coexpression of the UL5, UL8, and UL52 proteins is sufficient to enable the formation and nuclear localization of the tripartite helicase–primase complex. The presence of UL8 in both the nucleus and cytoplasm of triply transfected cells may occur because it is expressed at higher levels than UL5 or UL52 resulting in both free and complexed forms. Consequently, in these experiments, UL8 was considered to be a poor marker for the localization of the helicase–primase complex.

The plasmids expressing mutated UL8 proteins were tested in parallel for their ability to facilitate nuclear localization of UL52 in triply transfected cells. With the exception of the pNΔ005 and pCΔ004 products, none of the mutant UL8 proteins was able to localize UL52 to the nucleus. Examples of positive cells which received pNΔ023, pNΔ043, pCΔ033, pCΔ041, and p8ΔN1 are shown in Figs. 3D, 3E, 3H, 3I, and 3J, respectively. The pNΔ005 product was phenotypically indistinguishable from wt UL8 (Fig. 3C), whereas the pCΔ004 protein was unique in that individual cells were observed exhibiting either predominant nuclear or cytoplasmic UL52 localization. Examples of cells which received pCΔ004 and exhibited predominant nuclear or cytoplasmic UL52 staining are shown in Figs. 3F and 3G, respectively.

The phenotypes of the mutated UL8 proteins were essentially identical when similar transfection experiments were performed on BHK cells and the localization of either UL52 or UL5 was followed (data not shown).

Interaction of mutated UL8 proteins with UL5 and UL52

A possible explanation for the failure of the mutated UL8 proteins to enable nuclear localization of UL52 might be a failure to form a complex with the other two components of the helicase–primase complex. Four of the deleted UL8 genes were therefore transferred to baculovirus expression vectors and the mutated proteins examined for ability to interact with UL5 and UL52 in coimmunoprecipitation experiments as previously described (McLean *et al.,* 1994).

