

CONSTRUCTION AND CHARACTERIZATION OF HSV-1 MUTANTS
ENCODING TRUNCATED VP16 IN THE PRESENCE AND ABSENCE OF
FUNCTIONAL VHS.

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Abstract

VP16 is an integral element in the HSV-1 strategy to overturn normal cellular functions and use the host machinery for the production of new virus. VP16 enhances the expression of viral immediate-early genes in the nucleus of the infected cell. By binding to the virion host shutoff protein (vhs), VP16 is able to downregulate its activity and prevent destruction of the viral transcripts. Finally, VP16 has an essential but specifically undetermined role in virus assembly.

While a null mutation in VP16 correlates to a lethal defect in virus assembly, recent work has revealed that the C-terminal acidic activation domain of VP16 is not essential to virus survival. Viral mutants containing 422 and 379 amino acid tail-deleted derivatives of VP16 are viable.

We have constructed a viable viral mutant which encodes a 369 amino acid VP16, revealing that the N-terminal 369 amino acids of VP16 retain a function which is essential to virus survival. The viral mutants encoding truncated VP16 derivatives displayed a characteristic preference for growth on a cell line which supplied full length VP16 in *trans*. Furthermore, the preference for growth on the complementing

cell line became more apparent as VP16 was progressively truncated from the C-terminus.

To determine if full length VP16 was aiding these viruses in attenuating vhs activity or in performing a structural role in the presence of vhs, we constructed double mutant viruses encoding the truncated VP16s and containing an inactivating mutation in the vhs locus (Δ Sma). The elimination of vhs activity and/or vhs-VP16 binding alleviated the preference for growth on the complementing cell line in these viruses. Thus, while the acidic activation domain of VP16 is not essential for viral replication in tissue culture, it clearly confers a growth advantage to the virus. The viral mutants constructed here will prove useful in understanding the significance of the interaction between VP16 and vhs.

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s.a.	amino acid(s)
AD	activation domain
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BrdC 5-Bromo-2'-Deoxycytidine

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CTD carboxy-terminal domain

Da dalton

DBD DNA binding domain

DNA deoxyribonucleic acid

dsDNA double stranded DNA

EDTA ethylenediaminetetraacetic acid

EHV equine herpes virus

FBS fetal bovine serum

HEPES N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

HMA hexamethylene bisacetamide

hrs hours

HSV herpes simplex virus

IE immediate-early

Introduction

Herpes Simplex Virus Type 1

Herpesviruses are comprised of 4 structural elements: a core which contains the DNA molecule, a capsid of 162 identical capsomers surrounding the core, and an amorphous tegument region between the capsid and the outer envelope, the latter being derived from the host cell nuclear membrane. The genetic material of Herpes Simplex Virus type 1 (HSV-1) is 152 Kb of double-stranded DNA. The genome itself consists of 2 components, entitled the unique long (U_L) and the unique short (U_S) sequences, each of which are flanked by terminal and internal inverted repeat sequences (Figure 1). The genes encoded within the repeat regions of the genome are diploid and are involved in viral replication. Thus, they are essential for survival of the virus. Of note is the fact that the five immediate-early genes of the virus (some of which are essential) are

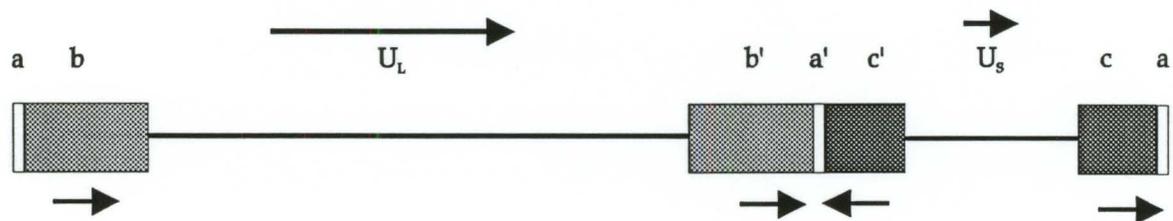


Figure 1:

The HSV-1 genome is comprised of two segments, designated long and short. Each segment contains a unique sequence (U_L and U_S) flanked by a pair of inverted repeats (eg. b, b'). There is a terminal redundancy, the 'a' sequence, which is a stretch of approximately 400 base pairs (adapted from McGeoch et al., 1988).

clustered around this region, perhaps to ensure adequate levels of expression (Steiner and Kennedy, 1993). HSV-1 fuses its envelope to the plasma membrane of the cell with the aid of interactions between its surface glycoproteins (gB and gC) and receptors on the cell surface (Ward and Roizman, 1994). The nucleocapsid of the virus enters the cell and travels to the nuclear pore where the viral DNA is released into the nucleus. Along with the nucleocapsid certain pre-existing viral proteins enter the cell. Two of the most important are the virion host shutoff protein (vhs) and VP16, a potent transactivator of the immediate-early genes of the virus. The virion host shutoff protein is responsible for effecting a shutdown of the host cell gene expression, enabling the virus to take over the host machinery to express its own genes (Strom and Frenkel, 1987). VP16 acts in the nucleus of the cell, initiating a tightly regulated cascade of viral gene expression, by stimulating the expression of the five viral immediate-early genes. The three classes of HSV-1 genes; immediate-early, early and late are expressed in a coordinated fashion and in general, the products of each class are a prerequisite for expression of the next temporal class. The immediate-early

