

# Mutational Analysis of the Virion Host Shutoff Gene (UL41) of Herpes Simplex Virus (HSV): Characterization of HSV Type 1 (HSV-1)/HSV-2 Chimeras

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**During lytic herpes simplex virus (HSV) infections, the half-lives of host and viral mRNAs are regulated by the HSV virion host shutoff (Vhs) protein (UL41). The sequences of the UL41 polypeptides of HSV type 1 (HSV-1) strain KOS and HSV-2 strain 333 are 87% identical. In spite of this similarity, HSV-2 strains generally shut off the host more rapidly and completely than HSV-1 strains. To examine type-specific differences in Vhs function, we compared the Vhs activities of UL41 alleles from HSV-1(KOS) and HSV-2(333) by assaying the ability of a transfected UL41 allele to inhibit expression of a cotransfected reporter gene. Both HSV-1 and HSV-2 alleles inhibited reporter gene expression over a range of *vhs* DNA concentrations. However, 40-fold less of the HSV-2 allele was required to yield the same level of inhibition as HSV-1, indicating that it is significantly more potent. Examination of chimeric UL41 alleles containing various combinations of HSV-1 and HSV-2 sequences identified three regions of the 333 polypeptide which increase the activity of KOS when substituted for the corresponding amino acids of the KOS protein. These are separated by two regions which have no effect on KOS activity, even though they contain 43 of the 74 amino acid differences between the parental alleles. In addition, alleles encoding a full-length KOS polypeptide with a 32-amino-acid N-terminal extension retain considerable activity. The results begin to identify which amino acid differences are responsible for type-specific differences in Vhs activity.**

Controls of the rate of mRNA decay play an important role in eukaryotic gene expression (1, 3, 33). During lytic herpes simplex virus (HSV) infections, the half-lives of both host and viral mRNAs are regulated by the product of the HSV virion host shutoff (*vhs*) gene (UL41) (30). At early times, copies of the Vhs polypeptide, which enter the cell as components of infecting virions, destabilize preexisting host mRNAs (10, 34, 38). This, together with the disruption of pre-mRNA splicing by the HSV immediate-early polypeptide ICP27 (15, 16), results in the shutoff of most host protein synthesis. In addition, following the onset of viral transcription, the Vhs protein ensures rapid turnover of viral mRNAs belonging to all kinetic classes of viral mRNA (22, 27, 28, 38). Thus, although it is apparently specific for mRNAs (20, 41), the Vhs protein is non-selective with regard to which mRNAs are targeted for degradation. As a generalized destabilizer of mRNAs, it plays two important roles during HSV infections: first, in redirecting the cell from synthesis of cellular to viral proteins; and second, in determining the levels of viral mRNAs and facilitating the sequential transition between expression of different classes of viral genes.

*vhs* (UL41) homologs have been identified in a number of alphaherpesviruses, including HSV types 1 and 2 (HSV-1 and -2) (6, 8, 9, 11, 25), varicella-zoster virus (5), pseudorabies virus (2), bovine herpesvirus, gallid herpesvirus, and equine herpesvirus 1 (7, 40). Of these, the most closely related, and also the most extensively studied, are the *vhs* genes of HSV-1 and HSV-2. Comparison of the predicted amino acid sequences of the UL41 polypeptides from HSV-1(17) and HSV-2(G) reveals that they are 87% identical (6). In spite of this similarity, HSV-2 strains generally exhibit more rapid and complete virion host shutoff than do HSV-1 strains (11, 17), and the

transfer of UL41 alleles between strains transfers the host shutoff phenotype (9). The most straightforward explanation for these results is that Vhs (UL41) proteins from HSV-2 strains possess a more potent mRNA degradative activity than the homologous HSV-1 polypeptides. However, at present, one cannot rule out the possibility that the more rapid virion host shutoff of HSV-2 is due to more rapid and efficient release of the UL41 polypeptide from infecting virions.

To further examine type-specific differences in Vhs function, we compared the Vhs activities of UL41 alleles from HSV-1 (strain KOS) and HSV-2 (strain 333) by using a transient expression assay in which Vhs activity is measured by the ability of a transfected UL41 allele to inhibit expression of a cotransfected reporter gene. This assay has the advantage that the activities of different UL41 polypeptides can be compared in the absence of other viral gene products, circumventing any complications due to differences between the proteins with regard to packaging or release from virions (19, 29). Both HSV-1 and HSV-2 alleles inhibited reporter gene expression over a range of transfected *vhs* DNA concentrations. However, 40-fold less of the HSV-2 allele was required to yield the same level of inhibition as HSV-1, indicating that the HSV-2 allele possesses a significantly more potent Vhs activity than its HSV-1 homolog. The cotransfection assay was also used to compare the inhibitory activities of a series of chimeric UL41 alleles containing various mixtures of HSV-1 and HSV-2 sequences. The results identify three regions of the UL41 polypeptide that contribute to type-specific differences in Vhs activity. Furthermore, they indicate that more than half of the differences between the *vhs* alleles are unrelated to their differences in activity.

## MATERIALS AND METHODS

**Cells.** Vero cells were purchased from the American Type Culture Collection and maintained in Eagle's minimum essential medium (GIBCO) supplemented with 10% (vol/vol) calf serum and antibiotics as described previously (28).

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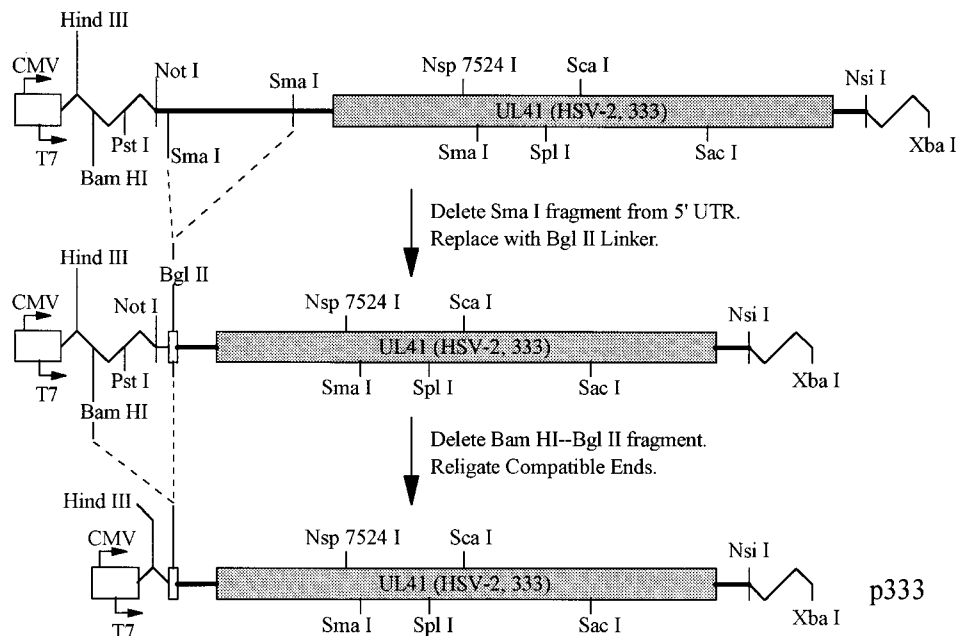


FIG. 1. Construction of p333. The UL41-containing *NotI*-*NsiI* fragment from HSV-2(333) was excised from plasmid pKC7BglN and inserted between the corresponding sites of the vector pcDNA1, to yield the plasmid diagrammed in line 1. The shaded rectangle represents the UL41 open reading frame, while the thick lines represent HSV-2 sequences from the 5' and 3' untranslated regions (UTR) of UL41. The zigzag lines represent the pcDNA1 polylinker, and the open rectangle represents the region of pcDNA1 containing the CMV immediate-early and T7 promoters. The CMV and T7 transcriptional start sites are depicted by arrows. The plasmid shown in line 1 was partially digested with *SmaI* to excise the short *SmaI* fragment from the UL41 5' untranslated region, and a *BglII* linker was inserted to generate the plasmid shown in line 2. This plasmid was digested with *BamHI* and *BglII* to remove the short *BamHI*-*BglII* fragment. The compatible ends generated by *BamHI* and *BglII* were then religated to yield the plasmid p333, which is shown in line 3. The conserved restriction sites within UL41 which were used to construct the HSV-1/HSV-2 chimeras are indicated. HSV-2 sequences are drawn to scale, while vector sequences are not.