Recombinant viruses AcUL8Δ1, AcUL8Δ2, AcUL8Δ3,

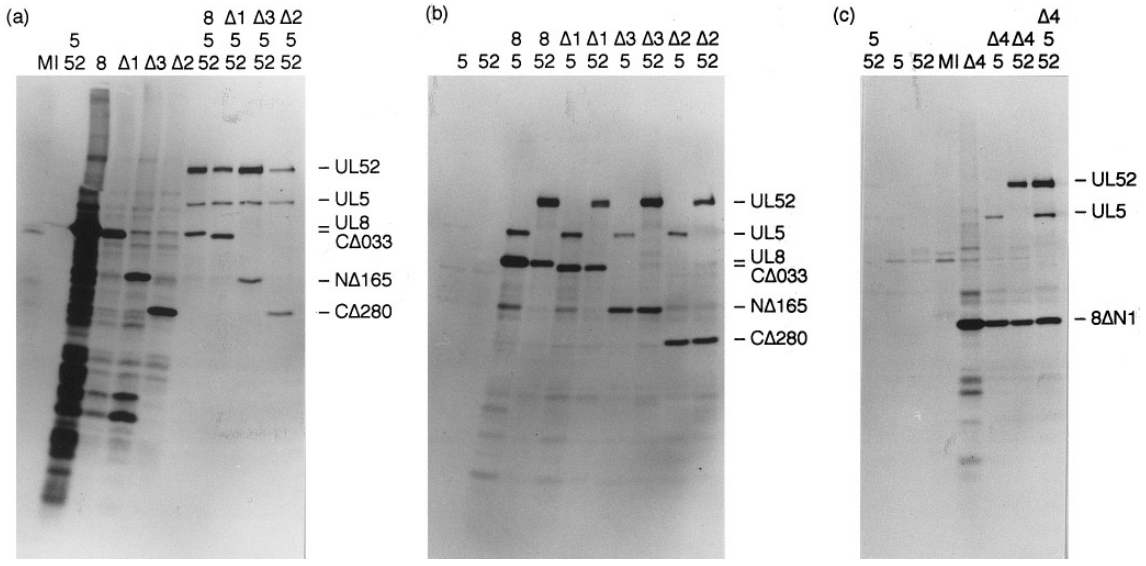

FIG. 4. Interaction of wt and mutated UL8 proteins with UL5 and UL52. ^{35}S-labeled extracts from insect cells infected with the indicated recombinant baculoviruses were immunoprecipitated with MAb 811 (a and b) or MAb 817 (c) [5, AcUL5; 52, AcUL52; 8, AcUL8; Δ1, AcUL8Δ1; Δ2, AcUL8Δ2; Δ3, AcUL8Δ3; Δ4, AcUL8Δ4; MI, mock-infected cells]. Proteins were separated on 8.5% SDS–PAGE gels and the positions of UL5, UL52, and UL8 proteins are shown.

and AcUL8Δ4 contain UL8 fragments from pCΔ033, pCΔ280, pNΔ165, and p8ΔN1, respectively. All four viruses efficiently express the deleted UL8 proteins which can be recovered in, and immunoprecipitated from, soluble extracts of infected Sf cells obtained after centrifugation at 100,000 *g* for 30 min (Figs. 4a, lanes 4–6, and 4c, lane 5).

Figure 4a shows the results of an experiment in which the products of recombinant viruses AcUL8Δ1, AcUL8Δ2, and AcUL8Δ3 were coexpressed with both UL5 and UL52 in triply infected Sf cells. Labeled extracts were precipitated with MAb 811. In agreement with previous results (McLean *et al.,* 1994), control experiments confirmed that the precipitation of UL5 and UL52 was dependent on the presence of UL8 (compare lanes 2 and 7). Each of the truncated proteins was precipitated from cells singly infected with the appropriate virus (lanes 4–6). In addition both UL5 and UL52 were coprecipitated with all three of the truncated proteins from the mixedly infected cells (lanes 8–10). The higher background seen with the immunoprecipitations from cells infected with baculoviruses expressing either wt or mutated UL8 protein alone is probably because of reduced efficiency of host cell shut off at the lower multiplicity of infection (5 PFU/cell for the single infections compared with 25 PFU/cell for the triple infections).

Since all three proteins appeared capable of forming a tripartite complex with UL5 and UL52, their ability to interact separately with these proteins was also tested in similar coinfection experiments (Fig. 4b). MAb 811 did not precipitate UL5 or UL52 from single infection controls (lanes 1 and 2), but, as previously reported (McLean *et al.,* 1994) both proteins were specifically coprecipitated with UL8 from coinfected cells (lanes 3 and 4). The three UL8 mutants also each coprecipitated both UL5 and UL52 from the mixedly infected cells (lanes 5–10).

Similar experiments performed with AcUL8Δ4, which expresses the fragment from p8ΔN1, are shown in Fig. 4c, although in this instance MAb 817 was used for immunoprecipitation. Neither UL5 nor UL52 were precipitated from control extracts of cells infected with AcUL5 and AcUL52 either alone or in combination (lanes 1–3). However, the truncated protein specifically coprecipitated both UL5 and UL52 from triply infected cells (lane 8). In addition UL5 and UL52 each precipitated with UL8 from extracts of doubly infected cells (lanes 6 and 7, respectively). All four deleted UL8 proteins examined therefore retain the ability to interact separately with UL5 and UL52.

DISCUSSION

Our approach to gaining further insights into the structure and function of UL8 involved the isolation and characterization of deletion mutants. As in most similar studies relying on deletion mutants and expression systems two important factors which should be considered in interpreting the results concern the overall structure and expression levels of the deleted proteins. Since grossly misfolded proteins frequently form insoluble aggregates, the solubilities of expressed products are often examined. We employed a rigorous criterion for solubility (presence in the supernatant following centrifugation at 100,000 *g* for 30 min) for proteins expressed in the mammalian and insect cell systems. Although the solubility of several of the proteins in transfected BHK cells was clearly reduced compared to wt UL8, the key mutants that

