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Analysis of Conserved Domains of UL41 of Herpes Simplex Virus Type 1 in Virion Host Shutoff and Pathogenesis

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The herpes simplex virus type 1 virion host shutoff protein has four domains whose sequences are conserved only among neurotropic herpesviruses. Mutant viruses with 29- and 31-amino-acid deletions in domains III and IV but outside of the domain required for interaction with VP16 were generated. The mutants failed to induce cellular RNA degradation and showed impaired virulence in mice. Domains III and IV are therefore required for both shutoff and virulence.

Rapid destabilization of host RNAs and translational arrest occur in herpes simplex virus (HSV)-infected cells through the action of a 58-kDa phosphoprotein coded for by the viral UL41 gene and known as the virion host shutoff (vhs) protein (2–6, 9, 10, 12, 13, 15, 23, 24). *vhs* is packaged into the tegument of the virus, and it therefore acts immediately upon infection (18). Mutational analyses are consistent with sequencing studies which show that the UL41 gene contains at least four domains whose sequences are conserved among five neurotropic herpesviruses (1, 7, 14). In addition, a domain in *vhs* which is necessary and sufficient for interaction with virion-associated transcriptional coactivator VP16 has been identified (17, 19). The precise mechanism of action of *vhs* and the role of *vhs*-VP16 interactions in infection and pathogenesis, however, remain to be determined.

Viruses containing lesions in the UL41 gene do not cause rapid shutoff or translational arrest upon infection (4, 15, 16, 18). Such mutants are viable and exhibit only modest reductions in growth in tissue culture (15, 20). However, two *vhs* mutants, UL41NHB and *vhs*-ΔSma, have been tested in a mouse model of latency and pathogenesis and have shown a profound attenuation of virulence (22). UL41NHB lacks the 246 amino acids constituting the carboxy-terminal half of the protein (part of domain III and all of domain IV), and *vhs*-ΔSma has a large in-frame deletion of 196 amino acids, including all of domain III (16). Both of these mutants lack the VP16 interaction domain (19, 24). These studies using large mutations are consistent with the idea that the conserved domains of UL41 are necessary for neuropathogenesis and shutoff but more targeted mutagenesis is required to confirm this idea. In the present study, additional viruses containing specific deletions in domains III and IV of UL41 were generated in order to further define functional domains for the vhs protein in terms of rapid destabilization of host RNA and virulence in mice. Importantly, the mutations made in this study were made in conserved domains III and IV but outside of the domain necessary for interaction with VP16 (Fig. 1A).

Virus ΔBNB was constructed as follows. pUL41 (22) was cut with *Bss*HII and *Eco*NI to yield a 1,519-bp fragment containing the UL41 open reading frame (ORF) which was cloned into the *Xmn*I site of vector pMAL-p2 (New England Biolabs) to generate pMAL41P. pMAL41P was digested partially with *Bsi*WI and then completely with *Nru*I, treated with mung bean nuclease, and religated to generate plasmid pΔBN containing a 93-bp in-frame deletion. This plasmid was sequenced to ensure the continuity of the UL41 reading frame. A 1,633-bp *Eco*RI-*Nco*I fragment of pΔBN was cotransfected into Vero cells with infectious BGS41 DNA (22), and the progeny were screened first by blue-white selection and subsequently by Southern blot analysis for an altered *Nru*I digestion pattern. ^{32}P-labeled pUL41 was used as a probe in all Southern blots. A virus yielding 4.1- and 2.6-kb bands rather than a 6.6-kb wild-type band (Fig. 1B) was plaque purified three times, and a high-titered stock was prepared and designated ΔBNB. ΔBNB therefore had a deletion from amino acids 207 to 238. The viral mutant ΔC4K was constructed by the insertion of a nonsense linker containing stop codons in all three reading frames and a unique *Hpa*I site into the *Eco*RV site of pUL41, yielding plasmid p4EH. A 3,632-bp *Hin*dIII-*Nco*I fragment of p4EH was cotransfected into Vero cells with infectious KOS DNA, and the progeny were screened by Southern blot analysis for an altered *Hpa*I digestion pattern. A virus yielding 10.5- and 2.5-kb bands rather than a 13-kb wild-type band (Fig. 1B) was plaque purified three times, and a high-titered stock was prepared and designated ΔC4K. ΔC4K therefore had a deletion from amino acids 460 to 489. Marker rescue for both *vhs* mutant viruses was accomplished by cotransfection of infectious mutant viral DNA with pUL41 and screening by Southern blotting (Fig. 1B) for restoration of wild-type restriction patterns, with viruses ΔC4K-R and ΔBNB-R being produced. Western blot (immunoblot) analysis of ΔC4K and ΔBNB yielded protein products with sizes of approximately 51 kDa, which is in agreement with the predicted sizes, while wild-type KOS, ΔC4K-R, and ΔBNB-R gave the expected 58-kDa protein products (16 and data not shown). The mutations introduced into the UL41 ORF therefore resulted in an appropriate truncation of the UL41 protein.