**Plasmids. (i) Plasmids encoding UL41 from HSV-2.** Plasmid pKC7BglN contains the 7.3-kb *BglII* N fragment from HSV-2(333) cloned into the *BglII* site of pKC7 (12). It was provided by Denise Galloway. Construction of p333, in which UL41 from HSV-2(333) is cloned downstream from the cytomegalovirus (CMV) immediate-early and T7 RNA polymerase promoters, is diagrammed in Fig. 1. Briefly, the UL41-containing *NotI*-*NsiI* fragment was excised from pKC7BglN and inserted between the corresponding sites of the vector pcDNA1 (Invitrogen). This plasmid was partially digested with *SmaI* to excise the *SmaI* fragment extending from 135 to 482 bp upstream from the UL41 start codon (6), and a *BglII* linker was inserted. The resulting plasmid was digested with *BglII* and *BamHI* to remove the short *BamHI*-*BglII* fragment from the upstream side of the gene; the plasmid was recircularized by ligation of the compatible *BamHI*- and *BglII*-generated ends. Sequencing of p333 confirmed that the authentic UL41 start codon is the first AUG from the 5' end of mRNAs produced in vivo from the CMV immediate-early promoter or by in vitro transcription using T7 RNA polymerase.

**(ii) Plasmids encoding UL41 from HSV-1.** Construction of pKOS, which contains UL41 from HSV-1(KOS) cloned downstream from the CMV immediate-early and T7 promoters, is diagrammed in Fig. 2. Plasmid pWT6, described previously (29), contains the UL41-containing *BstEII* fragment of HSV-1(KOS) cloned into a modified form of the vector pSP64(polyA) (Promega). This plasmid was digested with *SalI* and *BstEII* to excise the short *SalI*-*BstEII* fragment from the upstream side of UL41, treated with mung bean nuclease to generate blunt ends, and recircularized by blunt-end ligation. A UL41-containing *PstI*-*XbaI* fragment was excised and subcloned between the corresponding sites of pcDNA1 to yield pKOS. Sequencing of pKOS showed that UL41 is preceded on the upstream side by 89 bp of viral DNA, preceded by a single G:C base pair and then the *PstI* site from pcDNA1. Sequence analysis confirmed that the authentic UL41 start codon is the first AUG from the 5' end of mRNAs produced from the CMV immediate-early promoter or by in vitro transcription using T7 RNA polymerase.

**(iii) Plasmids encoding UL41 with an amino-terminal extension.** pXKOS was constructed by subcloning the UL41-containing *PstI*-*XbaI* fragment of pWT6 between the corresponding sites of pcDNA1 (Fig. 3). Sequencing of pXKOS revealed the presence of an in-frame AUG codon upstream from the authentic UL41 start codon, which was generated by the juxtaposition of viral and vector sequences. As a result, pXKOS is predicted to encode a Vhs polypeptide consisting of the full-length UL41 product of HSV-1(KOS) with a 32-amino-acid N-terminal extension (Fig. 3). Consistent with this, a 62-kDa Vhs polypeptide is produced by in vitro transcription and translation of pXKOS, as well as following transfection of cells with the pXKOS allele (data not shown). This is compared to

the 58-kDa product that is produced by in vitro transcription and translation of pKOS (data not shown) and which is seen in cells infected with wild-type HSV-1 or cells transfected with a plasmid encoding a wild-type UL41 polypeptide (29, 32).

**(iv) Plasmids encoding chimeric UL41 polypeptides.** Plasmids encoding chimeric UL41 alleles were constructed by exchanging fragments between p333 and pKOS or pXKOS, using restriction sites that are conserved between the HSV-2 and HSV-1 alleles. To exchange fragments encoding amino-terminal portions of the proteins, the plasmids were cleaved in the 5' polylinker with *HindIII* and with an enzyme that cleaves within UL41. To exchange carboxy-terminal fragments, the plasmids were digested in the 3' polylinker with *XbaI* and with an enzyme that cleaves within UL41. Appropriate restriction fragments were exchanged, and the plasmids were religated. Four restriction sites were used to cleave within UL41. Digestion with *Nsp7524I* allowed construction of chimeras with a recombination junction after codon 135 of the HSV-1 allele. Similarly, *SplI*, *ScaI*, and *SacI* were used to generate chimeric alleles with recombination junctions after amino acids 208, 243, and 365. Chimeric alleles were given designations that reflect the order of fragments from the parent alleles and the locations of the recombination junctions. For example, K/3/K(208,365) encodes a UL41 polypeptide containing KOS sequences from amino acids 1 through 208, 333 sequences from amino acids 209 to 365, and KOS sequences from amino acids 366 to 489. Since the KOS and 333 polypeptides differ in length by three amino acids, the coordinates used to designate recombination junctions refer to the numbering of amino acids in the HSV-1 sequence. Chimeric alleles were sequenced across the junctions and analyzed by in vitro transcription and translation to confirm that they encode polypeptides of the predicted size which can be immunoprecipitated with UL41-specific rabbit antisera (32).

**DNA isolation and sequencing.** Plasmids for use in transfections were prepared from bacterial lysates by using the MaxiPrep system as recommended by the manufacturer (Qiagen Corp.). For DNA sequencing, UL41-containing plasmids were denatured, annealed with appropriate primers, and sequenced by using an Applied Biosystems model 377 DNA sequencer in the Molecular Biology Core Facility of the University of Missouri—Kansas City. Sequencing primers were designed based on the published sequences for the UL41 alleles of HSV-1(17) (25) and HSV-2(G) (6) and purchased from Integrated DNA Technologies (Coralville, Iowa).