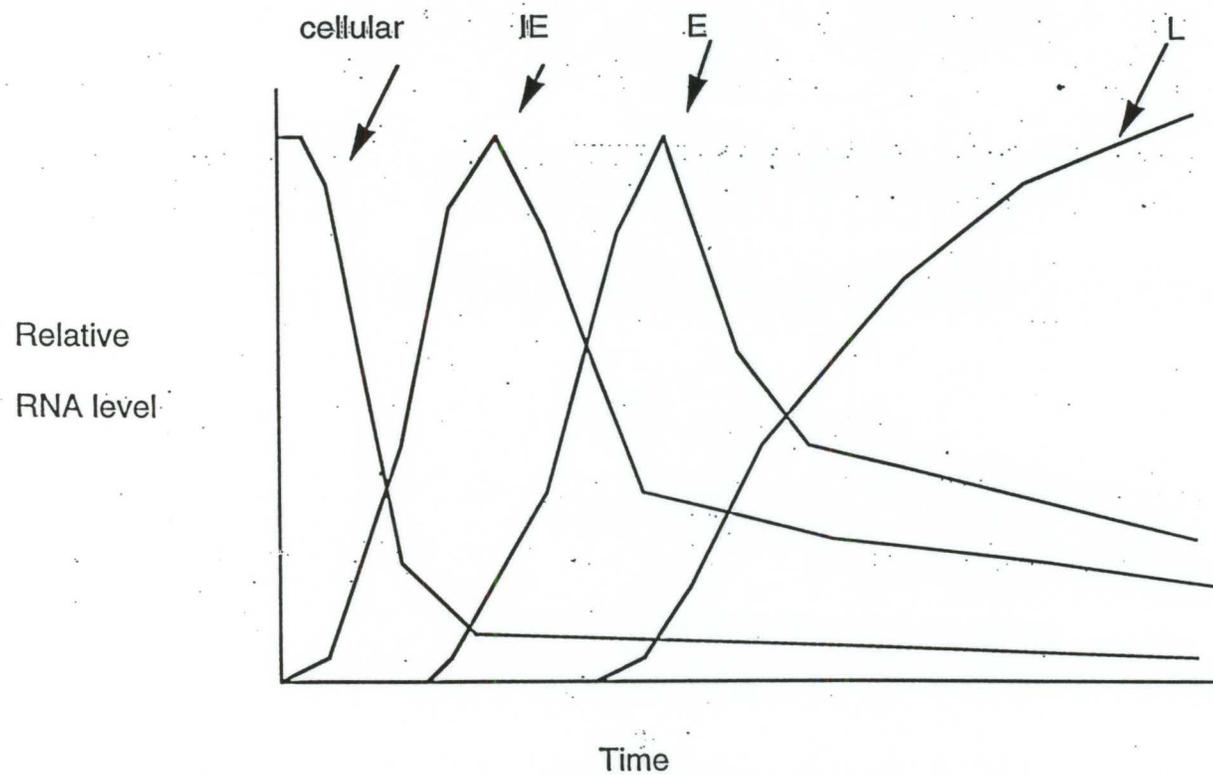


Figure 2:
Host and Viral RNA levels during infection by HSV-1.

genes have regulatory functions, the early proteins are largely involved in nucleic acid metabolism and the late proteins fulfil structural roles. VP16, a late protein, becomes incorporated into the tegument during virus assembly, which enables it to initiate the cascade of viral gene expression soon after it enters the infected cell. HSV-1 employs a strategy of tight control mediated via interactions between the viral proteins VP16 and vhs in appropriating the host cell gene expression machinery for its own use. Through vhs-induced termination of host transcription and translation coupled with the tight regulation of its own gene expression, HSV-1 is able to effect an efficient transition that diverts many cellular processes to the production of new virus particles (Figure 2).

HSV-1 Virion Host Shutoff Protein (vhs)

Infection by either HSV-1 or HSV-2 is characterized by a dramatic decline in the levels of host protein synthesis, due in part to the disruption of host polyribosomes (Sydiskis and Roizman, 1966). Early studies into this host shutoff phenomenon indicated that it was caused by a structural component of the infecting virion. Infection

with UV-light irradiated virus, or in the presence of cyclohexamide (which inhibits viral gene expression at the level of translation), still brought about the characteristic decline in host protein synthesis. As well, infection of enucleated cytoplasts resulted in rapid inhibition of protein synthesis. However, heat inactivation or neutralization with a virus specific antibody resulted in the elimination of this phenotype (Fenwick and Walker, 1978). Other early evidence into the rapid decline in host protein synthesis indicated that the overall event was a multistep process. The initial disaggregation of host polyribosomes was due to a viral component, however, the subsequent degradation of host mRNA required prior expression of viral genes (Nishioka and Silverstein, 1978).