To examine the RNA-destabilizing activity of the *vhs* mutant viruses, Northern (RNA) blot analysis of RNA from infected Vero cells was performed by probing for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA, with 28S being used as an internal loading control, as described previously (22). Vero cells were mock infected or infected at a multiplicity of infection of 20 with KOS, ΔC4K, ΔBNB, ΔC4K-R, or ΔBNB-R, and cytoplasmic RNAs were harvested 4 and 10 h postinfection. The level of GAPDH RNA for mock-infected

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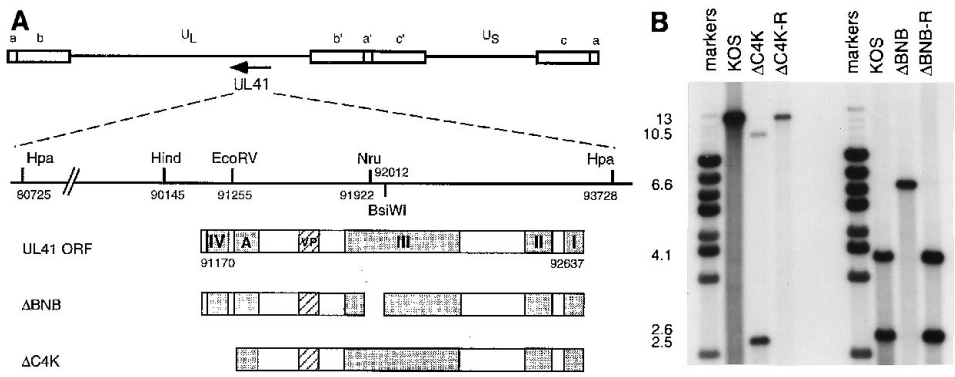

FIG. 1. (A) Map of the *vhs* (UL41) ORF, below which are shown the plasmids and viral mutants used in this study. The map shows a prototypical arrangement of the HSV-1 genome, with unique long (U_L) and unique short (U_S) segments flanked by internal (a′, b′, and c′) and terminal (a, b, and c) repeats. UL41 is transcribed in a leftward direction, as indicated by the arrow. The expanded view of the UL41 open reading frame (ORF) shows selected restriction enzyme sites with nucleotide numbers as described elsewhere (11). A map of the wild-type UL41 ORF shows conserved domains I to IV, the VP16 interaction domain (VP), and box A (1, 7, 16). The limits of the VP16 interaction domain (16) are amino acids 310 to 330. ΔBNB has a deletion of amino acids 207 to 238. ΔC4K has a deletion of amino acids 460 to 489. The limits of pUL41 (used as a probe in Southern blots) are from 90145 to 93728. (B) Southern blot analysis of KOS, ΔC4K, ΔC4K-R, ΔBNB, and ΔBNB-R, with ^{32}P-labeled pUL41 being used as a probe. The expected sizes of the *Hpa*I fragments are 10.5 and 2.5 kb for ΔC4K and 13 kb for ΔC4K-R and KOS. The expected sizes of the *Nru*I fragments are 4.1 and 2.6 kb for ΔBNB and 6.6 kb for ΔBNB-R and KOS. Sizes are indicated on the left in kilobases.

cells was set to 100% and compared with the 28S-normalized values. In three independent experiments with comparable results, infection with KOS or marker-rescued viruses led to a significant decrease in the amount of GAPDH RNA (Fig. 2 and Table 1). In contrast, ΔC4K and ΔBNB caused no significant degradation of GAPDH RNA, comparable to results seen for UL41NHB and *vhs*-ΔSma (22).

