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Truncation of the C-Terminal Acidic Transcriptional Activation Domain of Herpes Simplex Virus VP16 Produces a Phenotype Similar to That of the *in*1814 Linker Insertion Mutation

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We examined the phenotype of a herpes simplex virus (HSV) type 1 mutant (V422) in which the C-terminal acidic activation domain of the virion transactivator VP16 is truncated at residue 422. The efficiency of plaque formation by V422 on Vero cells was boosted by approximately 100-fold by including hexamethylene bis-acetimide (HMBA) in the growth medium, as previously observed with the *in*1814 VP16 linker insertion mutant isolated by Preston and colleagues. V422 displayed severely reduced levels of the immediate-early transcripts encoding ICP0 and ICP4 during infection in the presence of cycloheximide, and this defect was partially overcome by the addition of HMBA. The defect in plaque formation exhibited by V422 and *in*1814 was efficiently complemented in U2OS osteosarcoma cells, which had previously been shown to complement ICP0 null mutations. Taken in combination, these data confirm the key role of VP16 in triggering the onset of the HSV lytic cycle.

Herpes simplex virus (HSV) virions contain one or more factors that stimulate transcription of the viral immediate-early (IE) genes (27). Early studies demonstrated that the activity resides in the virion tegument (3) and that the major tegument protein VP16 activates IE promoters in transient cotransfection assays (7, 8). Intensive biochemical and molecular studies have since clarified the mechanism of action of VP16 as a transcriptional activator (reviewed in references 12 and 35); it bears a strong C-terminal acidic transcriptional activation domain (30, 36) and is targeted to the TAATGARATTC consensus sequence in IE promoters through interactions with the cellular proteins Oct1 and HCF (4, 5, 10, 11, 14–19, 21, 22, 24, 25, 28, 29, 33, 38, 39). VP16 also plays an essential but not yet defined role in virion assembly (1, 37) and binds to at least two other tegument proteins, the virion host shutoff protein vhs (31) and VP22 (9).

It seems plausible that the VP16 molecules delivered by the infecting virus particle play an important role in triggering the onset of IE transcription during HSV infection. Supporting this view, Preston and colleagues showed that a linker insertion mutation that disrupts the promoter-targeting function of VP16 (*in*1814) results in a greatly increased particle-to-PFU ratio in plaque assays and reduced levels of expression of some IE genes during infection at relatively high multiplicities (1, 2). The defect in plaque formation exhibited by *in*1814 is at least partially complemented by the drug hexamethylene bis-acetimide (HMBA [33]) or by expression of the IE protein ICP0 in *trans* (2). These data argue that loss of VP16 activation function greatly decreases the probability that cells infected with a single virus particle enter the lytic cycle and imply that VP16 plays a key role in launching IE transcription. However, in recent study using temperature-sensitive (*ts*) VP16 mutants, Poon and Roizman (26) were unable to detect any reduction in viral yields when VP16 was inactivated during the early phases of infection. Inasmuch as two of the *ts* mutants studied by Poon and Roizman displayed *ts trans*-inducing activity in vivo (R2604 and R2605), the results of their study could be regarded as conflicting with the conclusions of Ace et al. (2). In view of this apparent discrepancy, we examined the phenotype of a VP16 mutant which lacks the C-terminal activation domain and compared it to that of the *in*1814 linker insertion mutant.

We have previously described the isolation of V422, an HSV type 1 (HSV-1) strain KOS derivative in which the acidic activation domain of VP16 is truncated by an amber chain termination mutation following codon 422 (20). The mutant was generated by recombining a mutated VP16 gene into the VP16 locus of the VP16 null mutant 8MA (37). As previously described (20), V422 can replicate on noncomplementing Vero cells, but the virus stocks so produced exhibit a >100-fold-reduced titer in plaque assays on Vero cells compared to 8MAR (an 8MA rescue product which bears a wild-type VP16 gene). Standard plaque assays greatly underestimate the number of potentially infectious *in*1814 virions (2, 23). To determine if this is also the case with V422, we asked whether HMBA increased the number of plaques obtained in a plaque assay on Vero cells (Table 1). The apparent titers of V422 and *in*1814 stocks (grown on Vero cells) increased by approximately 100-fold when 3 mM HMBA was included in the plaque assay. In contrast, HMBA had no significant effect on the efficiency of plaque formation by 8MAR or the *in*1814 rescue product *in*1814R. Thus, V422 virions display a defect in initiating plaque formation comparable to that of *in*1814.

