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Varicella-Zoster Virus Complements Herpes Simplex Virus Type 1 Temperature-Sensitive Mutants

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Varicella-zoster virus (VZV) can complement temperature-sensitive mutants of herpes simplex virus. Of seven mutants tested, two, carrying mutations in the immediate-early ICP4 and ICP27 proteins, were complemented. This complementation was not seen in coinfections with adenovirus type 5 or cytomegalovirus. Following transfection into CV-1 cells, a DNA fragment containing the VZV short repeat sequence complemented the ICP4 mutant. These data demonstrate a functional relationship between VZV and herpes simplex virus and have allowed localization of a putative VZV immediate-early gene.

Varicella-zoster virus (VZV), the causative agent of chicken pox and shingles, contains a linear double-stranded DNA approximately 125,000 base pairs in size (14). The VZV genome, as determined by restriction endonuclease analysis (8, 15, 16, 29) and DNA sequencing (4, 5, 9), comprises short and long unique regions and repeat elements. The long unique region (U_L) of approximately 100,000 based pairs is flanked by an inverted repeat sequence of 88.5 base pairs. A short unique sequence (U_S) of 5,232 base pairs is surrounded by larger inverted repeat sequences of 7,320 base pairs.

In contrast to herpes simplex virus (HSV), VZV infectivity in cell culture is predominantly cell associated, and only relatively low titers of cell-free virus can be prepared. This has hampered the identification and mapping of specific VZV gene functions. However, DNA sequence analysis has suggested a number of possible open reading frames for VZV genes (4, 5, 7, 9). Furthermore, the VZV RNA transcript map has identified 58 discrete transcripts ranging in size from 0.8 to 6.5 kilobases (kb) (22). Yet only two VZV proteins have been definitively mapped; one is a viral envelope glycoprotein (gpI) (6, 18) and the other is the thymidine kinase (deoxypyrimidine kinase) gene (25).

Since the overall organization of VZV DNA is similar to that of HSV, there may also be functional similarities. This communication describes studies in which VZV was tested for its ability to complement well-characterized (26) temperature-sensitive (*ts*) mutants of HSV type 1 (HSV-1); VZV was found to complement two of these *ts* mutant strains.

Human embryonic fibroblasts (Flow 5000 cells) were used in the complementation studies. These cells were propagated in a 1:1 mixture of minimal essential medium and medium 199 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. The cells were seeded at near confluence into 75-cm^2 plastic flasks or 60-mm plastic plates. Twenty-four hours later, these cells were infected by addition of VZV-infected cells (VZV strain Ellen [28]) with a ratio of infected to uninfected Flow 5000 cells of 1:10. Twelve hours later, 2.5 PFU of wild-type HSV-1 (KOS) or one of seven *ts* mutants (HSV strains courtesy of P. A. Schaffer) per cell were added. The cultures were then maintained at the nonpermissive temperature of 39°C for an additional 24 h. Control cultures were infected with HSV in the absence of VZV or with VZV alone. Cultures were harvested by scraping, subjected to three cycles of freezing and thawing, and then clarified by centrifugation at 900 × *g* for 10 min. Virus yields were determined by plaquing on Vero (African green monkey kidney) cells at the permissive temperature of 34°C.

For each experiment, a complementation index (CI) (Table 1) was used to compare the yield from the VZV-HSV coinfection to the sum of yields from separate HSV and VZV control infections by analogy to complementation studies involving pairs of HSV mutants (26). Since V''V-infected cultures release relatively small amounts of ce -free virus and since VZV does not grow well in Vero cells, controls infected with VZV alone yielded no plaques when harvested and plated on Vero cells.

The HSV mutants used carried mutations in seven different complementation groups. For each HSV strain, the CI listed in Table 1 represents the mean of at least two experiments. The value for *ts*B21 of 82.7 ± 22.0 (standard error) represents a mean of five determinations and indicates a significant augmentation of yield by coinfection with VZV. Additionally, the value of 3.17 ± 0.44 for *ts*E6 also represents a mean of five determinations and is statistically significantly greater than that observed for wild-type HSV-1, although this difference is less compelling than that seen with *ts*B21. All other mutants tested, involving mutations in both early and late proteins, were characterized by CIs near 1. Based on these studies, we conclude that VZV is able to complement *ts*B21 and *ts*E6. In experiments testing the ability of HSV *ts* mutants to complement each other, CIs greater than 10 are considered positive, values less than 2 are considered negative, and values between 2 and 10 are intermediate and possibly reflective of intragenic complementation (11).

