Varicella-Zoster Virus Gene *51* Complements a Herpes Simplex Virus Type 1 *UL9* Null Mutant

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Varicella-zoster virus (VZV) gene *51* encodes a protein which is homologous to UL9, the origin of DNA replication-binding protein of herpes simplex virus type 1. No genetic information is available on VZV gene *51*, but its product has been shown to bind to virtually the same recognition sequence as does UL9 (D. Chen and P. D. Olivo, J. Virol. 68:3841–3849, 1994; N. D. Stow, H. M. Weir, and E. C. Stow, Virology 177:570–577, 1990). We report here that gene *51* can complement a *UL9* null mutant (*hr*94) (A. K. Malik, R. Martinez, L. Muncy, E. P. Carmichael, and S. K. Weller, Virology 190:702–715, 1992), but at a level which is only 20% of that of *UL9*. Quantitation of viral DNA synthesis suggests that this phenotype is due to a defect in viral DNA synthesis. Regardless, the ability of VZV gene *51* to complement *UL9* suggests that alphaherpesviruses have a highly conserved mechanism of initiation of viral DNA synthesis.

Varicella-zoster virus (VZV) is one of seven human herpesviruses, and like herpes simplex virus type 1 (HSV-1), VZV belongs to the alphaherpesvirus subfamily (14). HSV-1 and VZV have similar genomic organizations, and most of the 72 genes of HSV-1 have homologs in the VZV genome, including the seven HSV-1 genes that have been shown to be required for viral DNA replication (6, 15, 16, 23). VZV gene *51* is the homolog of the HSV-1 *UL9* gene, which encodes a protein that binds to the viral DNA origins of replication (8, 18). UL9 probably has an important role in the initiation of viral DNA synthesis, but how it functions during initiation remains undefined. Gene *51* protein has been shown to have VZV origin-binding activity by using a protein A-gene *51* fusion protein (21). Recently, it was found that the VZV and HSV-1 origin-binding proteins (OBPs) have virtually identical recognition sequences (3). However, there are no genetic data to support the idea that the VZV OBP is required for viral DNA synthesis. Recently, we found that a recombinant baculovirus that expresses gene *51* protein can substitute for a UL9-expressing baculovirus in an HSV-1 origin-dependent replication assay in insect cells (22). In this report, we describe results which demonstrate that gene *51* is able to complement an HSV-1 *UL9*-minus mutant. This complementation, however, is only partial, which may indicate that in this context gene *51* protein is defective relative to UL9 in its interaction with either the HSV-1 origin, one of the HSV-1 replication proteins, or a cellular factor.

VZV genes *51* and *52* share a predicted polyadenylation signal 3′ of the gene *52* open reading frame; therefore, we cloned a 3.2-kb fragment from the VZV genome (*Bam*HI D fragment from strain EF) containing the entire gene *51* and gene *52* coding sequences into a vector so that gene *51* was immediately downstream of the human cytomegalovirus immediate-early promoter (6, 17). To eliminate expression of gene *52*, a 648-bp deletion in the gene *52* open reading frame was made to generate pCMV51/52Δ. This same strategy was

used by Heilbronn and zur Hausen to clone the HSV-1 *UL9* gene, which is 3′ cotranscriptional with *UL8*, and generate pCMUL9 (9). pCMUL9 was used throughout this study for *UL9* expression. Gene *51* protein expression was detectable by immunoblotting extracts from BHK cells transfected with pCMV51/52Δ (data not shown).

Malik et al. generated a *UL9* null mutant of HSV-1, *hr*94, in which the *UL9* reading frame was disrupted by insertion of an ICP6 promoter::*lacZ* cassette (10). They showed that following infection of Vero cells, *hr*94 did not express UL9 or synthesize virus DNA and was unable to form plaques. However, in Vero cells transformed with a fragment of the HSV-1 genome containing the *UL9* gene (S22 cells), *hr*94 grew to wild-type levels. We performed transient complementation assays in which we transfected pCMUL9, pCMV51/52Δ, or a control plasmid (pHCMVIEP) into BHK cells and then superinfected these cells with *hr*94. Twenty-four hours later, the total *hr*94 yield was determined. Table 1 shows the results of two independent experiments in which the expression of pCMV51/52Δ in BHK cells complemented *hr*94. The complementation index of pCMV51/52Δ, however, was at least fivefold lower than the complementation index of pCMUL9.

