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Herpes Simplex Virus Type 1 Immediate-Early Protein Vmw110 Reactivates Latent Herpes Simplex Virus Type 2 in an In Vitro Latency System

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Reactivation of latent herpes simplex virus type 2 (HSV-2) by the immediate-early protein Vmw110 was studied by using an in vitro latency system. Adenovirus recombinants that express Vmw110 reactivated latent HSV-2. An HSV-1 mutant possessing a deletion in a carboxy-terminal region of Vmw110 reactivated latent HSV-2, whereas mutant FXE, which has a deletion in the second exon, did not. Therefore, Vmw110 alone is required to reactivate latent HSV-2 in vitro, and the region of Vmw110 defined by the deletion in FXE is important for this process.

Infection of an organism with herpes simplex virus (HSV) is usually followed by latency, a state in which viral DNA is retained in neuronal, and possibly other, tissues of the host (19, 27). In mouse model systems, latent DNA is found in an end-less form, probably as an episome, in brain stem and trigeminal ganglia after corneal inoculation (2, 10, 15). Viral gene expression in these tissues is restricted to the latency-associated transcript (LAT) derived from the long repeat region of the genome (14, 18, 20). The 3′ portion of LAT is complementary to the 3′ end of the mRNA encoding the immediate-early protein Vmw110 (otherwise named ICP0), suggesting that LAT might act as an antisense transcript to disrupt the expression of Vmw110 (20, 22, 23).

Although animal models reproduce certain aspects of HSV latency found in humans, some detailed experimental approaches are not available because of the complexity of the in vivo system. Tissue culture latency systems have been developed to attempt to overcome this limitation (16, 24–26). Infection of human fetal lung (HFL) cells with HSV type 2 (HSV-2) at the supraoptimal temperature of 42°C results in the establishment of a latent state that is stable upon downshift of cultures to 37°C (16). Latent virus can be experimentally reactivated at high efficiency by superinfection with HSV or human cytomegalovirus but not with adenovirus (16) or the HSV-1 mutant *dl*1403, which has a large deletion in the immediate-early gene that specifies Vmw110 (17, 21). These experiments indicated a role for Vmw110 in reactivation but left open two questions. First, the role of LAT was unclear, since the deletion in *dl*1403 removed large portions of both Vmw110 and LAT (21); and second, it was not possible to determine whether Vmw110 acted alone or in combination with other immediate-early or virion proteins. To resolve these issues, reactivation was investigated by using HSV-1 mutants with more precise deletions in Vmw110 (5) together with adenovirus recombinants that express Vmw110 upon infection of tissue culture cells (28). The adenovirus-Vmw110 recombinants were produced by cloning the Vmw110 coding sequences into the adenovirus type 5 (Ad5) E1 region under the control of either the Vmw110 promoter or the adenovirus major late promoter to yield recombinants 0_{PRO}-0 and MLP-0, respectively. Both recombinants expressed functional Vmw110 after infection of HeLa or 293 cells but were able to replicate only in 293 cells, which supply E1A gene products in *trans* (28).

The in vitro latency system described previously (16) was used. HFL cells were infected with 0.03 or 0.003 PFU of HSV-2 per cell and maintained at 42°C for 6 days, with a change of medium every 2 days. Monolayers were then transferred to 37°C, medium was changed after 2 days, and cells were infected or mock infected after a further 2 days. Cells were harvested after incubation at 37°C for 3 days (when extensive cytopathic effect was visible) and sonicated to release virus. Sonic extracts were either titrated on BHK cells or used to infect BHK cells for subsequent DNA analysis. DNA was extracted from cytoplasmic fractions of infected BHK cells, cleaved with *Hin*dIII, electrophoresed on 0.6% agarose gels, and transferred to GeneScreen Plus membranes (Dupont, NEN Research Products, Boston, Mass.) (17). Blots were probed with either HSV-2 *Hin*dIII fragment L or a 655-base-pair fragment derived from a 3′ portion of the HSV-2 glycoprotein G gene that has no homolog in the HSV-1 genome (9) and thus provides an HSV-2-specific probe.

HFL monolayers containing latent HSV-2 were superinfected with various viruses, and cell lysates were titrated on BHK cells (Table 1). As shown previously (16), HSV-2 was reactivated efficiently by superinfection with the HSV-1 mutant *ts*K or with human cytomegalovirus. Superinfection with the adenovirus recombinant 0_{PRO}-0 or MLP-0 also reactivated latent HSV-2, although overall yields of HSV were lower. Ad5 *dl*E1,3 (7) (kindly supplied by F. Graham), an appropriate control since 0_{PRO}-0 is deleted for E1 and MLP-0 is deleted for both E1 and E3, failed to reactivate latent HSV-2. Upon superinfection with Ad5, one plate in five contained a low level of HSV-2. This probably resulted from the infrequent spontaneous reactivation that has been documented previously (16), although it is also possible that Ad5 can reactivate HSV-2 at a low frequency that was not detected in earlier studies. Therefore, Vmw110, expressed by the adenovirus recombinants, can reactivate latent HSV-2 in the absence of any other known HSV proteins.