Six virus mutants constructed by Read and Frenkel (1983) were determined to be defective in the virion-associated shutoff of host protein synthesis because they did not display the characteristic pattern of decline during infection in the presence of actinomycin D (which inhibits protein expression at the level of transcription). One of these virion associated host shutoff (*vhs*) mutants also exhibited increased stability of the viral alpha or

immediate-early transcripts, implying the existence of a viral regulatory function which curtails the expression of these genes. Of note was the fact that each of the mutant viruses were able to induce a secondary shutoff of host protein synthesis, in the absence of actinomycin D. This result strongly supported the contention that there were two facets to the virion host shutoff function: one which was virion associated and one which required the expression of viral genes. By constructing single step growth curves for the mutant *vhs1*, they observed that the overall yield of the mutant relative to wild-type virus was only reduced three-fold. In light of this, they concluded that the *vhs* function was not essential to virus replication on Vero cells. However, the presence of functional virion host shutoff activity does confer a selective advantage to the wild-type virus over *vhs1*, as evidenced by the rapid outgrowth by wild-type virus in mixed infections (Kwong et al, 1988).

A further consequence of infection with either HSV-1 or HSV-2, the enhanced rapid turnover of cellular mRNA following infection, was reported by Schek and Bachenheimer (1985). By monitoring the levels of select cellular mRNAs

during infection by HSV-1, they observed a decrease in the cytoplasmic levels of each as infection progressed. They noted that the rate of cellular mRNA degradation was faster during HSV-2 infection than HSV-1 infection, which paralleled earlier observations into the rate of decline of protein synthesis induced by the two viruses (Pereira et al, 1977). The *vhs1* mutant was unable to induce this characteristic degradation of mRNA, indicating that a viral factor must be responsible for this effect. Under conditions of inhibition of *de novo* protein synthesis, inhibition of *de novo* RNA synthesis and infection with purified virions in the presence of actinomycin D, they were able to observe the induction of cellular mRNA degradation upon infection. They concluded that the rapid turnover of cellular mRNA associated with HSV-1 and HSV-2 infection was caused by a component of the infecting virion and not as the result of new synthesis of viral proteins.

In analyzing the properties of selected host proteins during virus infection, they noted for example, that while actin synthesis had decreased by 68% in one hour post-infection, the levels of actin mRNA were down by only 7%. This led them to conclude that the overall shutdown of host

protein synthesis was the result of a combined effort which targeted both the disruption of translation and the degradation of mRNA (Schenk and Bachemheimer, 1985).

Further investigation into the effect of the virion associated host shutoff phenomenon revealed that it functioned to regulate the levels of viral mRNA, as well as lower the levels of host mRNA. The *vhs1* mutant resulted in an increase in half-life of three immediate-early viral mRNAs, in a cyclohexamide block reversal experiment. Cytoplasmic RNA was extracted from infected cells either immediately after the removal of cyclohexamide or after a 5 hour incubation in the presence of actinomycin D. In the cells infected with wild type virus, the levels of each immediate-early mRNA were highest after the removal of the cyclohexamide block. This was followed by a progressive decrease in the levels of each mRNA over the next 5 hours of incubation with actinomycin D. The *vhs1* mutant had higher levels of the immediate-early mRNAs after removal of the cyclohexamide block which further increased after the subsequent 5 hour incubation (Oroskar and Read, 1987). To determine the effects of the mutation carried by *vhs1* on the accumulation of other viral mRNAs, Kwong *et al*, monitored

the levels of mRNAs representative of each temporal class of viral genes during infection. A 265 base-pair region corresponding to the mutation in *vhs1* which resulted in the degradation of host mRNA, shutoff of host protein synthesis and the destabilization of viral immediate-early, early and late mRNAs (Kwong *et al.*, 1988). They predicted that all of these functions were mediated by the actions of a single viral gene. The product of gene UL41, a 58 kDa virion phosphoprotein, was identified as the factor responsible for mediating the effects of the virion host shutoff phenomenon and for regulating viral gene expression during infection. It was given the name vhs (virion host shutoff) protein (Fenwick and Everett, 1990; Smibert *et al.*, 1992).

