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The Varicella-Zoster Virus Origin-Binding Protein Can Substitute for the Herpes Simplex Virus Origin-Binding Protein in a Transient Origin-Dependent DNA Replication Assay in Insect Cells

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We isolated two recombinant baculoviruses each of which expresses a varicella-zoster virus (VZV) homolog of one of the seven herpes simplex virus type 1 (HSV-1) genes required for DNA replication. We performed transient origin-dependent DNA replication assays in insect cells in which we substituted a baculovirus which expresses a VZV protein for a baculovirus which expresses its HSV homolog. VZV gene *51* protein was found to be able to support origin-dependent DNA synthesis when it was substituted for UL9, the HSV-1 origin-binding protein (OBP). This occurred whether an HSV-1 or a VZV origin-containing plasmid was used in the assay. These results suggest that VZV gene *51* protein is able to interact with the HSV replication machinery, and in light of the extensive structural divergence of these proteins, it suggests that initiation of VZV and HSV-1 DNA synthesis may involve a limited number of interactions between the OBP and other replication factors. Substitution of infected-cell protein 8 (ICP8), the major single-stranded DNA-binding protein of HSV-1, with VZV gene *29* protein, however, did not result in amplification of plasmids containing either an HSV-1 or a VZV origin. In the absence of ICP8, addition of both VZV gene *51* protein and gene *29* protein was also negative for origin-dependent replication whether or not UL9 was present. Although demonstration that our baculovirus-expressed VZV gene *29* protein is functional for DNA replication will await development of a VZV replication system, our results suggest that VZV gene *29* protein is unable to interact functionally with one or more of the HSV replication proteins. This approach should contribute to efforts to define the interactions among the alphaherpesvirus DNA replication proteins.

Herpesviruses, which have large linear DNA genomes, provide excellent model systems for eukaryotic DNA replication studies. Herpes simplex virus type 1 (HSV-1), the best characterized of the herpesviruses, has a 152-kbp genome which contains well-defined origins of replication and encodes seven genes which have been shown to be required for viral DNA synthesis (*1–4*). *UL30* and *UL42* encode the catalytic and accessory subunits of the DNA polymerase complex. *UL29* encodes the major single-stranded DNA-binding (SSB) protein, infected-cell protein 8 (ICP8). *UL5, UL8,* and *UL52* encode three polypeptides which make up the heterotrimeric helicase–primase complex. Finally, *UL9* encodes the origin of DNA replication-binding protein (OBP). Much of the biochemical characterization of these replication proteins has been done using protein made in insect cells infected with recombinant baculoviruses. Unfortunately, a cell-free HSV origin-dependent replication system has not yet been developed with which to test the ability of these insect cell-produced proteins to support origin-dependent DNA synthesis. This had raised concerns that one or more of these proteins may not be fully functional for DNA synthesis. This concern was recently eliminated when Stow demonstrated HSV-1 origin-dependent DNA replication in insect cells infected with a mixture of seven baculoviruses each of which expresses one of the seven essential replication genes (*5*). Stow suggested that this method would be valuable for testing the function of baculovirus-expressed mutant proteins. We decided to take advantage of this approach to test the ability of varicella-zoster virus (VZV) DNA replication proteins to substitute for their HSV-1 counterparts, both as a way of testing the function of the VZV proteins and as a means of comparing the two systems.

VZV is a human herpesvirus which, like HSV-1, belongs to the subfamily Alphaherpesvirinae (*6*). Despite significant differences in their biologic behavior, VZV and HSV-1 share many structural and molecular biological features. It has been reported, for example, that certain VZV immediate-early genes can complement their HSV-1 homologs (*7, 8*). The 125-kbp linear, double-stranded DNA VZV genome has a homolog to each of the seven essential HSV-1 replication proteins (*3, 9*). Unlike HSV-1, however, there have been very few genetic studies on VZV DNA replication genes, and there are no VZV mutants with lesions in the presumed essential replication genes. The VZV genome has no equivalent to the HSV-1 origin termed ori_L, but, like HSV, the VZV genome has two copies of ori_S in the inverted repeats flanking the short unique region of the genome (*9, 10*). Although VZV origin-dependent plasmid replication has been described in

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VZV-infected cells, there has been no report of origin-dependent replication using isolated VZV replication genes (*10*). Stow and Davison showed that a plasmid containing VZV ori_S was amplified in both VZV- and HSV-1 infected cells, but a plasmid containing an HSV-1 ori_S was not amplified in VZV-infected cells (*10*).