To analyze further the functional domains of Vmw110 that

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TABLE 1. Reactivation of latent HSV-2

Superinfecting virus[a]	No. of reactivated cultures[b]	Mean titer (PFU/ml)[c]
Mock	0	
Ad5	1	1.0×10^2
Ad5 *dl*E1,3	0	
HSV-1 *ts*K	5	1.1×10^5
HCMV (AD169)	5	1.1×10^5
Ad5 MLP-0	5	7.6×10^3
Ad5 0_{PRO}-0	5	2.8×10^3

[a] Cultures were infected with 0.003 PFU of HSV-2 per cell and superinfected with different viruses at a multiplicity of infection of 0.1 PFU per cell. Superinfection with *ts*K was carried out at 38.5°C. HCMV, Human cytomegalovirus.

[b] Total number of cultures in each case was 5.

[c] Superinfected HFL cell monolayers were harvested, sonicated, and titrated on BHK cells.

are important for reactivation, HSV-1 mutants containing small deletions in Vmw110 were examined. Mutant FXE lacks 45 amino acids which span a potential metal-binding site, whereas D14 has a deletion of 41 amino acids from the C-terminal region (4, 5) (Fig. 1). HFL cell monolayers containing latent HSV-2 were superinfected with wild type (wt) HSV-1, *dl*1403, D14, or FXE, and reactivation was analyzed by Southern hybridization. Figure 2A shows hybridization of HSV-2 *Hin*dIII fragment L to DNA from progeny virus after reactivation in HFL cells and growth in BHK cells. As expected, wt HSV-1 reactivated HSV-2 over a 100-fold range of superinfecting multiplicities of infection, whereas *dl*1403 did not (Fig. 2A, lanes 1 through 4 and 6 through 9, respectively). The presence of the HSV-1 *Hin*dIII fragments B, E, G, and N (which cross-hybridize with HSV-2 *Hin*dIII-L) confirmed that both wt HSV-1 and *dl*1403

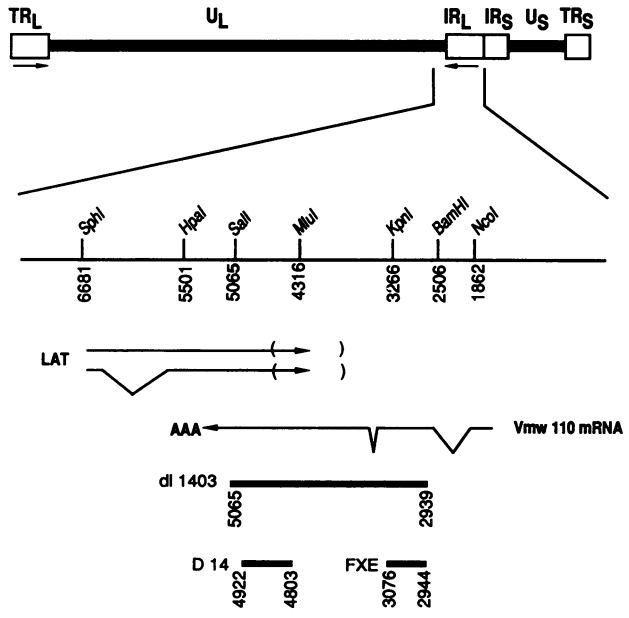

FIG. 1. Structure of HSV-1 mutants *dl*1403, D14, and FXE. Numbering of nucleotides starts at the junction of the internal long inverted repeat (IR_L) and the "a" sequence of strain 17 (13). The locations of Vmw110 mRNA and LAT are shown. Not all copies of LAT are spliced, and the position of the 3′ end is uncertain. Black boxes at the bottom represent the positions of the deletions.