Mechanism of Vhs Action

The mutant *vhs1* was isolated from a stock of virus which had been subjected to mutagenesis with 5-bromodeoxyuridine (Read and Frenkel, 1983). Cells infected with *vhs1* do not display the characteristic rapid decline in protein synthesis seen with wild-type HSV-1. There is, however, a secondary shutoff in host protein synthesis exhibited during infection with *vhs1* which is incomplete and delayed compared to wild-type (Kwong and Frenkel, 1987; Read

and Frenkel, 1983). Despite this, functional vhs is dispensable for virus growth in tissue culture, there being only a slight difference in growth between vhs1 and wild-type virus in Vero cells (Read and Frenkel, 1983). Another vhs mutant, termed Δ Sma, contains a deletion within the UL41 locus. Its effects on the virus resemble those incurred by the mutation in vhs1. Cells infected with Δ Sma do not display any evidence of host shutoff activity, and the expression of viral genes is unregulated as in the vhs1 mutant. The growth characteristics of Δ Sma infection are again very similar to vhs1, producing characteristically smaller plaques, and slightly lower levels of virus compared to wild-type HSV-1 on Vero cells (Read *et al.*, 1993). The crucial difference between the mutants vhs1 and Δ Sma is in the host shutoff phenotype seen during co-infection with wild-type virus. The mutant phenotype dominated when cells were co-infected with vhs1 and wild-type virus, but the wild-type host shutoff was observed when Δ Sma was co-infected with wild-type virus. The reason underlying this difference is that the vhs- Δ Sma protein is not packaged into assembling virus, although it is not yet known precisely why this is so. It is conceivable that the Δ Sma mutant is

disrupts host protein synthesis, or specifically how it causes mRNA degradation, have been complicated by the question of whether vhs acts directly, or with the aid of other viral or host factors (Jones *et al.*, 1995). Recent developments have indicated that the vhs protein may possess an intrinsic RNase activity (Zelus *et al.*, 1996). To eliminate the complication of accessory host factors contributing to the apparent RNase activity displayed by vhs, two approaches were taken. In the first, virion extracts were incubated with RNA and ribonucleoprotein (RNP) substrates. In these experiments, vhs dependent RNase activity required the presence of functional vhs within the virion extract. Neither the vhs1 nor Δ Sma virion extracts displayed this capability. Secondly, they used *in vitro* translated vhs and incubated RNA substrates with the translation product. Again, only lysates which contained functional vhs protein exhibited enhanced RNase activity. In each of these assays, antibodies directed against vhs abolished its activity. The RNase activity in the reaction mixtures was determined to be specific for mRNA (rRNA was spared) and thus, it was concluded that the vhs protein possesses highly specific intrinsic mRNAse activity. Unless

there is a co-factor common to the virion extracts and the reticulocyte lysate, it appears that the vhs protein can act independently. The model proposed by Zelus *et al*, suggests that vhs is a highly specific, low activity enzyme which cleaves host mRNA at certain sequences, making the mRNA more susceptible to destruction by more powerful endogenous RNases.

Vhs Interacts with the Virion Transactivator VP16

In attempting to identify proteins that might be involved in the regulation of vhs function during infection, another HSV-1 tegument protein, VP16, was found to interact specifically with vhs (Smibert *et al*, 1994). An investigation into the properties of this binding revealed that the transcriptional activation domain of VP16, which comprises its carboxy-terminal 80 amino acids, was dispensable for interaction with vhs. In fact, the interaction was sustained over progressive truncation down to the 369 N-terminal amino acids of VP16. Interestingly, vhs binding interfered with the ability of VP16 to recognize its native DNA binding site, indicating that it was binding VP16 on a region required for promoter recognition.

The residues between 238-344 amino acids of vhs were

initially shown to be sufficient for binding to VP16, but this has recently been narrowed down to a 21 amino acid sequence between residues 310 and 320. The critical residue required to sustain this interaction in vitro and in vivo within this region has been identified as tryptophan 321, however, mutation of surrounding residues significantly reduces the stability of the interaction (Schmelter *et al*, 1996).

The significance of this interaction during infection was recently proposed by Lam *et al* (1996), who demonstrated that VP16 downregulates vhs activity. The effects of vhs activity on host protein synthesis are well documented, and it is clear that the viral messages would suffer the same fate if VP16 did not intervene. The 8MA virus, which lacks VP16, displays a dramatic decline in protein synthesis and viral mRNA levels during infection which brings about an abrupt end to its translational activity. Several results demonstrate that this phenotype can be rescued and that it is VP16 which attenuates vhs activity, permitting the expression of viral transcripts. A revertant of 8MA, termed 8MAr displays nearly wild-type production of viral proteins, while an 8MA recombinant virus encoding a truncated 422

amino acid VP16, is also able to produce viral proteins, albeit at a lower level. Finally, if the vhs locus is inactivated within the context of the 8MA virus, as in the double mutant 8MA Δ Sma, viral transcripts are again saved. The manner in which VP16 downregulates vhs remains to be elucidated. However, because VP16 is in great molar excess of vhs within the cell, it is conceivable that the interaction between the two tegument proteins also functions to ensure proper packaging of vhs into new virus.