define the minimal regions whose removal inactivates replicative ability (proteins NΔ023, CΔ033, and 8ΔN1) nevertheless remained detectable in the soluble fraction, indicating that at least a proportion of the protein is potentially available to participate in DNA synthesis. Similarly the four deleted proteins expressed by baculovirus recombinants (CΔ033, CΔ280, NΔ165, and 8ΔN1) were also recovered in soluble extracts. The relative solubility of the CΔ280 protein appeared greater in insect than mammalian cells, perhaps because of the lower incubation temperature (28 versus 37°). In the absence of structural information, the strongest evidence that these proteins are not grossly misfolded is provided by the observation that they retain the ability to interact with both UL5 and UL52. The fact that three of these proteins contain large deletions also argues against the possibility that defects in the proteins carrying smaller deletions result because of global structural changes. The activity of proteins in various assays is also affected by their level of expression, and it should be noted that in this study the intracellular levels of baculovirus-expressed proteins were significantly higher than those achieved in transfected mammalian cells. Additionally, both the level of expression of a particular protein in individual cells and the solubility of the different proteins showed significant variation. For these reasons it is not possible to make rigorous quantitative statements regarding the activities of the deleted proteins, although a number of qualitative conclusions can be tentatively drawn.

The regions spanning amino acids 6–23 at the N-terminus, 718–750 at the C-terminus, and 78–339 internally all contain sequences whose removal destroys the ability of UL8 to support origin-dependent DNA synthesis (Fig. 2), suggesting that these regions may be important for UL8 activity. UL8, along with UL42, is the least well conserved of the six DNA replication proteins common to the alpha-, beta-, and gamma-herpesvirus subfamilies and lacks any obvious nonherpesviral homologues in the databases. Sequence alignments have been relatively unhelpful in identifying regions of the protein which may be important for its various activities. The internal deletion did, however, remove the one relatively small region (amino acids 272–318) which exhibits close similarity to portions of the EBV and HCMV homologues (Fixman *et al.,* 1992). Comparison of HSV-1 UL8 (McGeoch *et al.,* 1988) with its sequenced homologues in other alphaherpesviruses, i.e., varicella-zoster virus (VZV, Davison and Scott, 1986), equine herpesvirus 1 (EHV-1, Telford *et al.,* 1992), bovine herpesvirus 1 (BHV-1, Vlcek *et al.,* 1995), and pseudorabies virus (PRV, Dijkstra *et al.,* 1997), reveals that the important sequences at the termini of UL8 are relatively poorly conserved. Interestingly, it has been reported that the C-terminal 77 amino acids of the bovine herpesvirus 1 protein are not absolutely essential for virus growth in cell culture (Schmitt and Keil, 1996).

Our analysis was unfortunately unsuccessful in identifying any domain of UL8 required for a specific function. All of the mutants which were unable to support viral DNA synthesis also appeared defective in nuclear uptake of the helicase–primase complex. This observation is unlikely to be due to a failure of the replication-deficient UL8 proteins to interact with the other components of the complex since four of these proteins (CΔ033, CΔ280, NΔ165, and 8ΔN1), when expressed by recombinant baculoviruses, were able to coimmunoprecipitate with both the UL5 and UL52 proteins. The sequences involved in the UL8/UL5 and UL8/UL52 interactions are therefore probably confined to amino acids 340–470 (present in each of these four mutated proteins) or spread over multiple sites such that coprecipitation can still occur when certain interacting regions are deleted.

Because the mutants that are defective in DNA synthesis are also unable to generate a nuclear-located helicase–primase complex, possible contributions of various regions of UL8 to essential nuclear replicative functions cannot be determined from the present studies. It is quite possible that some of our mutants may be affected in modulation of the helicase or primase activities or binding to the UL9 or UL30 proteins and it will be interesting to analyze their biochemical properties. The observations that an antibody which recognizes an epitope within the C-terminal 29 amino acids of UL8 and a peptide representing amino acids 719–738 both block the interaction with UL30 (Marsden *et al.,* 1997) suggest that mutants with deletions encompassing this region may also be directly affected in DNA synthesis. UL8 mutants capable of localizing the helicase–primase complex to the nucleus will be required to address whether such activities are essential within the context of the transient replication assay or a viral infection.