VZV gene *51* encodes a protein which is homologous to UL9, the HSV-1 OBP, which is thought to play an important role in initiation of viral DNA synthesis (*11, 12*). Using a protein A fusion protein it was shown that, as with UL9, the origin-binding domain of the VZV OBP maps to the carboxyl terminus (*13*). It is likely that the VZV OBP plays the same role as UL9 during viral DNA replication, but there are no genetic data to support this idea, and origin-binding activity has not been identified in extracts of VZV-infected cells. Recently, we reported expression of the full-length VZV OBP by *in vitro* translation and showed that the VZV and HSV-1 OBP have virtually identical recognition sequences (*14*).

VZV gene *29* is the homolog of *UL29* which encodes ICP8, the major SSB protein of HSV-1 (*15–17*). ICP8 is probably involved in several stages of viral DNA synthesis. ICP8 can melt partially duplex DNA in the absence of ATP and magnesium in a manner similar to other SSBs (*18*). In doing so it may facilitate the activity of helicases such as the origin-binding protein, UL9, and the helicase–primase complex made up of UL5, UL8, and UL52. It has been shown to physically interact with and enhance the helicase activity of UL9 and thus may facilitate origin unwinding (*18, 19*). It also promotes movement of the HSV-1 DNA polymerase through hairpins on the template strand during elongation (*20, 21*). We have found that VZV gene *29* protein has similar single-stranded DNA-binding properties as ICP8, but there are no genetic or biochemical data about its interactions with other proteins or about any other possible functions (M. Horgan and P. D. Olivo, submitted for publication).

In this report we describe results of transient origin-dependent DNA replication assays in *Spodoptera frugiperda* (Sf9) insect cells infected with seven recombinant baculoviruses in which we substituted a baculovirus which expresses a VZV protein for a baculovirus which expresses its HSV homolog. Substitution of HSV-1 ICP8 with VZV gene *29* protein did not result in amplification of plasmids containing either the HSV-1 or VZV origin, but VZV gene *51* protein was found to be able to support origin-dependent DNA synthesis when it was substituted for UL9.

VZV genes *29* and *52* were subcloned from the *Bam*HI D and *Eco*RI B VZV genomic fragments, respectively, obtained from L. Gelb (Washington University) (*22*) and were cloned into vector pVL1392 (*23, 22*). Recombinant baculoviruses which express VZV gene *29* protein (AcNPV/29) and gene *51* protein (AcNPV/51) were constructed by using standard methods (*23*). Figure 1A is a fluorogram of an SDS–PAGE analysis of [^{35}S]methionine-labeled proteins made in recombinant baculovirus-infected insect cells illustrating the expression of gene *51* protein (lane 2) and gene *29* protein (lane 3) in insect cells infected with AcNPV/51 and AcNPV/29, respectively. Also shown is the expression of the HSV-1 homolog of gene *51* protein (UL9, lane 1) and the homolog of gene *29* protein (ICP8, lane 4) in insect cells infected with AcUL9 and AcUL29(ICP8) (*5*). Gene *51* protein produced by *in vitro* transcription/translation was previously shown to have a relative migration in SDS–PAGE consistent with a polypeptide of 85 kDa (*14*). This same size protein can be seen in AcNPV/51-infected cells (lane 2) and is absent in the AcUL9-infected cells which display the slower migrating UL9 protein (lane 1). Figure 1B shows that the 85-kDa gene *51* protein reacted with a rabbit anti-UL9 antisera generated with a carboxyl-terminal peptide (*2*) (Fig. 1B, lane 2). Both proteins also reacted with a rabbit anti-gene *51* protein antiserum generated with a carboxyl-terminal peptide (data not shown) (*14*). The cross-reactivity of both antisera is not surprising given the degree of predicted homology at the carboxyl termini of these proteins (*14*). Insect cell-expressed gene *51* protein did not react, however, with an antiserum generated with dodecapeptide derived from the predicted primary sequence of the amino terminus of UL9 (data not shown). Gene *29* protein migrates with a relative mobility in SDS–PAGE consistent with a polypeptide of 135 kDa, which is larger than the 130-kDa ICP8 (Fig. 1A, lane 4). Insect cell-produced gene 29 protein reacted with a rabbit antiserum generated against a peptide the sequence of which was derived from the predicted carboxyl terminus of gene *29* protein (*24*).