The Virion Transactivator VP16

The product of the gene UL48 is VP16 (also α -TIF, Vmw65, ICP25), a 490 amino acid protein. VP16 is a major component of the virus tegument, present in excess of 1000 copies (Roizman and Spector, 1991). The protein can be roughly broken down into two domains. The carboxy-terminal approximately 80 amino acids comprise a potent transcriptional activation domain (Greaves and O'Hare, 1989; Triezenberg *et al*, 1988). The acidic tail of VP16 can independently enhance transcription of an adjacent open reading frame when tethered to DNA by a heterologous DNA binding domain (Cousens *et al*, 1989). Though the acid tail of VP16 is generally only considered for its transcriptional

activation function, recent study revealed that it was required for a specific interaction with a structural protein, VP22 (Elliot et al, 1995). The relevance of this interaction remains to be determined, however, it appeared to result in co-localization of the complex to a novel location within the cell. The N-terminal approximately 380 amino acids of VP16 are known to be involved in mediating protein-protein interactions, as well as promoter recognition. Furthermore, in yeast systems, derivatives of VP16 lacking the acidic tail were able to significantly enhance transcription of a reporter gene (Popova et al, 1995).

As a component of the viral tegument, VP16 enters the cell upon infection ready to initiate its effects. It makes its way to the nucleus of the cell to stimulate the transcription of the five immediate-early genes of the virus (Batterson and Roizman, 1983). Though it functions as an important viral transcriptional activator, VP16 has little specificity for DNA by itself. Rather, it complexes with at least two host proteins found in the nucleus, Oct-1 (Xiao and Capone, 1990; Kristie and Sharp, 1990; Stern and Herr, 1991) and HCF (Kristie and Sharp, 1990) before it

specifically binds to the *cis*-acting consensus sequence TAATGARAT (where R is purine), located in the enhancers of immediate-early genes (Mackem and Roizman, 1982). Oct-1 is a member of the POU domain family of DNA-binding proteins. The POU domain is a bipartite DNA-binding domain which is comprised of an N-terminal POU-specific region and a C-terminal POU region linked together by an unknown structure (Wilson *et al.*, 1993). HCF can complex with VP16 independent of Oct-1 and TAATGARAT, however, it acts to stabilize the interaction between VP16 and Oct-1 on the consensus site (Xiao and Capone, 1990; Kristie and Sharp, 1990; Stern and Herr, 1991). Once VP16 is bound, its acidic tail interacts with the host's basal transcription machinery to enhance transcription of the viral genes (Triezenberg *et al.*, 1988).

Aside from its important function as the transactivator of viral immediate-early genes, VP16 plays a crucial role in virus assembly. The importance of this role is most evident in the VP16 null mutant virus, 8MA. The mutation in 8MA carries a lethal phenotype and as a result, the virus must be propagated on a cell line which supplies VP16 in *trans*. When grown on a complementing cell line, 8MA is able to overcome the effects of the mutation. Detailed

investigation of the 8MA virus revealed that the VP16 acquired from the complementing cell line is incorporated into the virus. This VP16 is sufficient to induce transcription of a transfected reporter gene driven by an immediate-early promoter, when those cells are superinfected with the 8MA virus. The growth characteristics of 8MA correlate well to KOS (wild-type) on the complementing cell line, however, there are drastic differences both in titre and ability to replicate on the non-complementing cell line. Though it is unable to replicate on a non-complementing cell line, 8MA still exhibits close to wild-type levels of DNA replication and encapsidation. The key difference between the KOS and 8MA viruses during infection of a non-complementing cell line can be visualized under electron microscope. The KOS virus appeared as numerous densely cored, enveloped, cytoplasmic particles which were often localized at the cell surface. The 8MA capsids were largely empty and did not appear to accumulate at the cell surface. The defect in 8MA clearly lies at the level of virus assembly (Weinheimer *et al.*, 1992).

Earlier implications of the multifunctional nature of VP16 during infection came from the study of another mutant,

in1814, which contains a 12-base pair insertion in VP16 at amino acid codon 379. The molecule was known to be defective in transcriptional activation, by virtue of the fact that it did not enter into the VP16-induced complex (VIC) on TAATGARAT in gel-shift analysis. However, intertypic marker transfer with an HSV-2 mutant, *ts2203* revealed that the *in1814* protein was able to rescue its defect in virus assembly (Ace *et al.*, 1988). Thus, it was evident that different regions of the VP16 protein were involved in these different functions. The virus containing the *in1814* mutant was severely hampered in plaque formation at low multiplicities of infection (MOI), however, at higher MOI no significant effects were seen. Unlike wild-type, the *in1814* virus is unable to induce transcription of a transfected reporter plasmid containing the immediate-early enhancer region. In cells infected with *in1814*, Northern analysis revealed that the levels of the immediate-early mRNAs for ICP0 and ICP27 are reduced by 4 to 5 fold, and ICP22/47 by 2-fold compared to wild-type. There was no effect on the levels of ICP4 compared to wild-type. The inability of this mutant virus to grow at low MOI appeared to result from a failure to produce significant levels of

the immediate-early proteins which would allow infection to proceed. Low activity levels of the early enzyme thymidine kinase (TK), compared to wild-type virus indicated that the early, and presumably, late gene expression in *in1814* were also dependent upon MOI during infection. Thus, with *in1814*, the requirement for transactivation of immediate-early genes by VP16 is crucial to virus survival at low MOI (Ace *et al.*, 1989).