Acute viral growth and establishment and reactivation of latency were studied in a mouse eye model of latency as described previously (22). Scarified corneas of CD-1 female mice (Charles River Breeding Laboratories, Inc., Kingston, N.Y.) were infected with 2×10^6 PFU of virus in a 5-μl volume. Acute replication in corneas was analyzed by daily eye swabbing out to 5 days postinfection. The titers of ΔC4K and ΔBNB in eye swabs were reduced by 10- to 100-fold compared with those of KOS and their marker-rescued viruses by 1 day postinfection, and the level of mutant viruses remained decreased by at least 10-fold out to 5 days postinfection (Table 1 and data not shown). Acute replication in trigeminal ganglia for ΔC4K and ΔBNB 3 days postinfection was reduced by at least 10,000-fold relative to that for wild-type and marker-rescued viruses (Table 1). External clinical symptoms during acute infection (e.g., fur loss and eyelid swelling) were notable for mice infected with KOS and marker-rescued viruses but were inapparent for ΔBNB- and ΔC4K-infected mice. Establishment of latency was measured by quantitative DNA PCR with a Molecular Dynamics PhosphorImager as described previously (8, 22). Levels of viral DNA in ganglia latently infected with the *vhs* mutants were reduced 20- to 50-fold, with means ($n = 4$) of 0.2 and 0.5 copies per ganglion cell equivalent for ΔC4K and ΔBNB, respectively, compared with a mean ($n = 4$) of 9.8 copies for KOS. In contrast, levels of viral DNA in ganglia latently infected with ΔC4K-R and ΔBNB-R were comparable to that for KOS and to each other (Fig. 3). These data are consistent with previous data for *vhs* mutants and marker-rescued viruses (22). Reactivation frequencies in explant cocultivation assays were 100% for KOS, ΔC4K-R, and ΔBNB-R, 17% for ΔBNB, and 0% for ΔC4K (Table 1). Viruses recovered from the ΔBNB latently infected ganglia were analyzed by Southern blotting, and the digestion patterns were consistent with ΔBNB, demonstrating that ΔBNB had indeed reactivated from both of these trigeminal ganglia (data not shown). The acute replication data shown in Table 1 were from a typical experiment for all five viruses in parallel. Previous independent experiments for ΔBNB ($n = 12$) and ΔC4K ($n = 12$) in parallel with that for KOS ($n = 12$) produced identical results for acute

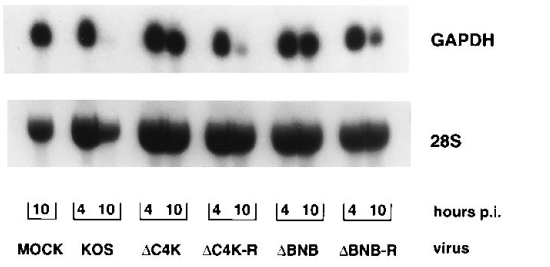

FIG. 2. RNA degradation assay by Northern blot analysis. Cytoplasmic RNA was extracted from KOS-, ΔC4K-, ΔBNB-, ΔC4K-R-, and ΔBNB-R-infected Vero cells at 4 and 10 h postinfection (p.i.). Above is an autoradiographic image of a Northern blot probed for GAPDH; below is an autoradiographic image of the blot shown at top after it had been stripped and reprobed for the 28S ribosomal subunit. The autoradiographs were scanned and analyzed with a Molecular Dynamics PhosphorImager.