We first wished to demonstrate HSV-1 origin-dependent DNA replication in insect cells using the assay developed by Stow (*5*). Sf9 cells were transfected with plasmid pUC19 or with pUC19 into which was inserted a 230-bp *Sma*I fragment containing the HSV-1 ori_S (pMC110), and the cells were coinfected with the complete set of HSV-1 replication protein-producing baculoviruses. Six hours after transfection the cells were infected with various mixtures of recombinant baculoviruses. Sixty-five hours after infection total cellular DNA was harvested and digested with *Bam*HI to linearize the plasmid. The sample was divided in two, and one half was digested with *Dpn*I to detect the presence of replicated (*Dpn*I-resistant) plasmid DNA. Following electrophoresis, the gel was transferred to a nylon membrane and hybridized using the ori_S plasmid as the probe. As can be seen in Fig. 2, pUC19 was not amplified under these conditions (lane 1). pMC110 was amplified following infection with the complete set of viruses (lane 3), but not if AcUL9 was left out of the baculovirus mixture (lane 5). We confirmed, therefore, the results of Stow that baculovirus-infected Sf9 cells do not exhibit nonspecific plasmid amplification

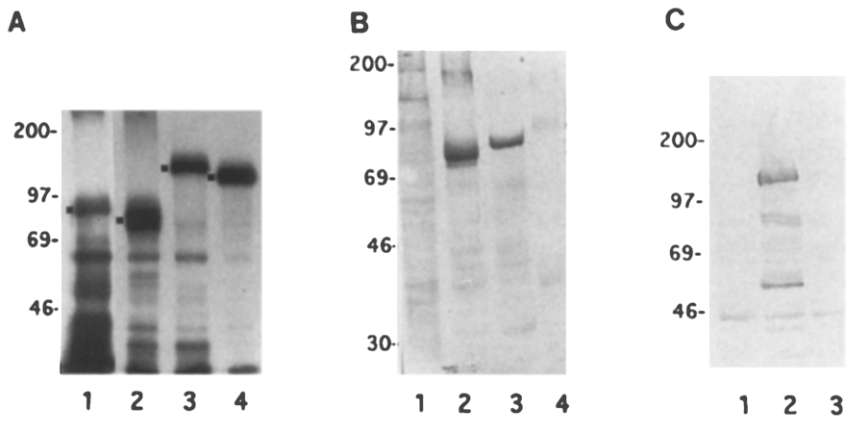

FIG. 1. Expression of gene *51* protein and gene *29* protein in Sf9 cells. (A) Fluorogram of [^{35}S]methionine-labeled proteins from recombinant baculovirus-infected cells analyzed by SDS–PAGE. The cells were infected with the indicated baculoviruses at an m.o.i. of at least 5. Sixty hours after infection the medium was removed and replaced with 1 ml methionine-free Grace's medium with 50 mCi [^{35}S]methionine (1000 Ci/mmol; Amersham, Arlington Heights, IL) for an additional 4 hr. The cells were then scraped into the medium, pelleted, washed two times in Grace's medium, and resuspended in SDS–PAGE sample buffer and an aliquot was submitted to SDS–PAGE. Following electrophoresis the gel was soaked in Amplify (Amersham), dried, and exposed to X-ray film. Lane 1, AcUL9-infected cells; lane 2, AcNPV/51-infected cells; lane 3, AcNPV/29-infected cells; lane 4, AcUL29(ICP8)-infected cells. Closed squares to the left of each lane indicate the recombinant proteins. (B) Western blot of protein produced in AcNPV/51- and AcUL9-infected insect cells using rabbit anti-UL9 carboxy-terminal peptide antisera. Lane 1, mock-infected cell extract; lane 2, AcNPV/51-infected cell extract; lane 3, AcUL9-infected cell extract; lane 4, wild-type AcNPV-infected cell extract. (C) Western blot of protein produced in AcNPV/29- and AcUL29(ICP8)-infected insect cells using rabbit anti-gene *29* protein carboxyl-terminal peptide antisera. Lane 1, mock-infected cell extract; lane 2, AcNPV/29-infected cell extract; lane 3, AcUL29(ICP8)-infected cell extract. Size markers are indicated on the left (in kDa).