In an effort to examine the role of VP16 during infection without deleting or distorting its structure, several point mutants were constructed (Poon *et al.*, 1995). It was discovered that doubly or triply substituting for any cysteine residues within the molecule resulted in a temperature sensitive phenotype. Each of the seven mutants was unable to enter into the VP16-induced complex at TAATGARAT in gel shift analysis at the non-permissive temperature. Viruses containing the temperature sensitive genes were severely hampered in growth at the non-permissive temperature, when compared to the wild-type virus. However, by varying the temperature during the growth cycle of the mutant viruses, the mutant viruses were able to grow to levels quite comparable to wild type virus. In fact, the

mutants could be grown at the non-permissive temperature during the first 10 hours of infection, but required the ambient temperature during the last 8 hours to obtain wild-type levels of virus. The first 10 hours of infection would include such events as virus entry and immediate-early and early gene expression. Virus assembly takes place at later times in the infection cycle (ie. during the remaining 8 hours). Thus, this evidence seemed to place even more emphasis on the role that VP16 plays during assembly of new virus and perhaps indicates that even though VP16 enhances immediate-early gene expression, it is possible for the virus to otherwise effect expression of these genes.

There has been no specific role in assembly assigned to VP16, and there are only a few reports that discuss specific interactions between VP16 and HSV-1 structural proteins, aside from vhs. In chemical cross-linking experiments, VP16 was shown to be one of three tegument proteins which interacted with the glycoproteins B,D and H (Zhu and Courtney, 1994). As mentioned previously, VP16 has been shown to interact with VP22, a structural protein which is another member of the amorphous tegument region that also contains VP16 (Elliot *et al.*, 1995).

VP16 Homologues Differ in the Carboxy Terminus

By comparison, VP16 homologues in other alphaherpesviruses exhibit significant homology to HSV-1 VP16. There are, however, notable differences between the proteins. Two equid herpesviruses, EHV-1 and EHV-4 encode proteins which are capable of enhancing the transcription of HSV-1 immediate-early genes (Purewal *et al.*, 1994). The homology between these proteins diverges greatly in the region of the carboxy-terminal transcriptional activation domain. This domain in HSV-1 VP16 is a stretch of 80 mostly acidic amino acids, while in the EHV homologues, it comprises only 36 amino acids and is not very acidic by nature.

Varicella-zoster virus (VZV), encodes a 410 amino acid protein, ORF10 which exhibits a high degree of homology to HSV-1 VP16. It is completely deficient for the region which corresponds to the acidic tail in VP16 (Moriuchi *et al.*, 1993). Despite this, ORF10 is capable of transactivating both VZV and HSV-1 immediate-early genes, using a transcriptional activation domain situated in the amino terminus of the protein. The ORF10 protein does not effect

transcription of the HSV-1 genes in the same fashion as VP16, for it does not enter into the immediate-early complex at TAATGARAT.

Both proteins are components of their respective viral teguments, however, unlike VP16, VZV ORF10 is dispensable for growth in tissue culture (Cohen and Seidel, 1994). The importance of VP16 function early in infection should be closer analyzed in light of these studies. Furthermore, that transactivation by tail-less derivatives of VP16 has been shown in yeast (Popova et al., 1995) and viral mutants encoding 422 and 379 (Lam et al., 1996 and Dr. J. Smiley, personal communication) amino acid proteins are viable, brings into question the essential requirement for the acidic tail of VP16 during infection. It is conceivable that mutants encoding tail-less VP16 derivatives of either 422 or 379 amino acids, are viable (Dr. J. R. Smiley, personal communication). The observation that tail-truncated derivatives of VP16 can activate transcription in yeast (Popova et al., 1995), begs the question of whether these truncated derivatives are sufficient to do the same in the virus. The tail-less VP16 viral mutants V422 and V379 are viable viruses and when grown on the 16-8 cell line, which provides full length VP16 in trans, produce wild-type levels of immediate-early proteins without VP16 or with a tail-less VP16 and that the presence of the acid tail only enhances transcription.

Project Overview

VP16 is an integral element in the HSV-1 strategy to overturn normal cellular functions and use the host machinery for the production of new virus. It is an essential component of the virus tegument, and deletion of VP16 correlates to a lethal defect in assembly (Weinheimer et al., 1992). Its transactivation of the viral immediate-early genes via its acidic tail has been shown to be important for virus survival at low MOI (Ace et al., 1993).