TABLE 1. Pathogenesis in mice and RNA degradation

Virus	% GAPDH RNA remaining[a]	PFU/ml of[b]:		Reactivation (%)[c]
		Eye swab	Ganglion homogenate	
KOS	14	1.1×10^3	5.8×10^5	8/8 (100)
ΔC4K	82	1.4×10^1	1.9×10^0	0/12 (0)
ΔC4K-R	8	6.9×10^2	5.4×10^4	14/14 (100)
ΔBNB	64	5.3×10^0	1.5×10^0	2/12 (17)
ΔBNB-R	11	3.4×10^2	4.7×10^5	14/14 (100)

[a] Measured at 10 h postinfection and calculated relative to 28S-normalized mock-infected cells set to 100%. Data are taken from a typical experiment shown in Fig. 2.

[b] Samples from individual eye swabs and ganglion homogenates were assayed on day 3 postinfection. Logarithmic mean values for at least four samples are shown.

[c] Number of explanted latently infected trigeminal ganglia yielding virus/total number of ganglia assayed.

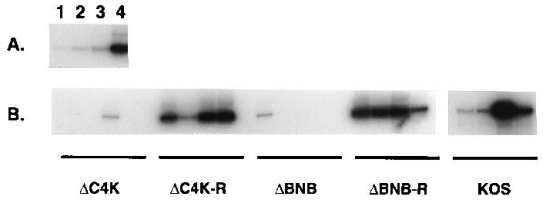

FIG. 3. Quantitative PCR analysis of HSV DNA extracted from latently infected trigeminal ganglia. The HSV-1 VP16 gene was used as a target for amplification, with the single-copy mouse adipsin gene being used as an internal loading control. PCR was performed for 35 cycles, and all probes were internal to the primers. (A) Quantitative PCR analysis of uninfected mouse tail DNA spiked with HSV DNA. Lanes: 1, 0.008 copy of viral DNA per mouse cell equivalent; 2, 0.04 copy; 3, 0.2 copy; 4, 1 copy. (B) PCR analysis of DNA from ganglia latently infected with ΔC4K, ΔC4K-R, ΔBNB, ΔBNB-R, and KOS. The autoradiographs were scanned and analyzed with a Molecular Dynamics PhosphorImager.

replication in corneas and ganglia and for establishment of and reactivation from latency (data not shown).

The mutations introduced into UL41 in viruses ΔC4K and ΔBNB rendered HSV-1 incapable of degrading host RNA upon a high multiplicity of infection. These mutations demonstrate that conserved domains III and IV are critical for *vhs* activity. The lack of shutoff by ΔBNB was expected, since the deletion included threonine 214, a residue which appears critical for *vhs* function (7, 10). Deletion of the 29 amino acids at the carboxy terminus that constituted domain IV, however, is novel and demonstrates the requirement of this domain for *vhs* function in the context of the virus. It should be noted that in three independent experiments, the RNA degradation induced by ΔBNB appears slightly higher than that induced by *vhs*-ΔSma, ΔC4K, or UL41NHB (22) (Table 1 and Fig. 2). ΔBNB also exhibits a slightly higher level of establishment of and reactivation from latency. These differences, although reproducible, cannot be shown to be statistically significant at this juncture ($P > 0.1$ by the t test), but the possibility that the loss of RNA degradation and loss of virulence may be separable for certain *vhs* mutants is under further study.

In addition, it has been demonstrated that the vhs protein contains distinct conserved domains (1). Perhaps most relevant to the study of *vhs* and pathogenesis is that amino acids 310 to 330 of the vhs protein are necessary and sufficient for interaction with virion-associated transactivator VP16 (17). The *vhs* mutants previously studied in pathogenesis, UL41NHB and *vhs*-ΔSma, both entirely lack this domain. Given that *in*1814, a VP16 mutant virus, has been shown to have reduced virulence in mice (21), it could be argued that the avirulence of UL41NHB and *vhs*-ΔSma was due to the inability of their mutant *vhs* polypeptides to interact with VP16 rather than to their inability to induce degradation of RNA. The present study, however, indicates that this argument is unlikely to be correct and that the *vhs* function itself is required for pathogenesis, since the deletions in ΔC4K and ΔBNB do not directly affect the VP16 interaction domain. The precise mechanism by which *vhs* contributes to pathogenesis remains obscure, but further studies of the functional domains of *vhs* should elucidate its role in RNA degradation, VP16 interaction, and pathogenesis.

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