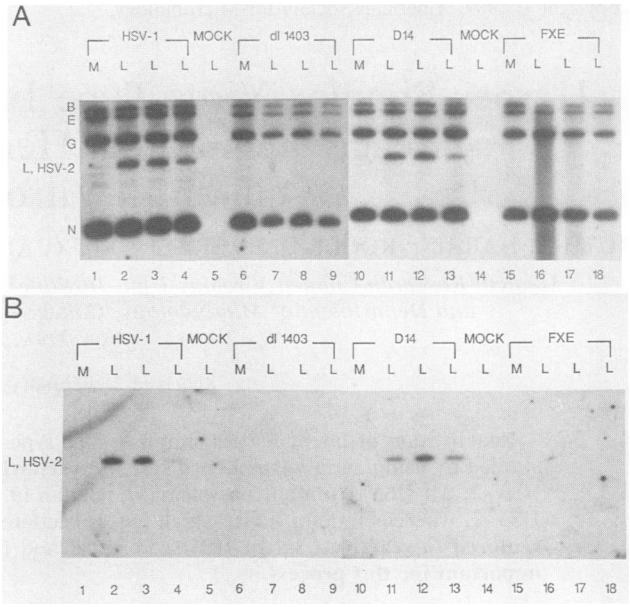

FIG. 2. Reactivation of latent HSV-2. HFL cells were initially infected with 0.03 PFU of HSV-2 per cell (lanes L) or mock infected (lanes M). Cultures were superinfected with 0.1 (lanes 2, 7, 11, and 16), 0.01 (lanes 3, 8, 12, and 17), or 0.001 (lanes 4, 9, 13, and 18) PFU of wt HSV-1, *dl*1403, D14, or FXE per cell. Four sets of cultures were mock infected and superinfected with 0.01 PFU of wt HSV-1 (lane 1), *dl*1403 (lane 6), D14 (lane 10), or FXE (lane 15) per cell, and two sets of cultures were infected with HSV-2 and mock infected (lanes 5 and 14). Progeny viruses were grown in BHK cells, and DNA was analyzed by Southern hybridization by using HSV-2 *Hin*dIII fragment L (A) or a 655-base-pair HSV-2-specific fragment (B) as probe. The positions of HSV-2 *Hin*dIII-L plus the cross-hybridizing HSV-1 *Hin*dIII fragments B, E, G, and N are indicated.

replicated during the course of the experiment. Mutant D14 also reactivated HSV-2, as shown by the presence of *Hin*dIII-L (Fig. 2A, lanes 11 through 13), but FXE did not (Fig. 2A, lanes 16 through 18), even though this virus itself replicated efficiently. Hybridization was repeated with a radiolabeled HSV-2-specific probe (Fig. 2B). In this case, as expected, minimal hybridization to HSV-1 bands was observed, but HSV-2 *Hin*dIII-L was detected in DNA samples from cultures superinfected with wt HSV-1 or D14 but not *dl*1403 or FXE.

The region of Vmw110 affected by the deletion in FXE must, therefore, be intact for reactivation of latent HSV-2 to occur.

The results demonstrate that production of LAT by the superinfecting virus is not necessary for reactivation and that Vmw110 is the only HSV gene product that is required for reactivation of HSV-2 in the in vitro latency system. Furthermore, the domain of Vmw110 deleted in FXE is involved. In transfection assays, Vmw110 is a potent transactivator of gene expression (3, 6, 12), but a plasmid bearing the FXE deletion was extremely deficient in this property (4), indicating that the events leading to reactivation are initiated by the activation of gene expression.

One of the remarkable features of the in vitro latency system is the insensitivity of the latent genome to reactivation upon superinfection with mutants lacking functional Vmw110. During superinfection with *dl*1403 or FXE, the immediate-early transactivator proteins Vmw175 (ICP4) and Vmw63 (ICP27) and the virion *trans*-inducing factor Vmw65 were readily available. In view of the demonstration (by

using 0_{PRO}-0 and MLP-0) that Vmw110 alone can reactivate latent HSV-2, it appears that the gene encoding HSV-2 Vmw118 (the homolog of Vmw110) is inaccessible to Vmw65, Vmw175, and Vmw63. This observation indicates that the promoter for the latent HSV-2 gene encoding Vmw118 (and, perhaps, others) is in a novel quiescent state, since Vmw65 can transactivate the homologous HSV-1 promoter when it is present in plasmids (11), transformed cells (8), or viral DNA (1). Reactivation therefore requires properties specific to Vmw110 that distinguish it from the other transactivators and enable it to act on the latent genome. Although the mechanism of transcription stimulation by Vmw110 is unknown, the protein is characterized by a lack of sequence specificity in the promoters that it activates during transfection assays. It is possible, therefore, that Vmw110 functions by increasing template availability in a relatively nonspecific manner, for example by displacing other bound proteins from a latent genome that is maintained in a state resembling inactive chromatin. A further possibility, however, is that Vmw110 acts on viral or cellular genes to trigger reactivation at a posttranscriptional stage. Further studies of the in vitro system should clarify the mechanism of action of Vmw110 and yield information relevant to the molecular basis of HSV latency.

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