HSV isolates bearing null mutations in the ICP0 gene resemble VP16 mutants in that they display a greatly increased particle-to-PFU ratio and exhibit a relatively normal phenotype at high multiplicities of infection (34). Yao and Schaffer (40) recently reported that U2OS osteosarcoma cells complement ICP0 null mutants in a plaque assay. Inasmuch as previous studies have shown that expression of ICP0 in *trans* at least partially overcomes the *in*1814 defect (2), it seemed possible that the VP16 mutants display a relatively normal phenotype in these cells. Therefore, we asked if U2OS cells support efficient

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TABLE 1. Effects of HMBA and cell type on plaquing efficiency of VP16 and ICP0 mutants[a]

Virus	Titer with the following cell lines:			
	Vero	Vero + HMBA	U2OS	U2OS + HMBA
V422	2.6×10^5	2.2×10^7	6.4×10^7	1.4×10^8
8MAR	7.6×10^9	8.6×10^9	3.4×10^9	4.2×10^9
*in*1814	1.0×10^6	9.8×10^7	7.6×10^7	2.2×10^8
*in*1814R	2.6×10^8	2.8×10^8	1.5×10^8	1.5×10^8
*n*212 (ICP0$^-$)	5.8×10^6	5.0×10^6	2.0×10^8	1.8×10^8

[a] The titers of virus stocks were determined by plaque assays on Vero and U2OS cells in the presence and absence of 3 mM HMBA. The V422 and *in*1814 stocks were produced on Vero cells in the presence of HMBA, and *n*212 was grown on U2OS cells.

plaque formation by V422 and *in*1814 in the absence of HMBA (Table 1). Both mutants produced substantially more plaques on U2OS cells than on Vero cells; in contrast, the corresponding wild-type strains exhibited a twofold-lower titer on U2OS cells. The increase in apparent titer observed with U2OS cells was similar to that induced by HMBA in Vero cells (ca. 100-fold) and comparable to that observed with the ICP0-deficient mutant *n*212 (6). Moreover, HMBA had only a relatively small stimulatory effect on the VP16 mutants in U2OS cells (ca. twofold). Thus, U2OS cells complement the VP16 mutants to approximately the same degree to which they complement an ICP0 mutant in a plaque assay. Although the mechanism of this complementation is unclear, one interpretation is that the cellular ICP0-like function proposed by Yao and Schaffer (40) partially bypasses the requirement for VP16 activation function, perhaps by facilitating IE gene expression at low multiplicities of infection.

We examined the effect of the V422 mutation on accumulation of the IE transcripts encoding ICP4 and ICP0 during infection of Vero and U2OS cells. In order to eliminate the potentially confounding effects of ICP4-mediated repression of IE transcription and differences in rates of progression through the lytic cycle, the assay was conducted in the presence of cycloheximide to prevent viral protein synthesis. Cells were infected with 10 PFU of V422 per cell (titer determined on Vero cells in the presence of HMBA) and 8MAR in the presence and absence of 8 mM HMBA, and total cellular RNA harvested at 6 h postinfection was analyzed by primer extension as previously described (32). V422 exhibited an approximately 20-fold reduction in ICP4 and ICP0 RNA levels relative to 8MAR in both cell types (Fig. 1). HMBA partially corrected the defect of V422 (three- to fivefold increase) but had no significant effect on 8MAR. These data demonstrate that the V422 mutation strongly inhibits accumulation of ICP4 and ICP0 transcripts under conditions that prevent viral protein synthesis and confirm previous reports that HMBA increases IE transcript levels in the absence of VP16 activation function (23). Yao and Schaffer (40) found that transfected ICP4 promoters are more active in U2OS cells than in Vero cells. However, V422 did not display obviously increased levels of ICP4 or ICP0 transcripts in U2OS cells compared to Vero cells under the conditions of our assays. The basis for this apparent discrepancy is not yet clear but may relate to the relatively high multiplicity of infection used in our experiment.