essential component of the virus tegument, and deletion of VP16 correlates to a lethal defect in assembly (Weinheimer *et al.*, 1992). Its transactivation of the viral immediate-early genes via its acidic tail has been shown to be important for virus survival at low MOI (Ace *et al.*, 1989). However, several lines of evidence downplay the importance of the C-terminal acidic amino acids during virus replication in tissue culture. Poon and Roizman revealed that temperature sensitive mutations in VP16 only affect viral growth during the last eight hours of infection. Disruption of VP16 transactivation by growth at the non-permissive temperature early in infection, had little effect on virus yield (Poon and Roizman, 1995). Furthermore, HSV-1 mutants encoding tail-less VP16 derivatives of either 422 or 379 amino acids, are viable (Dr. J. R. Smiley, personal communication). The observation that tail-deleted derivatives of VP16 can activate transcription in yeast (Popova *et al.*, 1995), begs the question of whether these truncated derivatives are sufficient to do the same in the virus. The tail-less VP16 viral mutants V422 and V379 are viable viruses and when grown on the 16-8 cell line, which provides full length VP16 *in trans*, produce wild-type levels

of virus. The VP16 provided by the cell line is sufficient to overcome the assembly defect in 8MA, permitting it to produce almost wild-type amounts of virus (Weinheimer et al., 1992). Presumably, V422 and V379 growth is enhanced by the presence of the endogenous VP16 during infection in 16-8 cells, because it can compensate for any functions that the tail-less VP16 proteins are unable to fully effect.

While deletion of VP16 correlates to a lethal phenotype in the 8MA virus, it is apparent that the acidic tail of VP16 is dispensable for growth in tissue culture. Our first goal was to identify further regions in the VP16 molecule which are sufficient to rescue the defect in 8MA. We constructed a viral mutant encoding a 369 amino acid VP16 derivative as a means to determine the regions in the N-terminus of VP16 that are able to carry out its essential functions in the virus. In attempts to construct a viral mutant encoding a 335 amino acid VP16, no viable recombinants were obtained and this mutation appears lethal (Dr. J.R. Smiley, personal communication). A viable VP16-369 encoding mutant would narrow down the regions in VP16 which are required to effect function(s) essential to the virus.

Two characteristics of VP16-369 *in vitro* purport to make it a relevant VP16 truncation to investigate. The limit to C-terminal truncation of VP16 which still permits interaction with vhs *in vitro* is 369 amino acids (Smibert *et al.*, 1994). VP16-369 is also the limit to truncation in tail-deleted derivatives which exhibit the ability to activate transcription of a reporter gene in yeast (Popova *et al.*, 1995).

The interaction between VP16 and another HSV-1 tegument protein, vhs, has been well documented (Smibert *et al.*, 1994). Vhs action brings about an end to host gene expression by disruption of polyribosomes and degradation of mRNA (Nishioka and Silverstein, 1977; Schek and Bachenheimer, 1985). The discovery that VP16 was required to downregulate the activity of vhs during infection solidified the relevance of the interaction between the two proteins (Lam *et al.*, 1996). Disruption of this interaction by deleting VP16 drastically effects viral gene expression, as evidenced by the complete attenuation of viral protein synthesis at early times, during infection by the 8MA mutant. The mutants V422 and V379 which encode tail-less derivatives of VP16, were able to sustain viral protein

synthesis to 9 and 12 hours, respectively (Lam, 1995; Lam et al., 1996).

The vhs null mutant virus, Δ Sma, is only slightly altered in its growth characteristics as a result of the deletion in its vhs locus (Read et al., 1993). To compare the capabilities of the tail-less VP16 derivatives in viruses containing wild-type vhs and in a null vhs background, we have constructed double mutant viruses encoding tail-deleted derivatives of VP16 and containing deletions within the vhs locus (Δ Sma). HSV-1 infection is contingent upon tight regulation facilitated by interactions between such viral factors as vhs and VP16. With an inactivated vhs protein and subsequent disruption of the VP16-vhs interaction in the double mutant viruses, our aim was to investigate how these mutations affected virus growth characteristics and the requirement for full-length VP16 during infection compared to the tail-less VP16 mutants encoding wild type vhs.

Materials and Methods

2.1. Materials

2.1.1. Chemicals and Reagents Used

Amplify™	Amersham Canada Ltd.
5-Bromo-2'-Deoxycytidine	Sigma Chemical Co.
calf serum, fetal bovine serum, fetal bovine serum (dialyzed)	Gibco/BRL
crystal violet	BDH Chemicals LTD.
geneticin (G418)	Gibco/BRL
L-glutamine (100X; 200mM)	Gibco/BRL
L-histidinol dihydrochloride	Sigma Chemical Co.
Hexamethylene bisacetamide (HMBA)	Sigma Chemical Co.
human immune serum globulin	
molecular weight standards	
(i) 1 Kb DNA ladder	Gibco/BRL
(ii) ¹⁴ C-labeled protein standards (high-range molecular weight 14.3- 200 kDa)	Gibco/BRL

(iii) ^{14}C -labeled protein standards (low-range molecular weight 14.3- 66 kDa)	Sigma Chemical Co.
penicillin(5000U/mL) -streptomycin (5000 $\mu\text{g/mL}$) solution	Gibco/BRL
phenylmethylsulphonylfluoride (PMSF)	Sigma Chemical Co.
protein A - Sepharose beads	Pharmacia Inc.
salmon testes DNA	Sigma Chemical Co.
Sephadex G-50	Pharmacia Inc.
trypsin-EDTA	Gibco/BRL

2.1.2. Radiochemicals

$[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (3000Ci/mmol; 10 $\mu\text{Ci}/\mu\text{L}$)	Amersham Canada Ltd.
L- $[\text{S}^{35}]$ -methionine (1151 Ci/mmol; 10 $\mu\text{Ci}/\mu\text{L}$)	Amersham Canada Ltd.