The observation that none of the UL8 mutants tested strongly inhibited viral DNA synthesis may also be attributable to their inability to localize the helicase–primase complex to the nucleus. In cells coexpressing both mutant and wt forms of UL8, even though the association of the mutant protein with UL5 and UL52 might cause their retention in the cytoplasm, sufficient wt helicase–primase complex would be expected to form and be transported into the nucleus to enable DNA synthesis, albeit at slightly reduced efficiency. It is possible that nonfunctional UL8 proteins capable of generating nuclear helicase–primase complexes might act as stronger inhibitors of replication by recruiting other components of the replicative machinery into nonfunctional assemblies.

The immunofluorescence experiments presented in this manuscript confirm and extended our previous observations on the nuclear uptake of the wt UL5, UL8, and UL52 proteins made using recombinant viruses (Calder *et al.,* 1992). It is now clear that UL8 plays an essential role in the nuclear localization of the helicase–primase complex and that no other herpesviral proteins are required for this process in either BHK (data not shown)

or Vero cells. Since neither UL8 nor a subassembly of UL5 plus UL52 is transported efficiently into the nucleus a feature specific to the tripartite complex is likely to be necessary for nuclear localization. This might represent a functional nuclear localization signal (NLS) formed from parts of each protein. Alternatively a conformational change to one or more of the proteins might occur when the tripartite complex is formed which unmasks a previously hidden NLS and/or prevents interaction with a cytoplasmic anchoring protein (reviewed by Silver, 1991; Nigg *et al.,* 1991). It is noteworthy, however, that none of the three proteins contains an obvious consensus NLS sequence of either the mono- or bipartite type described by Dingwall and Laskey (1991).

The analysis of the UL8 mutants indicates that whichever mechanism of nuclear uptake operates it is disrupted by removal of the 23 N-terminal or 33 C-terminal amino acids. Since neither of these regions contains any basic amino acids they are unlikely, either individually, or with other parts of the complex, to contribute directly to a NLS. The phenotype of the p8ΔN1 product also indicates that these two regions together are not sufficient for nuclear localization. A possible explanation is that an appropriate conformation of the tripartite complex is necessary to allow recognition by the cellular components involved in nuclear transport, and that this is critically dependent upon multiple interactions between the various subunits. Even removal of 4 amino acids from the C-terminus had an effect on cellular localization of the complex, although it is not clear why UL52 was detected in the nucleus of approximately 50% of the cells but remained cytoplasmic in the remainder. It is conceivable that cell to cell variation in expression level or solubility are responsible. An alternative possibility is that this property results from cell cycle differences among the cell population, perhaps related to reformation of a nucleus following mitosis. The reduction in DNA synthesis observed with the CΔ004 protein was nevertheless significantly greater than 50%, suggesting that nuclear uptake may still not be fully wild type even in those cells expressing nuclear UL52 protein or that the protein may be affected in a nuclear function. It is of interest to note that UL8 and the homologous proteins of VZV, BHV-1, EHV-1, and VZV are identical in three of these four C-terminal residues (FLFX).

ACKNOWLEDGMENTS

We thank D. J. McGeoch and H. S. Marsden for helpful comments and discussions. M. D. Challberg kindly provided antibody against the C-terminus of the UL52 protein. E.C.B. was the recipient of a Glaxo Group Research Studentship.

REFERENCES

Baradaran, K., Hardwicke, M. A., Dabrowski, C. E., and Schaffer, P. A. (1996). Properties of the novel herpes simplex virus type 1 origin-binding protein, OBPC. *J. Virol.* 70, 5673–5679.

Bishop, D. H. L. (1992). Baculovirus expression vectors. *Semin. Virol.* 3, 253–264.

Calder, J. M., and Stow, N. D. (1990). Herpes simplex virus helicase-primase: The UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities. *Nucleic Acids Res.* 18, 3573–3578.

Calder, J. M., Stow, E. C., and Stow, N. D. (1992). On the cellular localization of the components of the herpes simplex virus type 1 helicase-primase complex and the viral origin-binding protein. *J. Gen. Virol.* 73, 531–538.

Challberg, M. D. (1991). Herpes simplex virus DNA replication. *Semin. Virol.* 2, 247–256.