The magnitude of the reduction in IE transcript levels observed in the foregoing experiment was substantially greater than that reported by Ace et al. (2) in their experiments with *in*1814, in which ICP0 RNA levels were reduced four- to fivefold and ICP4 RNA levels were normal during infection of

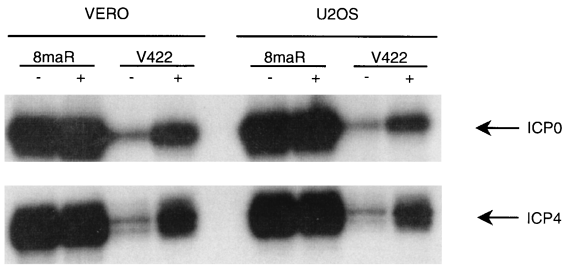

FIG. 1. ICP0 and ICP4 transcript levels during infection with V422. Vero and U2OS cells were infected with 10 PFU of V422 or 8MAR per cell in the presence of 200-μg/ml cycloheximide. An 8 mM concentration of HMBA was added to some cultures (+). Aliquots (10 μg) of total cellular RNA were then analyzed for ICP0 and ICP4 transcript levels by primer extension by using 5′ ^{32}P-labelled 25-mers. Primer extension products were resolved on an 8% sequencing gel. The ICP0 primer (5′-CGTAGGCGGGGCTTCTGTGGTGATG-3′) is complementary to residues 71 to 95 of ICP0 mRNA; the ICP4 primer (5′-CGAGCGTCT GACGGTCTGTCTCTGG-3′) is complementary to residues 49 to 73 of ICP4 mRNA.

BHK21 cells. Therefore, we compared the effects of the V422 and *in*1814 mutations on the accumulation of ICP0 and ICP4 transcripts under our experimental conditions. Vero cells were infected with 10 PFU of V422, *in*1814, 8MAR, and *in*1814R per cell in the presence of cycloheximide, and ICP0 and ICP4 transcript levels were examined by primer extension (Fig. 1). Signal intensities were then quantified by PhosphorImager analysis, and the results for each mutant were expressed as fold reduction relative to the corresponding wild-type strain (Table 2). The results demonstrate that *in*1814 displays a readily detectable defect in the accumulation of both ICP0 and ICP4 transcripts during infection of Vero cells in the presence of cycloheximide. We observed substantial variation in the wild-type-to-mutant ratios with each mutant over the course of three experiments (Table 2). However, in each experiment V422 displayed a defect greater than that of *in*1814. It is not clear whether this apparent difference stems from variation between HSV1 strains (KOS versus 17) or from the predicted ability of the V422 polypeptide to bind IE promoters (13) and perhaps to interfere with promoter function.

Taken in combination, these results provide strong support for the hypothesis that VP16 activation function stimulates IE gene expression during virus infection, thereby greatly increasing the probability that cells infected with a single HSV particle enter the lytic cycle (2). Why then did Poon and Roizman (26) observe no reduction in viral yields when VP16 was transiently inactivated during the early phase of infection with the R2604 and R2605 *ts* mutants? Although the explanation is not clear, one possibility is that the VP16 mutations in these isolates

TABLE 2. Fold reduction in IE transcript levels relative to wild-type level[a]

Expt. no.	Fold reduction			
	V422		*in*1814	
	ICP0	ICP4	ICP0	ICP4
1	48	65	20	18
2	24	35	6	5
3	76	106	7	14

[a] The levels of ICP4 and ICP0 transcripts produced by V422 and *in*1814 in the presence of 200-μg/ml cycloheximide were determined by primer extension and were expressed relative to the levels obtained with the corresponding wild-type strains in the same experiment.

partially impair VP16 activation function in vivo at the permissive temperature (33°C), thereby increasing the particle-to-PFU ratio relative to that of wild-type virus. Indeed, these mutations strongly reduced complex formation on TAATGA RATTC at 33°C in an in vitro assay (26). If so, then inhibitory effects on entry into the lytic cycle might have been masked at the relatively high multiplicities of infection used (1 PFU/cell, based on titers determined in the absence of HMBA).

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