**Transient expression assay for Vhs activity.** Vhs activity was measured by determining the ability of a transfected UL41 allele to inhibit expression of a cotransfected reporter gene. Transfections were performed as described previously (29) except that the reporter gene was *lacZ* rather than one encoding chloramphenicol acetyltransferase. Briefly, Vero cells were plated the day before

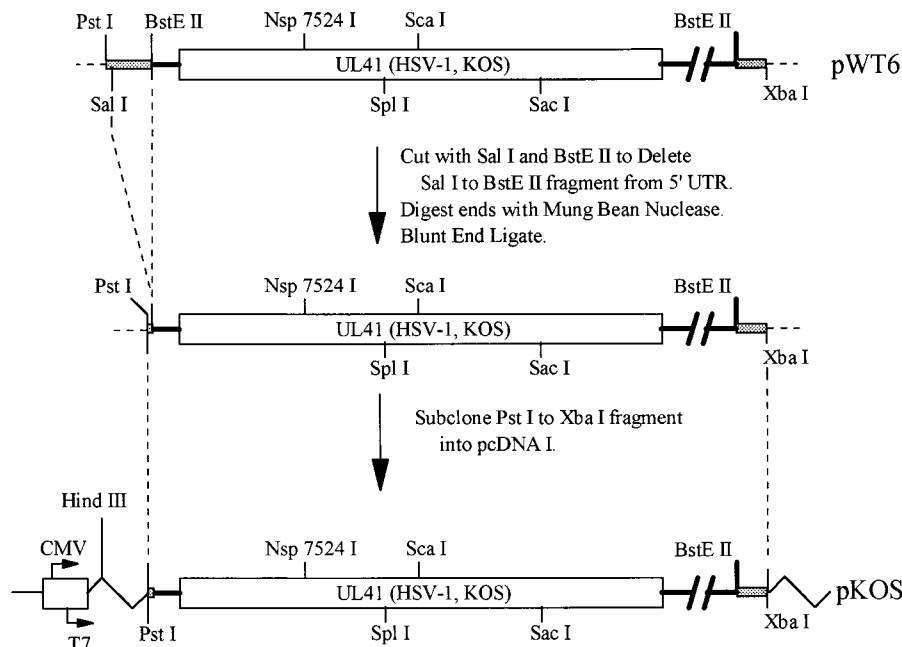


FIG. 2. Construction of pKOS. Plasmid pWT6 is diagrammed in line 1. The large open rectangle represents the UL41 open reading frame from HSV-1(KOS), and the thick lines represent viral sequences comprising the UL41 5' and 3' untranslated regions (UTR). The small shaded rectangles represent sequences from the modified polylinker of pWT6, and the dashed lines represent remaining vector sequences. pWT6 was cut to completion with *Sal*I and partially with *Bst*EII to delete the short *Sal*I-*Bst*EII fragment from the 5' untranslated region. The remaining plasmid was digested with mung bean nuclease and recircularized by blunt-end ligation to yield the plasmid shown in line 2. The UL41-containing *Pst*I-*Xba*I fragment was excised from this plasmid and subcloned between the corresponding sites of pcDNA1 to yield pKOS, which is shown in line 3. Zigzag lines represent the pcDNA1 polylinker, and the small open rectangle represents the region of pcDNA1 containing the CMV immediate-early and T7 promoters. The CMV and T7 transcriptional start sites are depicted by arrows. The conserved restriction sites within UL41 which were used to construct the HSV-1/HSV-2 chimeras are indicated. HSV-1 sequences are drawn to scale, while vector sequences are not.

transfection in 60-mm-diameter petri dishes at a density of  $2.5 \times 10^4$  cells/cm<sup>2</sup>. Three to four hours before transfection, the medium was replaced with fresh Eagle's minimum essential medium containing 10% (vol/vol) calf serum. Cultures were transfected with 0.6-ml aliquots containing calcium phosphate coprecipitates of 3  $\mu$ g of the reporter plasmid pSV- $\beta$ -Galactosidase (Promega) and various amounts of a UL41-containing effector plasmid. Each transfection mixture also contained enough pcDNA1 to maintain the amount of CMV promoter sequences (effector plasmid plus pcDNA1) equal to 0.73 pmol (3  $\mu$ g of pKOS is 0.73 pmol) and enough salmon sperm carrier DNA to bring the total amount of DNA to 12  $\mu$ g.

Cell extracts were prepared at 40 to 48 h after transfection and assayed for reporter gene expression by using a  $\beta$ -galactosidase enzyme assay system purchased from Promega. Briefly, cell extracts were aliquoted into 96-well trays and mixed with substrate, and the absorbance at 405 nm was determined at various times over a 2-h interval by using a Molecular Devices Thermo Max Microplate Reader. The  $\beta$ -galactosidase activity in each aliquot was determined from the initial velocity of the enzymatic reaction. For each experiment, triplicate cultures were transfected with each concentration of effector plasmid. For each culture, the amount of  $\beta$ -galactosidase activity was expressed as a fraction of that observed in transfections involving 0.73 pmol of pcDNA1 and no UL41-containing

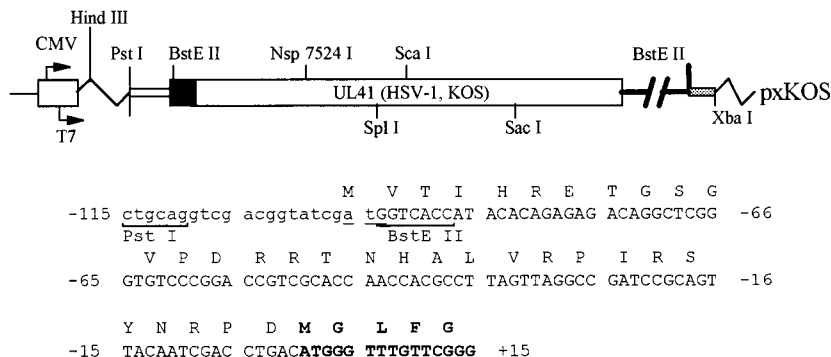


FIG. 3. Structure of pxKOS. The large open rectangle represents the wild-type UL41 open reading frame from HSV-1(KOS), and the solid rectangle represents the 32-amino-acid N-terminal extension which is present in the Vhs protein encoded by pxKOS. The thick line represents viral sequences comprising part of the UL41 3' untranslated region, while the narrow open and shaded rectangles represent sequences from the polylinker of pWT6. Zigzag lines represent the pcDNA1 polylinker, and the small open rectangle represents the region of pcDNA1 containing the CMV immediate-early and T7 promoters. The CMV and T7 transcriptional start sites are depicted by arrows. HSV-1 sequences are drawn to scale, while vector sequences are not. The nucleotide sequence from the *Pst*I site through the first five codons of the wild-type UL41 polypeptide is shown below the diagram, along with the predicted amino acid sequence of the UL41 polypeptide. The first five amino acids of the authentic UL41 polypeptide are shown in bold, preceded by the 32-amino-acid N-terminal extension in normal type. The ATG start codons utilized by the authentic UL41 polypeptide and by the polypeptide with the N-terminal extension are underlined, as are the *Pst*I and *Bst*EII sites. Nucleotides that are derived from the virus are shown in capital letters, while those derived from the plasmid vector are shown in lowercase. Nucleotide numbers are shown relative to the A of the start codon of the authentic UL41 polypeptide.