To assess whether recombination between HSV and VZV was occurring, plaque numbers were examined at the nonpermissive temperature of 39°C. A significant difference in HSV yield between VZV-HSV coinfections and controls at this temperature would provide evidence for recombinant VZV-HSV progeny. For each virus strain, titers were low in all cases (less than 10 PFU/ml), and no difference between coinfections and controls was found.

Determination of yield at various times after infection with *ts*A24 (a noncomplemented virus) demonstrated a progres-

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TABLE 1. Results of coinfections with VZV and various HSV *ts* mutants

HSV strain	Mutation	Map position (reference)	CI[a] (mean ± SE)
KOS	None (wild type)		1.01 ± 0.22
*ts*A24	DNA-binding protein	0.398–0.413 (31)	1.48 ± 0.04
*ts*B21	ICP4	0.988–1.000 (13)	82.7 ± 22.0[b]
*ts*C7	DNA polymerase	0.413–0.420 (3)	1.18 ± 0.25
*ts*D9	DNA polymerase	0.422–0.428 (3)	1.14 ± 0.01
*ts*E6	ICP27	0.753–0.761 (24)	3.17 ± 0.44[b]
*ts*G8	Major capsid protein	0.235–0.272[c]	0.98 ± 0.35
*ts*J12	Glycoprotein B	0.357–0.360 (12)	1.50 ± 0.46

[a] Yield from VZV-HSV coinfection at 39°C/yield from HSV infection alone at 39°C + yield from VZV infection alone at 39°C.
[b] Significantly greater than the value for KOS, $P < 0.001$ (two-tailed t test).
[c] S. K. Weller, personal communication.

sive decline in virus titer after 24 h; coinfection with VZV did not lead to an appreciable increase in yield at 36, 48, or 72 h (data not shown). Thus, complementation for this mutant is not time dependent. For mutants *ts*B21 and *ts*E6, the degree of complementation (CI) at 36 h was greater than that seen at 24 h due to a decrease in yield in the noncoinfected dishes.

The mutation in *ts*B21 affects the 175-kilodalton (kDa) immediate-early protein ICP4, which is encoded by a 4.2-kb transcript from the short repeats of HSV-1 (1, 21). VZV

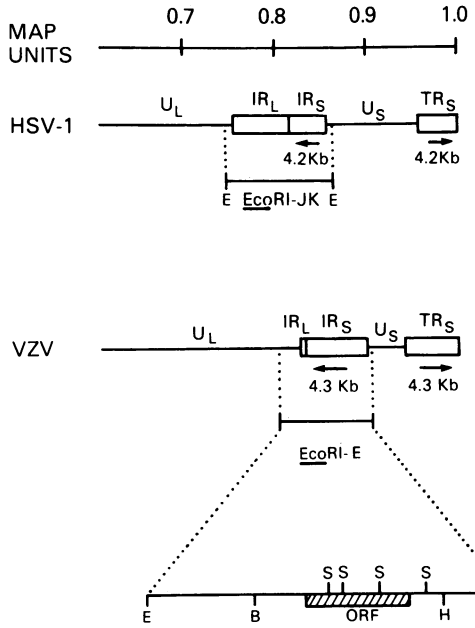

FIG. 1. Comparison of the right-hand portions of the HSV (I_L [inversion of L component prototype] configuration) and VZV genomes. The locations of the 4.2-kb ICP4 transcript of HSV and the corresponding 4.3-kb transcript of VZV are shown (arrows) relative to the internal and terminal short repeat elements (IR_S, TR_S). Plasmid pSG1 containing the *Eco*RI JK fragment of HSV-1 (map coordinates 0.744 to 0.865) in pBR325 includes a copy of the ICP4 gene. Plasmid pVZVEcoE contains the *Eco*RI E fragment of VZV in pBR325 and also contains the gene region equivalent to ICP4, as determined by sequence and transcript mapping data (22). The locations of restriction sites for the enzymes *Eco*RI (E), *Sal*I (S), *Bgl*II (B), and *Hpa*I (H) within the VZV fragment as well as the location of the 140-kDa open reading frame (shaded area) are based on published sequence data (9). Hatched box, Open reading frame (ORF).

transcript mapping studies have localized a 4.3-kb transcript to the short repeat sequence (Fig. 1) corresponding to the site of the HSV 4.2-kb transcript (22). This VZV DNA sequence contains an open reading frame that can encode a polypeptide of at least 140 kDa (9). DNA sequence data predict that three separate regions of the HSV ICP4 and the VZV equivalent protein have significant amino acid homology. Two of these regions, one of 28 amino acids and the other of 187 amino acids, have approximately 50% homology. A third region mapping near the carboxy-terminal end of the protein shows 54% homology over 442 amino acids, with many long domains of nearly 100% homology (7). Additionally, molecular hybridization studies between VZV and HSV-1 DNA have indicated some limited DNA homology in this region (10).