and that HSV-1 origin-dependent plasmid replication requires coinfection with all seven of the baculoviruses which express the required HSV-1 replication proteins.

Having reproduced HSV-1 origin-dependent replication in insect cells, we wished to determine whether baculoviruses expressing the VZV replication proteins could substitute for those which express their HSV-1 homologs. As shown in Fig. 2, *Dpn*I-resistant pMC110 was produced in Sf9 cells coinfected with six baculoviruses expressing HSV-1 proteins, but with AcNPV/51 added in place of AcUL9 (lane 7). AcNPV/29, however, was not able to substitute for AcUL29(ICP8) (lane 11).

In order to demonstrate that VZV gene *29* protein was expressed in the coinfected cells, we [^{35}S]methionine-labeled coinfected cells and analyzed cell extracts made from multply infected insect cells by SDS–PAGE and fluorography. We were able to demonstrate a band corresponding to each of the seven HSV-1 replication proteins in the insect cells infected with all seven HSV-1 recombinant baculoviruses, and in the instances in which AcNPV/51 or AcNPv/29 was substituted for AcUL9 or AcUL29(ICP8), a band comigrating with gene *51* protein or gene *29* protein was seen (data not shown). The expression of gene *51* protein and gene *29* protein in the multiply infected cells was also directly demonstrated by Western blotting (data not shown). The absence of HSV-1 origin-dependent replication in experiments in which AcUL29(ICP8) is replaced by AcNPV/29 protein is not, therefore, due to lack of expression of gene *29* protein or of any of the HSV-1 replication proteins.

ICP8 is a multifunctional protein which probably has several functions during viral DNA replication. It has been shown to specifically interact with UL9 and the DNA polymerase and probably interacts with the UL5/8/52 helicase–primase. One possible reason that gene *29* protein is unable to functionally substitute for ICP8 may be that it can not productively interact with one or more of the HSV-1 replication proteins. We, therefore, asked whether the presence of gene *51* protein, with or without the presence of UL9, could overcome the inability of gene *29* protein to replace ICP8. Figure 3 demonstrates that pMC110 was converted to *Dpn*I resistance in insect cells only when ICP8 was included in the virus mixture. Lane 1 demonstrates that the seven HSV-1 baculovirus system is functional, and lane 11 shows that AcNPV/51 virus stock used in this set of experiments is able to replace AcUL9. However, we detected no pMC110 amplification when ICP8 was left out of the mixture (lane 3), when gene *29* protein was substituted for ICP8 (lane 5), when both UL9 and ICP8 were replaced with gene *51* protein and gene *29* protein, respectively (lane 7), and when ICP8 was replaced with gene *29* protein and both UL9 and gene *51* protein were added to the mixture (lane 9).