	1														60
HSV-1 (17)	-----	-----	-----	<b>-R-</b>	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HSV-1 (KOS)	MGLFG	MMKFA	HTHHL	VKRQG	LGAPA	GYFTP	IADVL	WNVMY	TLVVK	YQRRY	PSYDR	EAITL			
HSV-2 (333)	-----	-----	<b>Q</b> ----	-----	-R-E	-----	-----	-----	-----	-----	-----	-----			
HSV-2 (G)	-----	-----	<b>Q</b> ----	-----	-R-E	-----	-----	-----	-----	-----	-----	-----			
	61														120
HSV-1 (17)	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----			
HSV-1 (KOS)	HCLCR	LLKVF	TQKSL	FPIFV	TDRGV	NCMEP	VVFGA	KAILA	RTTAQ	CRTDE	EASDV	DASPP			
HSV-2 (333)	-----	<b>S</b>	M-R-	-----	-----	E-T-	-----	-----	-----	-----	-----	-----			
HSV-2 (G)	-----	<b>S</b>	M-R-	-----	-----	E-T-	-----	-----	-----	-----	-----	-----			
	121														180
HSV-1 (17)	-----	-----	-----	-----	-----	-----	**	-----	-----	-----	-----	-----			
HSV-1 (KOS)	PSPIT	DSRPS	SAPSN	MRRRG	TSLAS	GTRGT	**AGSGA	ALPSA	APSKP	ALRLA	HLFCI	RVLRA			
HSV-2 (333)	-----	-----	F-----	-----	HAF-P	-D---	FA-P-P	-A-WG	-----	-----	-----	-----			
HSV-2 (G)	-----	-----	F-----	-----	HAF-P	-D---	FA-P-P	-A-G	-----	-----	-----	-----			
	181														240
HSV-1 (17)	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----			
HSV-1 (KOS)	LGAYY	INSGQ	LEADD	ACANL	YHTNT	VAYVY	<b><u>TTD</u></b> D	LLLMG	CDIVL	DISAC	YIPTI	NCRDI			
HSV-2 (333)	-----	-----	-----	-----	-----	-----	<b><u>H</u></b>	-----	-----	-----	-----	-----	H---	L	
HSV-2 (G)	-----	-----	-----	-----	-----	-----	<b><u>H</u></b>	-----	-----	-----	-----	-----	H---	L	
	241														300
HSV-1 (17)	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----			
HSV-1 (KOS)	LKYFK	MSYPQ	FLALF	VRCHT	DLHPN	NTYAS	VEDVL	RECHW	TPPSR	SQTRR	AIRRE	HTSSR			
HSV-2 (333)	-----	-----	-----	-----	-----	-----	-----	-----	A-	A-	A-	RAN-			
HSV-2 (G)	-----	-----	-----	-----	-----	-----	-----	-----	A-	A-	A-	RAN-			
	301														360
HSV-1 (17)	-----	-----	-----	<b>-T-</b>	-----	-----	-----	-----	-----	-----	-----	-----			
HSV-1 (KOS)	STETR	PPLPP	AAGGT	<b>EMRVS</b>	WTEIL	TQQIA	GGYED	DEDLP	LDPRD	VTGGH	P*GPRS	SSSEI			
HSV-2 (333)	-L-SM	-T-TA	-FV-L	-T-I-	-----	A-----	-EDDY	E-P-	-Q-P-	-A-P	RD-A-	-----			
HSV-2 (G)	-L-SM	-T-TA	-FV-L	-T-I-	-----	A-----	-EDDY	E-P-	-Q-P-	-A-P	RD-A-	-----			
	361														420
HSV-1 (17)	-----	-----	-----	-----	-----	<b>-NP</b>	-----	-----	-----	-----	-----	-----			
HSV-1 (KOS)	LTPPE	LVQVP	NAQLL	EEHRS	YVASR	RRHVI	HDAPF	SLDWL	PDFMT	ITELV	EHRYI	KYVIS			
HSV-2 (333)	-----	-----	---RV	A---G	---G-	-----	-----	A-----	-----	A-----	-----V	-----			
HSV-2 (G)	-----	-----	---RV	A---G	---G-	-----	-----	A-----	-----	A-----	-----V	-----			
	421														480
HSV-1 (17)	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----			
HSV-1 (KOS)	LIGPK	ERGPW	TLLKR	LPIYQ	DIRDE	NLARS	IVTRH	ITAPD	IADRF	LEQLR	TQAPP	PAFYK			
HSV-2 (333)	-----	-----	-----	-----	-L---	D-----	-----	-----	-----	A--W	AH--	-----			
HSV-2 (G)	-----	-----	-----	-----	-L---	D-----	-----	-----	-----	A--W	AH--	-----			
	481	489													
HSV-1 (17)	-----	-----													
HSV-1 (KOS)	DVLAK	FWDE													
HSV-2 (333)	-----	-----													
HSV-2 (G)	-----	-----													

FIG. 4. Comparison of amino acid sequences of UL41 polypeptides encoded by various strains of HSV-1 and HSV-2. The predicted amino acid sequence of the UL41 polypeptide encoded by HSV-1(KOS) is shown in line 2 and compared to the published sequences of the UL41 polypeptides of HSV-1(17) (line 1) (25) and HSV-2(G) (line 4) (6), as well as to the sequence predicted for HSV-2(333) (line 3). Dashes in the sequences for strains 17, G, and 333 represent amino acids that are identical to those in KOS. Amino acids which differ between the UL41 polypeptides of KOS and strain 17 are shown in bold. The threonine at position 214 of the KOS polypeptide, which is changed to an isoleucine in the mutant *v*h<sub>s</sub> 1, is shaded and underlined. Asterisks are introduced at three locations in the sequences of the HSV-1 strains to maintain maximal alignment with the polypeptides encoded by the HSV-2 strains, which are three amino acids longer. The amino acid numbers refer to the HSV-1 polypeptides. The single amino acid which differs between the UL41 polypeptides of HSV-2 strains 333 and G is shown in bold.

effector plasmid. For each type of effector allele, the data were plotted to yield a dose-response curve showing inhibition of reporter gene expression as a function of the concentration of effector DNA. A curve was fitted to the data by third-order regression analysis using SigmaPlot version 2.02 (Jandel) and used to determine the concentration of effector DNA required to reduce reporter gene expression to 30% of the control value ( $[DNA]_{0.3}$ ). Replicate experiments were performed on different days, each yielding a separate dose-response curve and value of  $[DNA]_{0.3}$ . These values were then averaged to yield the values of  $[DNA]_{0.3}$  that are reported in Fig. 6 and 7 and to calculate the standard errors of the mean.

**Nucleotide sequence accession numbers.** The DNA sequences of the UL41 alleles of HSV-1(KOS) and HSV-2(333) have been deposited in GenBank with accession no. AF007815 and AF007816, respectively.

## RESULTS

**Comparison of the UL41 sequences of HSV-1(KOS) and HSV-2(333).** The sequences of the UL41 alleles of HSV-1(17) and HSV-2(G) have been published elsewhere (6, 25). Nevertheless, before comparing the activities of the UL41 alleles from HSV-1(KOS) and HSV-2(333), it was necessary to de-

termine their sequences. On the basis of the nucleic acid sequence, HSV-1(KOS) is predicted to encode a 489-amino-acid UL41 polypeptide that is identical to that of HSV-1(17) except at amino acids 19, 317, 384, and 385 (Fig. 4). Interestingly, strain 17 has been reported to encode a particularly weak *v*h<sub>s</sub> function (9), suggesting that some of these changes may affect activity. In addition to the single-nucleotide changes which give rise to these differences in the protein sequence, the KOS allele contains six single-nucleotide changes that do not alter the amino acid sequence from that predicted for strain 17 (Table 1).

HSV-2 strains 333 and G each encode a 492-amino-acid UL41 polypeptide (Fig. 4). The additional three amino acids of the HSV-2 polypeptides are due to the insertion of two amino acids between residues 150 and 151 of the HSV-1 polypeptide and an insertion of one amino acid between HSV-1 amino acids 351 and 352. The strain 333 polypeptide is identical to that encoded by strain G except for the presence of a trypto-

TABLE 1. Differences between the UL41 sequences of HSV-1(KOS) and HSV-1(17)<sup>a</sup>

KOS		17	
Nucleotide	Amino acid	Nucleotide	Amino acid
<b>A(56)</b>	Q(19)	G(56)	R(19)
C(93)	I(31)	T(93)	I(31)
A(315)	Q(105)	G(315)	Q(105)
A(636)	T(212)	G(636)	T(212)
T(922)	L(308)	C(922)	L(308)
<b>T(950)</b>	M(317)	C(950)	T(317)
<b>G(1151)</b>	S(384)	A(1151)	K(384)
<b>G(1154)</b>	R(385)	C(1154)	P(385)
T(1353)	I(451)	C(1353)	I(451)
C(1405)	L(469)	T(1405)	L(469)

<sup>a</sup> Nucleotide numbers (in parentheses) are given relative to the A of the UL41 start codon. Amino acid numbers are shown in parentheses. Nucleotide differences that result in amino acid differences are shown in bold. The strain KOS sequence was determined in this study; the strain 17 sequence has been published elsewhere (25).

phan instead of a serine at amino acid 161 (Fig. 4). In addition to the single-nucleotide change giving rise to this difference, the strain 333 allele contains three single-nucleotide changes that do not alter the protein sequence from that predicted for strain G (Table 2). Comparison of the amino acid sequences of the UL41 polypeptides encoded by HSV-1(KOS) and HSV-2(333) reveals that they are 87% identical. While single amino acid substitutions are scattered throughout the proteins, two regions, those from amino acids 141 to 160 and from amino acids 282 to 353, contain particularly high concentrations of nonidentical amino acids (Fig. 4).