Stably transformed BHK cell lines which contain either gene *51* or *UL9* were isolated by cotransfecting BHK cells with a plasmid (pMamNeo; Clontech, Inc., Palo Alto, Calif.) which contains a geneticin resistance gene and with either pCMV51/52Δ or pCMUL9. Geneticin-resistant clones were tested for expression of gene *51* and *UL9* by immunoblotting with rabbit antisera directed against peptides derived from the predicted primary sequence of gene *51* protein and UL9 (Fig. 1). As can be seen in Fig. 1A, extracts from a BHK/UL9 cell line and two BHK/51 cell lines reacted with antiserum R250, raised against a UL9 carboxyl-terminal peptide. This was expected because of homology at the carboxyl termini of gene *51* protein and UL9 (3, 6, 16). The observed relative mobilities of UL9 (88 kDa) and gene *51* protein (85 kDa), as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, are consistent with the results obtained with baculovirus-expressed proteins (22). Both UL9 and gene *51* protein were also detected by antiserum R309, directed against the gene *51* protein carboxyl terminus, but in contrast to the results shown in Fig. 1A, gene *51* protein produced the more intense band (data not shown).

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TABLE 1. Transient complementation of *hr*94
(*UL9*-minus HSV-1 mutant) by VZV gene *51*

Plasmid[a] and expt	Titer (PFU/ml)[b]	Complementation index[c]
Vector		
1	1.0×10^3	1
2	1.0×10^3	1
pCMUL9		
1	7.6×10^5	760
2	5.6×10^5	560
pCMV51/52Δ		
1	1.3×10^5	130
2	1.1×10^5	110

[a] Plasmids used to transfect BHK cells are described in Materials and Methods.
[b] On BHK/UL9 cells.
[c] *hr*94 titer from cells transfected with *UL9* or gene *51* plasmid/titer from cells transfected with vector.

However, antiserum raised against an amino-terminal peptide of UL9 (R216) reacted only with UL9 (Fig. 1B). To demonstrate that these two gene *51* cell lines do not contain the *UL9* gene, a Southern blot was performed. DNAs from the gene *51* cell lines hybridized with a gene *51* probe, but not with a *UL9* probe. By using plasmid standards, the copy number of gene *51* was calculated to be 5 to 10 copies per cell in both BHK/51 cell lines (data not shown).

BHK/51, BHK/UL9, and BHK/neo cells were then infected with *hr*94 at a multiplicity of infection (MOI) of 3, and progeny virus was collected at various times after infection and titers were determined on a complementing cell line. Figure 2 shows an example of one such single-step growth curve of *hr*94 for each cell line. The production of progeny *hr*94 from BHK/51 cells was delayed relative to that from BHK/UL9 cells, and the final yield of *hr*94 from BHK/51 cells was about one-third of the yield from BHK/UL9 cells. *hr*94 also had a lower plaquing efficiency on BHK/51 cells than on BHK/UL9 cells (Table 2). In addition, although *hr*94 formed plaques on BHK/51 cells, these plaques were barely visible microscopically. Since the *hr*94 genome contains *lacZ* and expresses β-galactosidase,
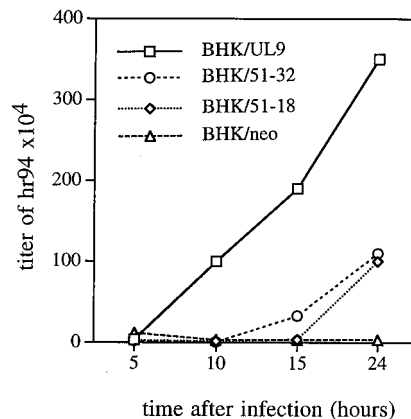
FIG. 2. Single-step growth curve of *hr*94 on BHK cells. BHK/UL9, BHK/51-32, BHK/51-18, and BHK/neo cells were infected with *hr*94 at an MOI of 3. At the indicated times after infection, cells were scraped into the medium and subjected to three freeze-thaw cycles. Virus titers (PFU/ml) were determined by plaque assays with BHK/UL9 and S22 cells.

plaques can be histochemically stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Figure 3 shows that *hr*94 infection of BHK/neo cells causes individual stained cells, not plaques. Plaques on BHK/UL9 cells are large, whereas plaques on BHK/51 cells are very small and difficult to visualize without X-Gal staining. Incubation for as long as 4 days did not significantly increase the sizes of plaques.