Another possible reason that gene *29* protein is unable to functionally replace ICP8 could be because we have been using an HSV-1 origin rather than a VZV origin. It seemed unlikely that a nonspecific single-stranded DNA-binding protein such as gene *29* protein would exhibit origin specificity, particularly since the origin-binding proteins were interchangeable for HSV-1 origin-depen-

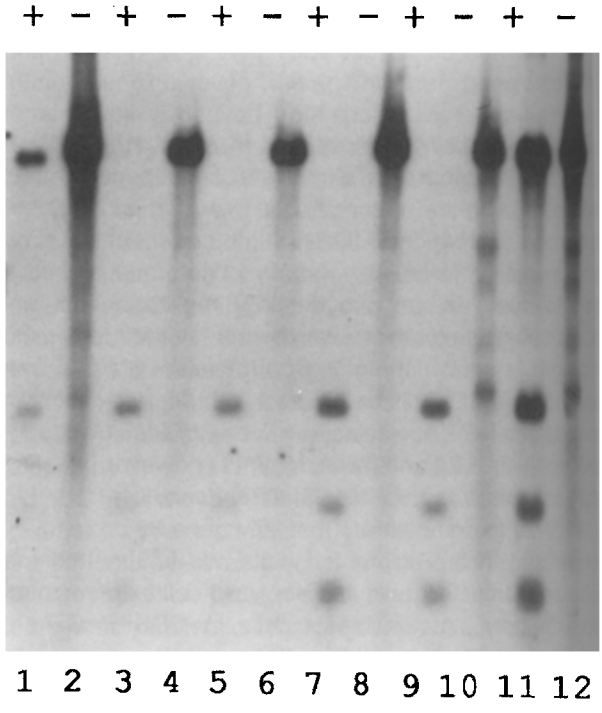

FIG. 2. Plasmid amplification in recombinant baculovirus-infected insect cells demonstrating the HSV-1 origin-dependence of replication and the ability of VZV gene *51* protein to substitute for UL9. Monolayers of Sf9 cells in six-well tissue culture plates (approximately 1×10^6 cells/well) were transfected with 1 μg of either pUC19 or pMC110 using 5 μg Lipofectin (Gibco BRL, Inc., Bethesda, MD) in Grace's medium. Six hours after transfection the cells were coinfected with the indicated recombinant baculoviruses in a volume of 0.5 to 1.4 ml complete medium using a m.o.i. of at least 5 for each virus. After 2 hr at 27°, the inoculum was replaced with 2 ml complete Grace's media and the cells were incubated at 27° for 63–65 hr. Total cellular DNA was extracted by the method of Challberg (*4*). One-fourth of the total DNA extracted from each well was cleaved with *Bam*HI alone or with *Bam*HI and *Dpn*I. The fragments were separated by electrophoresis in a 1% agarose gel and transferred to Magnagraph membrane (MSI, Westboro, MA). The DNA was hybridized and detected using the Genius System (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's instructions. The probe was pMC110 which was labeled using a random primer protocol with digoxigenin-tagged nucleotides. Hybridized probe was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody and visualized with Lumi-Phos 530, a chemiluminescent substrate, followed by exposure to X-ray film. The "+" and "−" above each lane indicates that the sample was digested (+) with the restriction enzyme *Dpn*I or not digested (−). Lanes 1 and 2, insect cells were transfected with pUC19; lanes 3–12, insect cells were transfected with pMC110 (HSV-1 ori_S); lanes 1–4, infection with the complete mixture of baculoviruses expressing the seven essential HSV-1 replication proteins; lanes 5 and 6, omission of AcUL9; lanes 7 and 8, omission of AcUL9 and addition of AcNPV/51; lanes 9 and 10, omission of AcUL29 (ICP8); lanes 11 and 12, omission of AcUL29(ICP8) and addition of AcNPV/29.

dent replication. However, it was a formal possibility which was easily testable. We, therefore, evaluated the replication of a plasmid (pVO2) containing VZV ori_S in insect cells (*10*). Figure 4 demonstrates that pVO2 was replicated in insect cells infected with a mixture of baculoviruses only when ICP8 was included (see lanes 1 and 4). Therefore, using a plasmid containing a VZV origin did not overcome the inability of gene *29* protein to replace ICP8 (lane 11).