**Comparison of the activities of UL41 alleles.** During virus infections, interactions with other viral proteins are likely to influence the activity of the UL41 polypeptide. In particular, UL41 has been shown to interact with the viral transcriptional activator VP16 (32, 35, 36), and this interaction has been implicated as important for controlling the mRNA degradative activity of newly synthesized copies of the UL41 protein (24). Interaction with VP16 may be important for packaging of the UL41 polypeptide (32, 35, 36), and disruption of the interaction at early times may be important for the release of Vhs from infecting virions. A domain which is sufficient for interaction with VP16 has been mapped to between amino acids 310 and 330 of the HSV-1 UL41 polypeptide (35). Comparison of the UL41 sequences from HSV-1(KOS) and HSV-2(333) revealed the presence of seven nonidentical amino acids within this region (Fig. 4), raising the possibility that differences between the virion host shutoff activities of HSV-1 and HSV-2

TABLE 2. Differences between the UL41 sequences of HSV-2(333) and HSV-2(G)<sup>a</sup>

333		G	
Nucleotide	Amino acid	Nucleotide	Amino acid
<b>G(482)</b>	W(161)	C(482)	S(161)
C(1044)	V(348)	T(1044)	V(348)
C(1296)	P(432)	A(1296)	P(432)
C(1306)	L(436)	T(1306)	L(436)

<sup>a</sup> Nucleotide numbers (in parentheses) are given relative to the A of the UL41 start codon. Amino acid numbers are shown in parentheses. Nucleotide differences that result in amino acid differences are shown in bold. The strain 333 sequence was determined in this study; the strain G sequence has been published elsewhere (6).

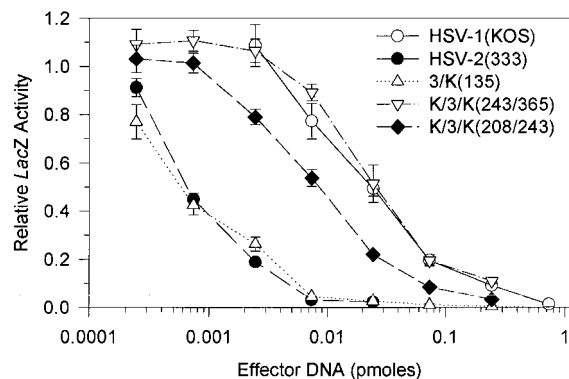


FIG. 5. Dose-response curves for the inhibition of reporter gene expression by wild-type and chimeric UL41 alleles. Replicate cultures were transfected with 3  $\mu$ g of a plasmid carrying the *lacZ* reporter gene and the indicated amounts of various UL41 effector genes. LacZ expression was determined at 40 to 48 h after transfection and expressed as a fraction of that observed for transfections involving the pcDNA1 vector and no UL41 effector gene. Error bars represent standard errors of the mean. For data points where no error bars are seen, the error bars were smaller than the data point.

were due, at least in part, to differences in the interaction of UL41 with VP16 or other viral proteins. In view of this, it was desirable to compare the activities of UL41 alleles by using an assay that allows measurement of Vhs activity in the absence of other viral gene products.

To this end, cells were transfected with a constant amount of a *lacZ* reporter gene and increasing amounts of the UL41 alleles from HSV-1(KOS) and HSV-2(333). Vhs activity was monitored by assaying the ability of the transfected UL41 allele to inhibit reporter gene expression. Both KOS and 333 alleles inhibited *lacZ* expression in a dose-dependent fashion (Fig. 5). However, 40-fold less of the 333 allele was required to inhibit *lacZ* expression to the same extent as the KOS allele (Fig. 5 and 6), indicating that the HSV-1 and HSV-2 polypeptides differ considerably in mRNA degradative activity and that type-specific differences in host shutoff activity cannot be attributed entirely to differences in the interactions of the UL41 polypeptides with other viral proteins.

The cotransfection assay was next used to compare the Vhs activities of a number of chimeric UL41 alleles containing different mixtures of sequences from KOS and 333. Dose-response curves were determined for each of the chimeras, some examples of which are shown in Fig. 5, and used to determine  $[DNA]_{0.3}$  values. These values are shown in Fig. 6 and 7 and offer a convenient measure of relative Vhs activity. The chimera 3/K(365), which encodes a polypeptide with the first 368 amino acids from strain 333 fused to amino acids 365 through 489 of strain KOS, inhibited *lacZ* expression as well as the parental strain 333 allele (Fig. 6, line 3). The same was true of 3/K(208) and 3/K(135), which encode UL41 polypeptides with the first 210 and 135 amino acids of strain 333 fused to KOS sequences from the recombination point to the carboxyl terminus (Fig. 5; Fig. 6, lines 4 and 5). An important control was provided by 3/K<sub>1214</sub>(208), which is identical to 3/K(208) except that it encodes an isoleucine instead of threonine at position 214 in the KOS portion of the chimera (Fig. 7, line 4). This is due to the same point mutation as that carried by the mutant *vhs* 1 (23, 31). When present in a KOS background, this mutation inactivates Vhs activity both during virus infections (20, 28, 31) and in the cotransfection assay (29). As expected, 3/K<sub>1214</sub>(208) did not inhibit *lacZ* expression in the cotransfection assay (Fig. 7, line 4). The data indicate that differences