Although UL9 and, by analogy, gene *51* protein are known to play essential roles in viral DNA synthesis, it has not been ruled out that they might be involved in other stages of the viral life cycle. We wished to determine whether the decreased production of *hr*94 by BHK/51 cells is due to a defect in the ability of gene *51* protein to support viral DNA synthesis or a defect in another stage of viral growth. Viral DNA synthesis was tested in both BHK/51 and BHK/UL9 cell lines following infection with *hr*94. Figure 4 shows that the level of *hr*94 DNA synthesis in BHK/51 cells was only 30% of the level in BHK/UL9 cells 24 h after infection. This is commensurate with the relative levels of virus yield for these two cell lines. Viral DNA synthesis in *hr*94-infected BHK/51 cells was also delayed, relative to the kinetics of viral DNA synthesis in BHK/UL9 cells.

Since gene *51* does not fully complement a *UL9*-minus virus, it seemed possible that it interferes with wild-type UL9 function, thus exhibiting a dominant-negative phenotype. To address this possibility, BHK/51, BHK/UL9, and BHK/neo cells were infected with wild-type HSV-1 (KOS strain) at a low
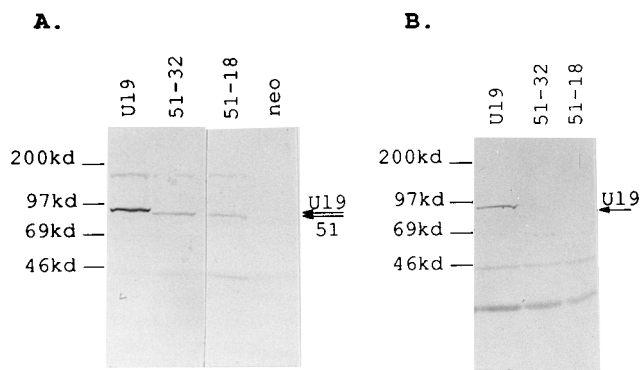
FIG. 1. Western blot (immunoblot) demonstration of gene *51* protein and UL9 expression in stably transformed BHK cells. Extracts from *hr*94-infected BHK/UL9, BHK/51-32, BHK/51-18, and BHK/neo cells were made 18 h after infection. The cell line from which the infected-cell extract was made is indicated above each lane. The sizes (in kilodaltons) and positions of molecular markers are indicated on the left of each blot. Arrows indicate the positions of UL9 and gene *51* protein. (A) The primary antibody was R250, raised against a decapeptide which is identical to the carboxyl terminus of UL9 and 90% identical to the carboxyl terminus of gene *51* protein (3, 19). (B) The primary antibody was R216, raised against a 20-amino-acid peptide which is identical to the amino terminus of UL9 and exhibits no homology with the amino terminus of gene *51* protein.

TABLE 2. Growth and plaquing efficiencies of *hr*94 and KOS on BHK/UL9 and BHK/51 cells

Cell line	Virus growth (PFU/ml)[a]		Plaquing efficiency (PFU/ml)[b]	
	*hr*94	KOS	*hr*94	KOS
BHK/neo	3.3×10^4	4.8×10^6	$<10^2$	5.5×10^7
BHK/UL9	3.5×10^6	4.5×10^6	3.8×10^6	5.3×10^7
BHK/51-18	1.0×10^6	3.9×10^6	1.5×10^6	4.0×10^7
BHK/51-32	1.1×10^6	4.3×10^6	1.7×10^6	5.1×10^7