VZV gene *51* protein shares structural features with UL9. Their predicted amino acid sequences have 44% identity, they have several sequence motifs in common, and the site-specific origin-binding domain of both proteins map to the carboxyl terminus (*13, 14, 25, 26*). They also have been shown to have virtually identical origin-recognition sequences (*14*). It is still somewhat surprising, given the degree of sequence divergence of the two proteins, that gene *51* protein can substitute for UL9. Gene *51* protein extracted from baculovirus-infected insect cells is insoluble, and thus, we have not been able to use it as a source of material to perform a biochemical characterization of gene *51* protein (D. Chen and P. D. Olivo, unpublished observation). The origin–replication results presented here are, therefore, our only evidence

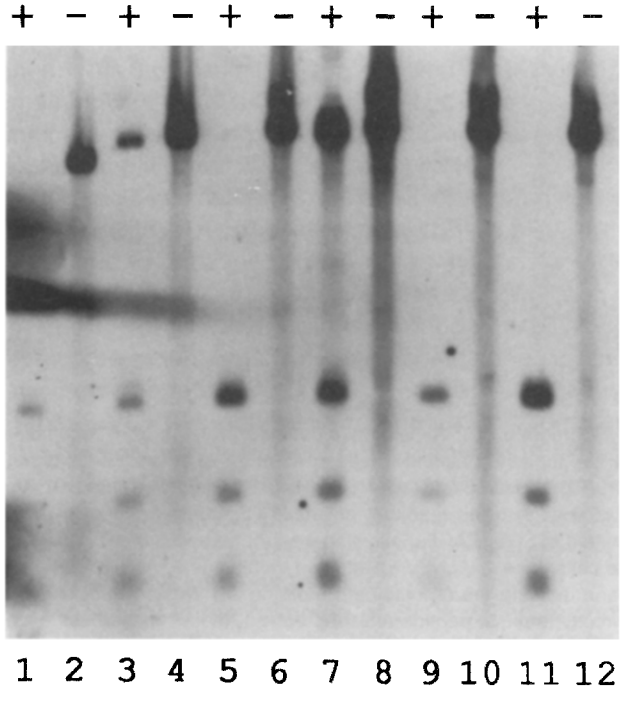

FIG. 3. Plasmid amplification in recombinant baculovirus-infected insect cells demonstrating that the inability of VZV gene *29* protein to replace ICP8 cannot be overcome by the presence of VZV gene *51* protein. The experimental methods were performed as described in the legend to Fig. 2 and the plasmid transfected was pMC110. The "+" and "−" above each lane indicates that the sample was digested (+) with the restriction enzyme *Dpn*I or not digested (−). Lanes 1 and 2, complete mixture of baculoviruses expressing the seven essential HSV-1 replication proteins; lanes 3 and 4, omission of AcUL29(ICP8); lanes 5 and 6, omission of AcUL29(ICP8) and addition of AcNPV/29; lanes 7 and 8, omission of both AcUL29(ICP8) and AcUL9 and addition of both AcNPV/29 and AcNPV/51; lanes 9 and 10, omission of AcUL29(ICP8) and addition of both AcNPV/29 and AcNPV/51; lanes 11 and 12, omission of AcUL9 and addition of AcNPV/51 and AcNPV/29.