Crute, J. J., Bruckner, R. C., Dodson, M. S., and Lehman, I. R. (1991). Herpes simplex-1 helicase-primase: Identification of two nucleoside triphosphatase sites that promote DNA helicase action. *J. Biol. Chem.* 266, 21252–21256.

Davison, A. J., and Scott, J. E. (1986). The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* 67, 1759–1816.

Davison, A. J. (1993). Herpesvirus genes. *Rev. Med. Virol.* 3, 237–244.

Dijkstra, J. M., Fuchs, W., Mettenleiter, T. C., and Klupp, B. G. (1997). Identification and transcriptional analysis of pseudorabies virus UL6 to UL12 genes. *Arch. Virol.* 142, 17–35.

Dingwall, C., and Laskey, R. A. (1991). Nuclear targeting sequences—A consensus? *Trends Biochem. Sci.* 16, 478–481.

Dodson, M. S., and Lehman, I. R. (1991). Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase-primase composed of the UL5 and UL52 gene products. *Proc. Natl. Acad. Sci. USA* 88, 1105–1109.

Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1992). *Trans*-acting requirements for replication of Epstein–Barr virus ori-Lyt. *J. Virol.* 66, 5030–5039.

Inoue, N., Dambaugh, T. R., Rapp, J. C., and Pellett, P. E. (1994). Alphaherpesvirus origin-binding protein homolog encoded by human herpesvirus 6B, a betaherpesvirus, binds to nucleotide sequences that are similar to *ori* regions in alphaherpesviruses. *J. Virol.* 68, 4126–4136.

Kitts, P. A., Ayres, M. D., and Possee, R. D. (1990). Linerization of baculovirus DNA enhances recovery of recombinant virus expression vectors. *Nucleic Acids Res.* 18, 5667–5672.

Lawrence, G. L., Nicholas, J., and Barrell, B. G. (1995). Human herpesvirus 6 (strain U1102) encodes homologues of the conserved herpesvirus glycoprotein gM and the alphaherpesvirus origin-binding protein. *J. Gen. Virol.* 76, 147–152.

Liptak, L. M., Uprichard, S. L., and Knipe, D. M. (1996). Functional order of assembly of herpes simpex virus DNA replication proteins into prereplicative site structures. *J. Virol.* 70, 1759–1767.

Lukonis, C. J., and Weller, S. K. (1996). Characterization of nuclear structures in cells infected with herpes simplex virus type 1 in the absence of viral DNA replication. *J. Virol.* 70, 1751–1758.

Marsden, H. S., Cross, A. M., Francis, G. J., Patel, A. H., MacEachran, K., Murphy, M., McVey, G., Haydon, D., Abbotts, A., and Stow, N. D. (1996). The herpes simplex virus type 1 UL8 protein influences the intracellular location of the UL52 but not the ICP8 or POL replication proteins in virus-infected cells. *J. Gen. Virol.* 77, 2241–2249.

Marsden, H. S., McLean, G. W., Barnard, E. C., Francis, G. J., MacEachran, K., Murphy, M., McVey, G., Cross, A., Abbotts, A. P., and Stow, N. D. (1997). The catalytic subunit of the DNA polymerase of herpes simplex virus type 1 interacts specifically with the C-terminus of the UL8 component of the viral helicase-primase complex. *J. Virol.,* in press.

Matsuura, Y., Possee, R. D., Overton, H. A., and Bishop, D. H. L. (1987). Baculovirus expression vectors: The requirements for high level expression of proteins, including glycoproteins. *J. Gen. Virol.* 68, 1233–1250.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E., and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* 69, 1531–1574.

McLean, G. W., Abbotts, A. P., Parry, M. E., Marsden, H. S., and Stow, N. D. (1994). The herpes simplex virus type 1 origin-binding protein interacts specifically with the viral UL8 protein. *J. Gen. Virol.* 75, 2699–2706.

Nicholas, J. (1996). Determination and analysis of the complete nucleotide sequence of human herpesvirus 7. *J. Virol.* 70, 5975–5989.