[a] Virus yield 24 h after infection with *hr*94 at an MOI of 3 or 72 h after infection with KOS at an MOI of 0.1. Yield was determined on BHK/UL9 and S22 cells for *hr*94 and on Vero and BHK cells for KOS.
[b] Determined by standard plaque assay. *hr*94 plaques were histochemically stained to allow visualization of microplaques.
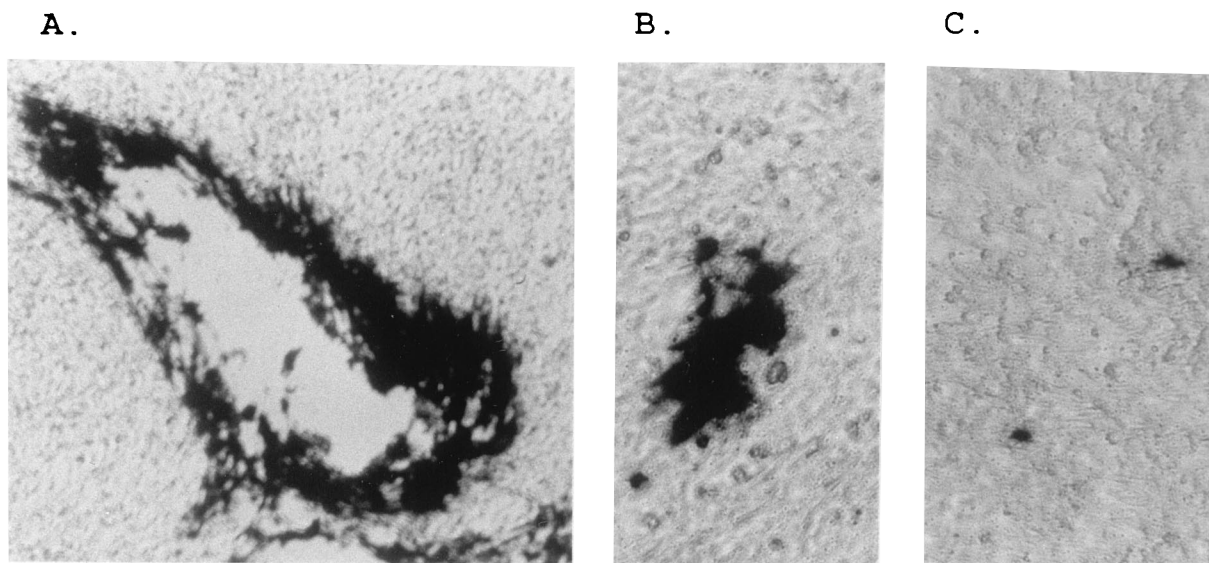
FIG. 3. Photomicrographs of plaques formed by mutant virus *hr*94 on BHK/UL9 (A), BHK/51-32 (B), and BHK/neo cells (C). *hr*94 was inoculated onto monolayers of each cell line, and after 1 h, medium containing 0.25% pooled human immunoglobulin was added. After 3 days, plaques were histochemically stained with X-Gal.

MOI (0.1) and progeny virus was collected and titers were determined on BHK cells. The results shown in Table 2 indicate that HSV-1 replicated to the same level and exhibited the same plaquing efficiency on all three cell lines. Therefore, VZV gene *51* protein does not have a transdominant inhibitory effect on UL9 function.

Gene *51* protein and UL9 were predicted to have closely related functions on the basis of overall homology (44% identity and 53% similarity), the presence of a number of common sequence motifs and conserved sequence blocks, the mapping of their origin-binding domains to the carboxyl terminus, and their binding to virtually identical recognition sequences (3, 12, 13, 21). However, given the complex nature

of viral DNA synthesis, gene *51* was not totally expected to substitute for *UL9*. Although the details of initiation of HSV-1 and VZV DNA synthesis have yet to be elucidated, they are likely to involve multiple protein-DNA and protein-protein interactions. Presumably, the structural bases for the most critical of these interactions have been conserved between HSV-1 and VZV. Recently, the region of the UL9 primary sequence which is important for interaction with the HSV-1 single-stranded DNA-binding protein ICP8 was mapped to a homologous region at the extreme carboxyl terminus of each protein (1, 3, 7). This observation, taken together with the data in this report, predicts that gene *51* protein can interact productively with ICP8. In fact, this functional conservation
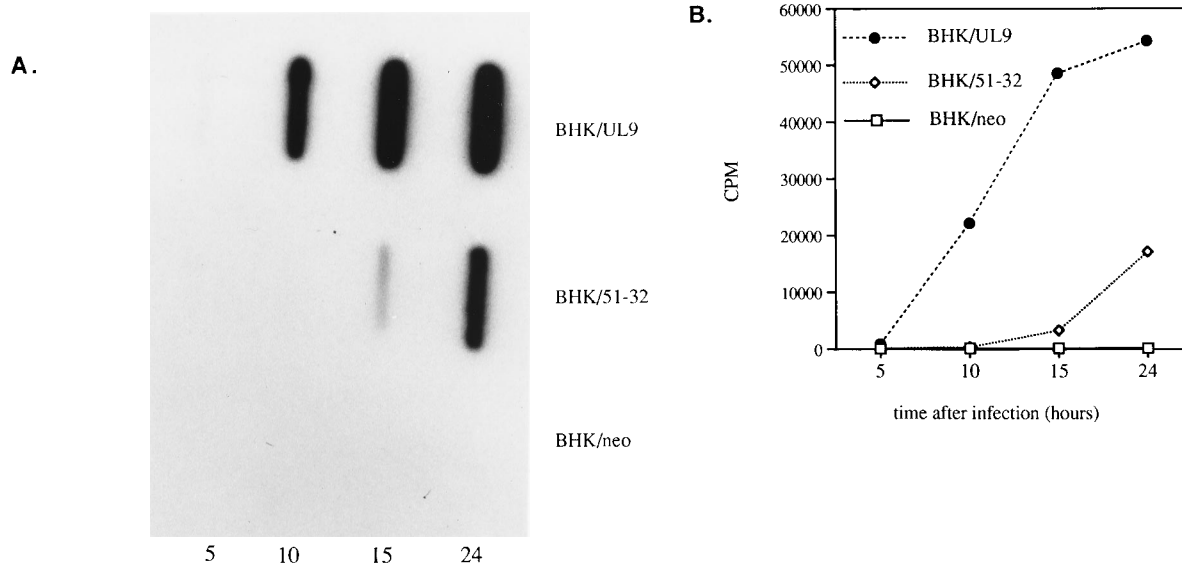