The mutation in *ts*E6 involves the 63-kDa immediate-early protein ICP27 (24). VZV DNA sequence data indicate that VZV may also encode a similar protein from a structurally related region of U_L (A. Davison, personal communication). However, VZV complementation involving this mutant did not occur at the level seen with *ts*B21. It is possible that the VZV equivalent to ICP4 is more closely related to its HSV counterpart than is ICP27, although biological factors such as an additional mutation in *ts*E6 could also lead to a low CI.

The lack of complementation seen with mutations involving the major DNA-binding protein, DNA polymerase, major capsid protein, and glycoprotein B suggest that the equivalent gene products in VZV may be less closely related to those of HSV. It is noteworthy that *ts*J12, a glycoprotein B mutant, was not complemented, despite recent data showing that a cross-reactive monoclonal antibody can recognize both glycoprotein B and a 63-kDa VZV envelope glycoprotein (17).

To exclude the possibility that a nonspecific mechanism involving VZV activation of cellular or viral genes underlies the VZV-induced complementation reported here, studies were conducted to examine the ability of both adenovirus and cytomegalovirus (CMV) to complement some of the HSV *ts* mutants (Table 2). Wild-type adenovirus can activate transcription from the promoters of HSV-1 early genes (20) and cellular genes (30). This activation appears to be mediated by the product of the E1A gene, a protein that is expressed early in infection and regulates the transcription of later genes.

In these experiments, confluent Flow 5000 cells were coinfected with 2.5 PFU of an HSV strain and adenovirus type 5 (courtesy of H. S. Ginsberg) per cell and incubated at

TABLE 2. Results of coinfections with HSV *ts* mutants and adenovirus type 5 or CMV

Coinfecting virus (PFU/cell)	HSV strain	CI[a]
Adenovirus		
2.5	KOS	0.98
25.0	*ts*B21	1.22
2.5	*ts*B21	0.94
2.5	*ts*E6	0.86
2.5	*ts*J12	1.03
CMV		
2.5	KOS	0.77
2.5	*ts*B21	1.48
2.5	*ts*E6	0.83
2.5	*ts*J12	0.90

[a] Calculated as described in Table 1, footnote *a*.

39°C for 24 h. For CMV studies, cells were infected with human CMV (strain AD169, courtesy of A. Rook) 24 h prior to coinfection with an HSV strain and then incubated at 39°C for an additional 24 h. All cultures were harvested, and titers were determined as described above. Yields from virus coinfections were again compared with the sum of yields from control infections involving separate viruses. No CI value greater than 2 was seen. Control cultures infected with adenovirus alone (2.5 PFU/cell) or CMV alone (2.5 PFU/cell) at 39°C yielded no plaques when harvested and replated on Vero cells with our plaquing system. Additionally, *ts*B21 did not grow better on 293 cells (these cells constitutively express the E1A gene) than on Vero cells at 39°C (data not shown). Together, these experiments argue against a nonspecific mechanism of activation being responsible for complementation of *ts*B21.

It is known that the HSV ICP4 has more functions than that demonstrated in *trans* activation assays and plays an essential role throughout the viral replicative cycle, regulating the expression of immediate-early, early, and late genes (11, 20). Similarly, the lack of complementation seen with CMV would argue that under these experimental conditions CMV immediate-early gene expression is unable to complement *ts*B21, even though a cloned CMV fragment containing the major immediate-early gene was able to activate transcription from an HSV-1 early promoter (19). The major CMV immediate-early protein differs in size from ICP4 (75 versus 175 kDa [27, 32]) and is a relatively weak activator of HSV-1 early gene transcription (19).

To further locate the gene responsible for the complementation of *ts*B21 by VZV, we transfected plasmid DNAs (containing the HSV ICP4 or the putative corresponding VZV gene) into CV-1 cells and then infected them with *ts*B21 at 39°C (Tables 3 and 4, Fig. 1). The ability of transfected cloned HSV DNAs to complement *ts*B21 and KOS1.1 *ts*18, a DNA-binding protein mutant, has been reported by others (23, 24). Transfection of the HSV-1 DNA plasmid pSG1 (Fig. 1) into CV-1 cells led to a 5- to 25-fold increase in yield of *ts*B21. When the plasmid pVZVEcoE was used, an approximately 3- to 11-fold increase in yield was observed, indicating that this region of the VZV genome can complement *ts*B21 and providing further evidence that the VZV ICP4 equivalent is located in this region. The DNA-binding protein mutant *ts*A24 was not complemented by these two fragments, as expected. In an additional experiment, a plasmid containing the *Eco*RI-B (map units 0.39 to 0.51) fragment of VZV was unable to complement *ts*B21 under similar conditions (data not shown).