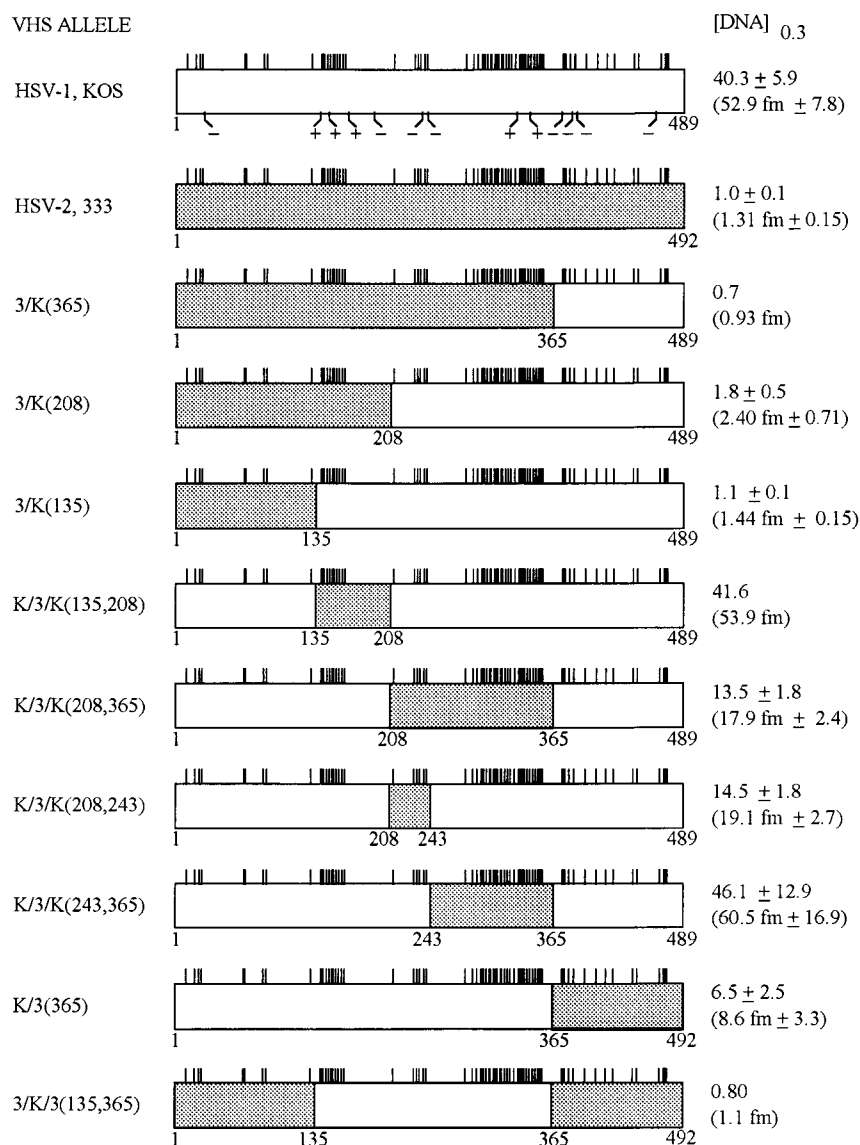


FIG. 6. Structures and activities of chimeric UL41 alleles. The UL41 polypeptide encoded by HSV-1(KOS) is represented by the open rectangle in line 1, and that encoded by HSV-2(strain 333) is represented by the shaded rectangle in line 2. The vertical lines at the top of each rectangle indicate the sites at which the sequences of the KOS and 333 polypeptides differ. The vertical lines below the rectangle for HSV-1(KOS) indicate the locations at which linker insertion mutations have been constructed previously for UL41 of HSV-1(KOS) (19, 29). + indicates linker insertion mutations which maintain Vhs activity, as measured in the cotransfection assay; - indicates linker insertion mutations which inactivate activity. The structures of the polypeptides encoded by a series of chimeric UL41 alleles are depicted in lines 3 through 11, with the portion contributed by KOS represented by an open rectangle and that contributed by 333 represented by a shaded rectangle. The  $[DNA]_{0.3}$  for each protein, expressed as a fraction of the  $[DNA]_{0.3}$  for UL41 from HSV-2(333) is shown at the right. For each allele, the  $[DNA]_{0.3}$  is also expressed (in parentheses) as the number of femtomoles of UL41 needed to transfect a 60-mm-diameter petri dish seeded with  $7 \times 10^5$  Vero cells.

within the first 135 amino acids of UL41 from strains KOS and 333 play a major role in the greater inhibitory activity of the strain 333 polypeptide. This region of the polypeptides contains only 10 nonidentical amino acids (Fig. 4).

The chimera K/3/K(135,208) encodes a UL41 polypeptide containing the first 135 amino acids from strain KOS fused to amino acids 136 through 210 of strain 333, followed by amino acids 209 to 489 of KOS (Fig. 6, line 6). This chimera exhibits an inhibitory activity that is indistinguishable from that of its KOS parent. Thus, the introduction of amino acids 136 and 210 from strain 333 into a KOS background does not increase the activity of the KOS polypeptide. This region contains a cluster of 11 nonidentical amino acids within a stretch of 20 residues,

implying that these differences are unrelated to the difference in Vhs activity of KOS and 333.

The chimera K/3/K(208,365) encodes a UL41 polypeptide containing the first 208 amino acids from strain KOS fused to amino acids 211 through 368 of strain 333, followed by amino acids 366 to 489 of KOS (Fig. 6, line 7). The inhibitory activity of this allele was intermediate between those of its KOS and 333 parents, indicating that the introduction of residues between amino acids 211 and 368 of strain 333 is able to increase the activity of KOS somewhat. This improvement was due to residues between amino acids 210 and 245 of strain 333 because the chimera K/3/K(208,243) exhibited an inhibitory activity that was indistinguishable from that of K/3/K(208,365)

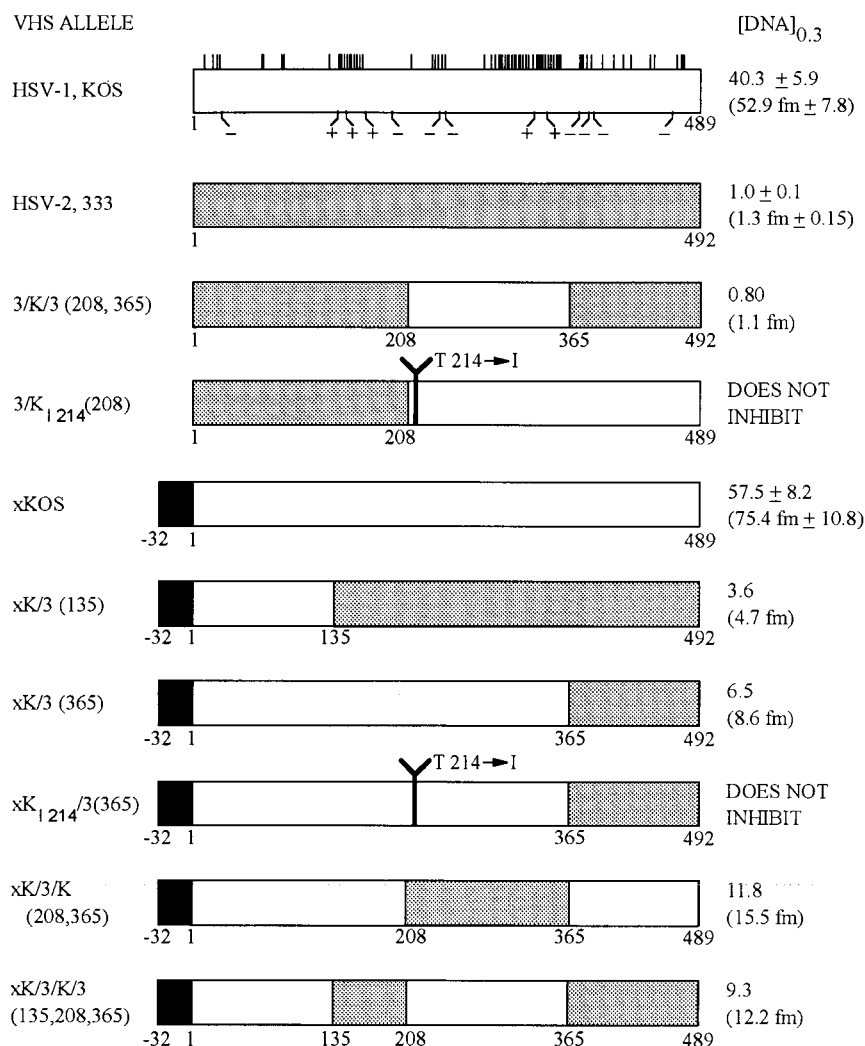


FIG. 7. Structures and activities of chimeric UL41 alleles. The UL41 polypeptides encoded by HSV-1(KOS), HSV-2(333), and a number of intertypic chimeras are depicted, with the portion of each protein encoded by KOS represented by an open rectangle and that encoded by 333 represented by a shaded rectangle. The alleles diagrammed in lines 5 through 10 encode polypeptides with a 32-amino-acid N-terminal extension, the sequence of which is shown in Fig. 3 and which is depicted by a solid rectangle. The alleles shown in lines 4 and 8 contain the point mutation carried by the mutant *vhs* 1, which changes a threonine at position 214 to an isoleucine. The position of this mutation is indicated by a thick vertical line with a "V" on top. The [DNA]<sub>0.3</sub> for each protein, expressed as a fraction of the [DNA]<sub>0.3</sub> for UL41 from HSV-2(333), is shown at the right. For each allele, the [DNA]<sub>0.3</sub> is also expressed (in parentheses) as the number of femtomoles of UL41 needed to transfect a 60-mm-diameter petri dish seeded with  $7 \times 10^5$  Vero cells.