FIG. 4. Viral DNA synthesis in *hr*94-infected BHK/UL9, BHK/51-32, and BHK/neo cells. Each cell line was infected with *hr*94 at an MOI of 5. Total cellular DNA was extracted at the indicated times after infection, and viral DNA was detected by slot blot with 5 μg of DNA and a ^{32}P-labelled HSV fragment. (A) Autoradiogram; (B) quantitation of the radioactivity in each slot.

of the HSV-1 and VZV OBPs may be representative of a highly conserved mechanism of initiation of DNA replication among alphaherpesviruses, such as HSV-1, HSV-2, VZV, and equine herpesvirus 1, and certain other herpesviruses, such as Marek's disease virus (2). A recent report by Martin and Deb, which demonstrated that the product of equine herpesvirus 1 gene *53* is similar to UL9 and gene *51* protein in both primary structure and site-specific DNA binding, supports this idea (11).

The reason that gene *51* does not fully complement *UL9* is not clear. Malik et al. reported that a high copy number of *UL9* in stably transformed cell lines resulted in reduced replication of *hr*94 (10). This effect, however, was seen only with very high gene copy numbers (e.g., 50 to 200 copies per cell), and it was not specifically correlated with the level of UL9 protein expression. The BHK/51 cell lines used for our studies have only 5 to 10 copies of gene *51*, which is comparable to the copy number of our BHK/UL9 cell line; in addition, the level of gene *51* protein expression is comparable to the level of UL9 expression in BHK/UL9 cells and more than that in wild-type-infected cells. Moreover, several independently isolated clones exhibit very different levels of gene *51* protein expression by immunoblot but no differences in their complementation phenotypes (data not shown). A cellular factor that might interact with UL9 has been reported, and gene *51* protein may interact poorly with such a factor from nonprimate cells (4, 5). However, we performed transient complementation assays with monkey kidney cells (Vero) and a human melanoma cell line (Mewo) and obtained results that were similar to the results with BHK cells (data not shown). It is also possible that gene *51* protein interacts poorly with the HSV-1 origin of replication, relative to UL9. The UL9 and gene *51* protein binding sites in HSV-1 oriS and VZV oriS are virtually identical, so any differences in binding affinity have to be due to intrinsic differences in the DNA binding properties of these two proteins. Gene *51* protein may be less efficient at forming the necessary preinitiation complex on an HSV-1 origin than on a VZV origin because of either an intrinsic property of this protein or a structural difference between HSV-1 and VZV origins (20). Alternatively, gene *51* protein may interact poorly, relative to UL9, with other HSV-1 replication proteins, such as ICP8. These possibilities are not mutually exclusive, and the defect may involve problems with both protein-protein and protein-DNA interactions. Finally, gene *51* protein may be an intrinsically less active replication enzyme than UL9 is. A direct biochemical comparison of these two proteins is necessary to address this possibility. Nevertheless, in a manner analogous to that of comparisons between products of mutant and wild-type genes, a comparison between gene *51* protein and UL9 should help us decipher which properties of these OBPs are essential for viral DNA synthesis.

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