TABLE 3. Complementation of *ts*B21 by transfection with cloned DNA fragments[a]

HSV mutant	Expt	Plasmid	Virus yield (PFU/ml)	Fold increase
*ts*B21	1	pBR325	3.5×10^2	1.0
		pSG1	7.6×10^3	21.7
		pVZVEcoE	4.0×10^3	11.4
	2	pBR325	4.5×10^3	1.0
		pSG1	3.3×10^4	7.3
		pVZVEcoE	1.7×10^4	3.8
*ts*A24	1	pBR325	4.7×10^3	1.0
		pSG1	4.1×10^3	0.87
		pVZVEcoE	4.6×10^3	0.98
	2	pBR325	2.1×10^4	1.0
		pSG1	1.6×10^4	0.76
		pVZVEcoE	1.5×10^4	0.71

[a] CV-1 cells were seeded (6×10^5 cells per dish) and after 24 h were transfected in duplicate with 0.5 μg of plasmid DNA and 10 μg of carrier DNA (salmon sperm) in 0.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline. Four hours later, cells were treated with 15% glycerol in HEPES-buffered saline for 2 min. The cells were infected 24 h later with the indicated virus at a multiplicity of 2 PFU/cell. After 18 h at 39°C, cultures were harvested and titers were determined.

TABLE 4. Complementation of *ts*B21 by transfection with endonuclease-digested pVZVEcoE[a]

Plasmid	Digestion	Expt 1		Expt 2	
		Yield (PFU/ml)	Fold increase	Yield (PFU/ml)	Fold increase
pBR325		3.1×10^2	1.0	8.5×10^2	1.0
pSG1		4.4×10^3	14.2	4.6×10^3	5.4
pVZVEcoE		1.5×10^3	4.8	2.5×10^3	2.9
pVZVEcoE	*Eco*RI	1.3×10^3	4.2	2.0×10^3	2.4
pVZVEcoE	*Sal*I	3.5×10^2	1.1	1.0×10^3	1.2
pVZVEcoE	*Bgl*II-*Hpa*I	9.3×10^2	3.0	2.1×10^3	2.5

[a] Plasmid pVZVEcoE was digested with restriction enzymes for 1 h, followed by phenol and chloroform extractions and precipitation with ethanol. The resulting DNA was then used for transfection by the method outlined in the text. For each experiment, the increase in yield relative to that with pBR325 is noted.

To localize the VZV DNA sequences responsible for complementation, pVZVEcoE was digested with various restriction enzymes (Table 4). Digestion with *Eco*RI separated plasmid DNA from the 12.1-kb *Eco*RI E fragment; no substantial loss of complementing activity occurred. The enzyme *Sal*I has five recognition sites within the short repeat sequence (Fig. 1), including three within the 140-kDa open reading frame that may encode the VZV ICP4 equivalent (9). Digestion with this enzyme abolished the complementing activity. Double digestion with *Bgl*II and *Hpa*I led to fragments of 6.5 and 11.5 kb. The 140-kDa open reading frame was contained entirely within the 6.5-kb fragment (Fig. 1). Two other open reading frames with coding capacities of 20 and 30 kDa were found in the short repeat sequence, with the *Hpa*I site located within the latter (9). Complementing activity remained after digestion with *Bgl*II and *Hpa*I. Although the 20-kDa open reading frame was left intact by the double digestion, none of the *Sal*I sites were present in this sequence or in the U_L portion of pVZVEcoE. Therefore, abolition of complementing activity by *Sal*I digestion argues against the presence of complementing genes in these locations. Together, these results localize the complementing activity to the 6.5-kb fragment which contains the entire 140-kDa open reading frame.

The complementation data presented here represent functional evidence for the relatedness of HSV and VZV and provide a technique by which additional VZV genes may be mapped. Among the genes investigated, the strongest candidates for evolutionarily conserved genes between these two viruses are the VZV genes analogous to ICP4 and ICP27. For the ICP4 gene, the technique involving complementation by cloned gene fragments has localized the complementing activity to a specific region of the VZV genome (Fig. 1).

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