(Fig. 5; Fig. 6, line 8), while the activity K/3/K(243,365) was the same as that of the KOS parent (Fig. 5; Fig. 6, line 9). Interestingly, the region of the 333 polypeptide (amino acids 210 to 245) responsible for the improved activity contains only six amino acids that are nonidentical to those of KOS (Fig. 1). In contrast, the region from amino acids 246 to 368, which does not improve KOS activity, contains 31 nonidentical residues.

Analysis of the 3/K(365) chimera revealed another region of the strain 333 allele which improves the activity of the KOS allele when inserted into a KOS background. This allele encodes a UL41 polypeptide containing amino acids 1 through 365 from KOS fused to amino acids 369 through 492 of strain 333 (Fig. 6, line 10). It inhibited *lacZ* expression more efficiently than either the KOS parent or K/3/K(208,243) but not as well as strain 333, requiring six times as much transfected UL41 to achieve the same level of inhibition. Taken together, the data on the UL41 chimeras identify three regions of the strain 333 allele (encoding amino acids 1 to 135, 210 to 245,

and 369 to 492) which improve the Vhs activity of the KOS allele when inserted into a KOS background. These are separated by two regions (encoding amino acids 136 to 209 and 246 to 368) which are unrelated to the greater Vhs activity of strain 333.

**UL41 alleles encoding Vhs polypeptides with a 32-amino-acid N-terminal extension are active.** During early attempts to clone the KOS gene downstream from the CMV immediate-early promoter, we inadvertently constructed a UL41 allele encoding a Vhs polypeptide with a 32-amino-acid N-terminal extension. This was due to the generation of an upstream AUG codon by the juxtaposition of viral and vector sequences (Fig. 3). When tested in the cotransfection assay, this allele inhibited reporter gene expression but required 50% higher concentrations of transfected UL41 than did KOS (Fig. 7, lines 1 and 5). KOS-333 chimeras that contained the N-terminal extension exhibited predictable Vhs activities. For example, xK/3(135), which encodes a UL41 polypeptide containing the 32-amino-

acid extension fused to amino acids 1 through 135 of KOS, followed by amino acids 136 through 492 of 333, inhibited *lacZ* expression at concentrations that were only fourfold higher than those required by the wild-type strain 333 allele (Fig. 7, lines 2 and 6). Similarly, xK/3(365) inhibited *lacZ* expression to the same extent as K/3(365) (Fig. 6, line 10; Fig. 7, line 7), while the activities of xK/3/K(208,365) and K/3/K(208,365) were indistinguishable (Fig. 6, line 7; Fig. 7, line 9). Furthermore, as predicted, the mutation changing threonine to isoleucine at position 214 abolished the activity of alleles containing the N-terminal extension (Fig. 7, line 8). Taken together, the data indicate that UL41 alleles containing the 32-amino-acid N-terminal extension retain considerable Vhs activity.

## DISCUSSION

In this study, we demonstrate that UL41 from HSV-2(333) is significantly more active than the UL41 allele of HSV-1(KOS) at inhibiting expression of a cotransfected reporter gene in a transient expression assay in which no other viral gene products are present. This difference in UL41 activity parallels the type-specific difference in host shutoff that is observed during virus infections, in which HSV-2 strains generally shut off the host more rapidly and completely than HSV-1 strains. This implies that type-specific differences in host shut off are due primarily to intrinsic differences in the activities of the UL41 polypeptides rather than to differences in their interactions with other viral proteins or in the speed or efficiency with which they are released from infecting virions.

The transient expression assay also was used to characterize the Vhs activities of a series of chimeric UL41 alleles containing various mixtures of HSV-1 and HSV-2 sequences. The results identify three regions of the 333 polypeptide (amino acids 1 through 135, 210 through 245, and 369 through 492) which increase the activity of KOS when substituted for the corresponding amino acids of the KOS protein. These are separated by two regions of the 333 allele (amino acids 136 through 209 and 246 through 368) which have no effect on KOS activity when introduced into a KOS background. Although the KOS and 333 proteins differ at sites scattered throughout UL41, two clusters of amino acids (141 through 160 and 282 through 353) contain particularly high concentrations of nonidentical residues, accounting for 43 of the 74 differences between alleles. Interestingly, both clusters fall within the portions of the 333 polypeptide which have no effect on KOS activity, suggesting that these amino acid differences are unrelated to type-specific differences in activity. On the other hand, differences in relatively few amino acids in other portions of the protein can result in major type-specific differences in activity. Of particular note, a chimera encoding a UL41 polypeptide with the first 135 amino acids from strain 333 and the remainder from KOS exhibits a Vhs activity that is indistinguishable from that of 333, even though it differs from KOS at only 10 amino acids. Similarly, a chimera with amino acids 210 through 245 from 333 and the rest from KOS differs from KOS at only six amino acids yet exhibits a Vhs activity that is intermediate between those of KOS and 333.

These results are consistent with those of previous studies which examined the effects of various linker insertion mutations on the activity of UL41 from HSV-1(KOS) (19, 29). Those experiments (summarized in Fig. 6, line 1) identified three regions of UL41 in which linker insertion mutations abolish activity. These regions roughly correspond to the three portions of UL41 which were shown in the present study to be responsible for the type-specific differences in Vhs activity and which are highly conserved among the UL41 homologs of

other alphaherpesviruses (2). The earlier studies also identified two regions of UL41 which tolerate linker insertion mutations without the loss of activity (19, 29); these fall within the two portions of UL41 which are unrelated to type-specific differences in activity (Fig. 6, line 1). In this light, it is interesting that the additional three-amino-acid length of the strain 333 polypeptide relative to that of KOS is due to the insertion of two amino acids after residue 150 and of one amino acid after residue 351 (Fig. 4). Both of these sites fall within the regions of UL41 which tolerate linker insertions and are unrelated to type-specific differences in activity. These regions are also entirely missing from the UL41 homologs of some alphaherpesviruses (2), further supporting the notion that their precise sequences are not critical to Vhs activity.

These experiments also demonstrate that UL41 alleles encoding polypeptides with a 32-amino-acid N-terminal extension retain considerable activity. Thus, although addition of the N-terminal extension to the KOS polypeptide increased the  $[DNA]_{0.3}$  by 50%, other alleles containing both the N-terminal extension and key regions of the 333 allele exhibited activities which were considerably greater than that of KOS. This result may be of practical importance for the purification of active Vhs protein, since it suggests that many UL41 polypeptides which have been modified by the addition of an epitope tag at the amino terminus will remain active.