Nigg, E. A., Baeuerle, P. A., and Luhrmann, R. (1991). Nuclear import-export: In search of signals and mechanisms. *Cell* 66, 15–22.

Olivo, P. D., Nelson, N. J., and Challberg, M. D. (1989). Herpes simplex virus type 1 gene products required for DNA replication: Identification and overexpression. *J. Virol.* 63, 196–204.

Pari, G. S., and Anders, D. G. (1993). Eleven loci encoding *trans*-acting factors are required for transient complementation of human cytomegalovirus *ori*Lyt-dependent DNA replication. *J. Virol.* 67, 6979–6988.

Parry, M. E., Stow, N. D., and Marsden, H. S. (1993). Purification and properties of the herpes simplex virus type 1 UL8 protein. *J. Gen. Virol.* 74, 607–612.

Perry, H. C., Hazuda, D. J., and McClements, W. L. (1993). The DNA binding domain of herpes simplex virus type 1 origin binding protein is a transdominant inhibitor of virus replication. *Virology* 193, 73–79.

Rose, J. K., Buonocore, L., and Whitt, M. A. (1991). A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *BioTechniques* 10, 520–525.

Sarisky, R. T., and Hayward, G. S. (1996). Evidence that the UL84 gene product of human cytomegalovirus is essential for promoting *ori*Lyt-dependent DNA replication and formation of replication compartments in cotransfection assays. *J. Virol.* 70, 7398–7413.

Schmitt, J., and Keil, G. M. (1996). Identification and characterization of the bovine herpesvirus 1 UL7 gene and gene product which are nonessential for virus replication in cell culture. *J. Virol.* 70, 1091–1099.

Sherman, G., Gottlieb, J., and Challberg, M. D. (1992). The UL8 subunit of the herpes simplex virus helicase-primase complex is required for efficient primer utilization. *J. Virol.* 66, 4884–4892.

Silver, P. A. (1991). How proteins enter the nucleus. *Cell* 64, 489–497.

Smith, J. A., and Pari, G. S. (1995). Human cytomegalovirus UL102 gene. *J. Virol.* 69, 1734–1740.

Stow, N. D., and McMonagle, E. C. (1983). Characterization of the TRS/IRS origin of DNA replication of herpes simplex virus type 1. *Virology* 130, 427–438.

Stow, N. D., and Wilkie, N. M. (1976). An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. *J. Gen. Virol.* 33, 447–458.

Stow, N. D., Hammarsten, O., Arbuckle, M. I., and Elias, P. (1993). Inhibition of herpes simplex virus type 1 DNA replication by mutant forms of the origin-binding protein. *Virology* 196, 413–418.

Tanguy Le Gac, N., Villani, G., Hoffmann, J.-S., and Boehmer, P. E. (1996). The UL8 subunit of the herpes simplex virus type-1 DNA helicase-primase optimizes utilization of DNA templates covered by the homologous single-strand DNA-binding protein ICP8. *J. Biol. Chem.* 271, 21645–21651.

Telford, E. A. R., Watson, M. S., McBride, K., and Davison, A. J. (1992). The DNA sequence of equine herpesvirus-1. *Virology* 189, 304–316.

Tenney, D. J., Hurlburt, W. W., Micheletti, P. A., Bifano, M., and Hamatake, R. K. (1994). The UL8 component of the herpes simplex virus helicase-primase complex stimulates primer synthesis by a subassembly of the UL5 and UL52 components. *J. Biol. Chem.* 269, 5030–5035.

Tenney, D. J., Sheaffer, A. K., Hurlburt, W. W., Bifano, M., and Hamatake, R. K. (1995). Sequence-dependent primer synthesis by the herpes simplex virus helicase-primase complex. *J. Biol. Chem.* 270, 9129–9136.

Vlcek, C., Benes, V., LU, Z., Kutish, G. F., Paces, V., Rock, D., Letchworth, G. J., and Schwyzer, M. (1995). Nucleotide sequence analysis of the bovine herpesvirus 1 genome which exhibits a colinear gene arrangement with the UL21 to UL4 genes of herpes simplex virus. *Virology* 210, 100–108.