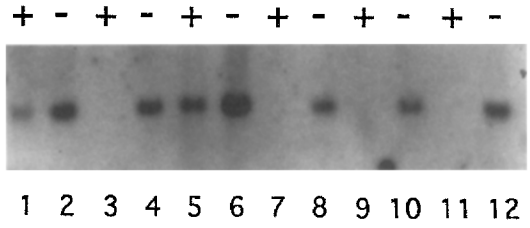

FIG. 4. Plasmid amplification in recombinant baculovirus-infected insect cells demonstrating replication of a VZV origin-containing plasmid when gene *51* was substituted for UL9, but lack of replication when VZV gene *29* protein was substituted for ICP8. The experimental methods were performed as described in the legend to Fig. 2 except the cells were transfected with pVO2 (VZV ori_S). The "+" and "−" above each lane indicates that the sample was digested (+) with the restriction enzyme *Dpn*I or not digested (−). Lanes 1 and 2, complete mixture of HSV recombinant baculoviruses; lanes 3 and 4, omission of AcUL9; lanes 5 and 6, omission of AcUL9 and addition of AcNPV/51; lanes 7 and 8, omission of AcUL29(ICP8); lanes 9 and 10, omission of AcUL29 (ICP8) and addition of AcNPV/29; lanes 11 and 12, omission of AcUL29 (ICP8) and addition of both AcNPV/29 and AcNPV/51.

that gene *51* protein expressed in insect cells is functional.

Stow and Davison showed that an HSV-1 origin-containing plasmid was not replicated in VZV-infected cells (*10*). This may have been because of the relative inefficiency of the VZV infectious process or for other trivial reasons. However, it also may have reflected an intrinsic inability of the VZV replication machinery to utilize an HSV-1 origin. The OBP-binding sites of the HSV-1 and VZV origins are very similar, but there are sufficient structural differences between the origins to support this idea (*10*). The results presented in this report argue against a functional difference in the HSV and VZV origins since both OBPs can use either origin as a substrate for initiation of DNA synthesis.

The ability of gene *51* protein to substitute for UL9 demonstrates that gene *51* protein is capable of functionally participating in all of the UL9–protein and UL9–DNA interactions required for origin-dependent DNA synthesis. It has been reported that UL9 and ICP8 physically and functionally interact and that UL9 may interact with a cellular factor during origin-binding (*18, 27*). It is not known whether UL9 physically interacts with any of the other HSV replication proteins. In addition, since there are no published data which suggest that UL9 is involved in the elongation phase of DNA replication, there is no compelling reason to invoke specific interactions of UL9 with elongation factors such as the HSV DNA polymerase, etc. Our data lend support to the idea that UL9 is likely to be involved in a limited number of protein–protein interactions during DNA synthesis.

We did not attempt to quantitate the amount of plasmid replication that results in the presence of gene *51* protein relative to the amount that occurs with UL9. There are too many factors which could influence the level of replication in this assay to enable us to determine whether gene *51* protein functions as efficiently as UL9. In multiple assays, however, we did not get the impression that experiments with gene *51* protein resulted in markedly reduced levels of *Dpn*I-resistant material after taking into account transfection efficiency. Recently, we have found that gene *51* can complement a UL9-minus HSV-1 mutant virus (D. Chen, E. Stabell, and P. D. Olivo, manuscript in preparation). The level of viral DNA synthesis and virus yield, however, was approximately 10-fold lower with gene *51* than with *UL9*.

The inability of VZV gene *29* protein to functionally replace ICP8 in the origin-dependent DNA synthesis assay in insect cells is not surprising. Interestingly, however, gene *29* protein extracted from insect cells is fully soluble and characterization of its single-stranded DNA-binding properties reveals that it binds ssDNA in a manner quite similar to that of ICP8 (M. Horgan and P. D. Olivo, manuscript submitted). It cannot be ruled out that the inability of gene 29 protein to substitute for ICP8 is due to expression of a nonfunctional protein because of a mutation that arose during generation of the recombinant baculovirus or for some other trivial reason. Although the ssDNA-binding activity of gene *29* protein produced in insect cells supports the idea that it is a functional protein, it is possible that a mutation arose which affects a function unrelated to its ssDNA-binding activity. Definitively demonstrating that gene *29* protein is fully functional for DNA synthesis will depend on construction of baculoviruses which express the remaining five essential VZV replication proteins and development of VZV origin-dependent replication system in insect cells.

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