The present results should be interpreted with the caveat that, because different UL41 alleles may express different amounts of the Vhs polypeptide in transfected cells, the transient expression assay does not provide a quantitative measure of the specific mRNA degradative activities of different UL41 polypeptides. For example, some chimeric proteins may differ in stability, leading to differences in the amounts that are present in transfected cells. In addition, the transient expression assay probably underestimates the difference between the activities of the KOS and 333 polypeptides. Because the UL41 polypeptide accelerates degradation of all mRNAs (30), it should be autoregulatory, degrading its own mRNA and decreasing the amount of UL41 protein that is produced in transfected cells. This effect should be greatest for the most active UL41 alleles. That this actually is the case is suggested by previous experiments which found several times more of the Vhs polypeptide in cells transfected with the inactive UL41 allele of the mutant *vhs* 1 than in cells transfected with the wild-type KOS allele (29). A qualitatively similar result has been obtained in studies using indirect immunofluorescence to visualize the UL41 polypeptide in cells transfected with the wild-type KOS allele and with the chimera 3/K(208), which exhibits an activity similar to that of 333. In these experiments, which used antisera raised against a UL41-LacZ fusion protein containing amino acids 239 through 489 of the KOS polypeptide (29) [a region that is common to both the KOS and 3/K(208) polypeptides], staining of cells transfected with 3/K(208) was much less intense than staining of those transfected with KOS (29a).

The three regions of the 333 polypeptide which increase KOS activity do so when substituted for the corresponding regions of KOS either individually or in combination. This finding is consistent with the possibility that these regions encode functionally independent domains of the protein that can be interchanged to yield functional UL41 proteins containing different combinations of KOS and 333 domains. In contrast, recent studies using site-directed mutagenesis of the KOS allele to change individual amino acids to their strain 333 counterparts have revealed one residue at which changing from the KOS to the 333 amino acid abolishes UL41 activity. However, if this change is made in conjunction with five other type-



MEAEA	QCAIL	DLTDQ	TSGTI	TDSDS	IWLFG	XPG-Xenopus
MEAEA	QCAVIL	DLSDD	TSGTI	TDSDS	IWLFG	XPG-Mouse
MEAEA	QCAIL	DLTDQ	TSGTI	TDSDS	IWLFF	XPG-Human
MEAEA	QCAEL	LQLNL	VDGII	TDSDS	VPLFG	RAD 2
QEAEA	QCSKL	LELKL	VDGIV	TDSDS	VPLFG	RAD 13
EADA	QLVYL	EKENI	IDGII	TEPDS	MLVFG	Exonuclease I. <i>S. pombe</i>
EAEA	SCAAI	VKAGK	VYAAA	TEPDS	CLTFG	Flap Endonuclease
MEADD	VCANI	FHTNT	VAHIY	TTDSD	MILMG	BHV
MEADD	VCANI	YHTNT	VAQVH	TTDSD	MILTG	PRV (Kaplan)
LEADD	VCANI	YHTNT	VAQVY	TTDSD	LLIMG	EHV-1 (AB4P)
LEADD	ACANI	FHTRT	VALVY	TTDSD	LLFMG	VZV (DUMAS)
LEADD	ACANI	YHTNT	VAVYH	TTDSD	LLIMG	HSV-2 (333)
LEADD	ACANI	YHTNT	VAVYV	TTDSD	LLIMG	HSV-1 (KOS)
191		201		211	220	

FIG. 8. Similarity between sequences of a conserved region of the UL41 polypeptides from alphaherpesviruses and sequences found in a number of cellular nucleases. The sequence of the UL41 polypeptide of HSV-1(KOS) from amino acids 191 through 220 is shown in line 13, aligned with the homologous sequences from the UL41 polypeptides of other alphaherpesviruses (lines 8 through 12). Homologous sequences from a number of cellular nucleases are shown in lines 1 through 7. Amino acids identical to those in the UL41 polypeptide of HSV-1(KOS) are shaded, while conservative changes are boldfaced and underlined. The location of the point mutation carried by the mutant *vhs* 1, which changes a threonine to an isoleucine at position 214 of the HSV-1(KOS) polypeptide and which inactivates Vhs activity, is shown by the arrow below line 13. BHS, bovine herpesvirus; PRV, pseudorabies virus; EHV-1, equine herpesvirus 1; VZV, varicella-zoster virus.

specific changes within the same putative domain, the result is a chimeric protein with an activity that is significantly greater than that of KOS (6a), suggesting that the six type-specific residues are part of the same functional domain of the protein.

If the UL41 polypeptide does, in fact, contain separate functional domains, the biochemical activities of those domains remain to be elucidated. Although obvious UL41 homologs exist in a number of alphaherpesviruses, computer searches of protein sequence databases have not revealed large-scale homologies between UL41 and known cellular proteins. However, comparison of short stretches of the UL41 polypeptide to cellular sequences reveals a region of 30 amino acids that is contained within the central conserved domain of UL41 and which is strikingly similar to sequences found in a number of cellular nucleases. Figure 8 compares the sequences of this region of the UL41 polypeptides from strains KOS and 333 to those of the corresponding regions of the UL41 homologs of other herpesviruses and to similar sequences in the XPG proteins from humans, mice, and *Xenopus laevis*; to those of the RAD2, RAD13, and Flap endonuclease proteins of *Saccharomyces cerevisiae*; and to that of exonuclease I from *Schizosaccharomyces pombe* (4, 13, 14, 18, 26, 39). Even cursory examination reveals that this region of UL41 is highly conserved among the UL41 homologs of alphaherpesviruses, with the only difference between KOS and strain 333 being a histidine instead of tyrosine at position 210 of the 333 protein. The similarity of the UL41 polypeptides to the cellular nucleases is particularly strong for two stretches of 10 amino acids corresponding to amino acids 191 through 200 and 211 through 220 of the KOS protein. Within each of these 10-amino-acid sequences, most of the cellular nucleases are identical to the KOS UL41 at five positions and have conserved residues at two others. Data indicating that this region of UL41 is functionally important come from the fact that the point mutation which inactivates the Vhs activity of the KOS mutant *vhs* 1 changes the threonine at position 214 to isoleucine (21). Interestingly, six of the seven cellular nucleases contain a serine at this position, an amino acid difference that is highly conservative.

In view of the fact that Vhs induces RNA degradation, it is curious that most of these cellular proteins are DNases. One

possible explanation is that the homologous sequences in UL41 and the cellular proteins are motifs common to a number of DNases and RNases and that the domain responsible for the specificity of UL41 for RNA lies elsewhere. One candidate for such a domain is a region of UL41 centered on amino acid 180, which is similar to the RNA recognition or RNP motif found in yeast and human poly(A) binding proteins (7, 37). Although there are no biochemical data indicating that the Vhs protein binds RNA, it is tempting to speculate that this motif is important for recognition of the target mRNA. In addition, although the Vhs activity degrades mRNAs nonselectively, it is specific for mRNAs, leaving ribosomal and other nonmessenger RNAs untouched both in vivo and in vitro (20, 27, 28, 41). This specificity may be due to interaction of the UL41 protein with the cap or some other feature that is unique to mRNAs. Alternatively, Vhs may recognize mRNAs through interactions with cellular proteins that associate specifically with mRNAs. These might be cap binding proteins, translation initiation factors, or proteins that are components of messenger RNPs. Whatever the mechanism, the domains responsible for the specificity of Vhs are likely to be within the regions that are conserved among UL41 homologs and which are responsible for the type-specific differences in Vhs activity. The answers to these and other questions should be illuminated by further genetic studies of UL41.

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