2.1.3. Plasmid Vectors Used for Marker Transfer and Southern Blotting

ptkSb-x and p14a were obtained from Dr. J.R. Smiley, Dept. of Pathology, McMaster University. ptkSB-x consists of a mutant form of the HSV-I TK PvuII fragment containing an XbaI linker at the BamHI site of ptkSB (Jones et al, 1995). p14a contains the VP16 insert from pVP16KOS, flanked by XbaI sites, cloned into the XbaI site within the HSV-1 thymidine kinase coding region of ptkSB-x, antisense to TK

(Lam *et al.*, 1996). Two plasmids encoding mutant derivatives of VP16 were also obtained from Dr. J.R. Smiley: 14a/AB3405 was constructed by inserting an NheI linker (containing stop codons in all three frames) at the SacI site in VP16 at codon 422. 14a/PH379 was constructed similarly, with a linker inserted at codon 379. p14a/369 was constructed by inserting a C-terminal deletion of VP16, encoding the 369 amino-terminal amino acids into p14a. The truncated VP16 was excised from pCADVP369 (Popova *et al.*, 1995) using *KpnI*. p14a was cut with *KpnI*, collapsing the site and then re-ligating, created p14a(-kpn). The truncated derivative was then cloned into p14a(-kpn) and screened to ensure correct orientation.

2.1.4. Host Bacterial Strains

E.coli host strain DH5 α was used in all areas of cloning.

2.1.5. Mammalian Cell Lines

Three mammalian cell lines were employed in my project: Vero cells, 16-8 cells and QL10 cells. The 16-8 cells were obtained from Dr. J.R. Smiley but were constructed by Weinheimer *et al.* 16-8 cells are transformed Vero cells

containing VP16 under the control of a Moloney murine sarcoma virus LTR with termination polyadenylation signals from the HSV-1 TK gene (Weinheimer *et al*, 1992). QL10 cells are transformed Vero cells containing the VP16 ORF under control of the HSV-1 gD promoter (Lam *et al*, 1996). Vero cells (African green monkey kidney cells) were obtained from the American Type Culture Collection.

2.1.6. Mammalian Viruses

HSV-1 8MA is a mutant of KOS which has its VP16 coding sequences deleted and replaced with an expression cassette for β -galactosidase (Weinheimer *et al*, 1992). The wild type strain KOS (Smith *et al.*, 1964), was used as an experimental control. Δ Sma contains an inactivating mutation in the vhs gene. (Read *et al*, 1993) V379 and V422 are derivatives of 8MA which encode VP16 that is truncated at residues 379 and 422, respectively. In these viruses, the mutant VP16 coding sequences have been targeted into the VP16 locus, replacing the β -galactosidase gene. 8MA Δ Sma contains both the VP16 mutation of 8MA and the deletion in vhs of Δ Sma (Lam *et al*, 1996). All of the above viruses were obtained from Dr. J.R. Smiley.

2.2. Methods

2.2.1. Plasmid Construction

New plasmids were constructed according to standard molecular biology protocols as outlined in Sambrook *et al*, (1989). Enzymes used were purchased from New England Biolabs, Pharmacia Inc. and Promega Corp. Other relevant techniques or materials employed include Qiagen columns (Qiagen Inc.) for large scale preparation of DNA. DNA concentration was determined by fluorescence spectroscopy (Sambrook *et al.*, 1989).

2.2.2. Mammalian Cell Line Maintenance

Monolayers of each cell line were maintained at 37° C and 5% CO₂. Vero cells were maintained in Dulbecco's Modified Eagle Medium (MEM) supplemented with 10% calf serum and 1% each of penicillin/streptomycin and L-glutamine. 16-8 cells

were maintained in α -MEM supplemented with 10% fetal bovine serum, 1% each of penicillin/streptomycin and L-glutamine and 450 μ g/mL of G418. QL10 cells were kept in Dulbecco's Modified Eagle Medium (minus histidine) supplemented with 10% fetal bovine serum, 1% each of penicillin/streptomycin and L-glutamine and 0.15mM Histidinol. All media was purchased from the Cancer Research Group, Dept. of Pathology, McMaster University. Cell monolayers were detached from the plates by first washing in PBS (136.8mM NaCl, 2.7mM KCl, 8mM Na₂HPO₄.7H₂O, 1.5mM KH₂PO₄), then treatment with 1X trypsin (diluted in PBS). For experimental use (ie. purposes other than maintenance), QL10 cells and 16-8 cells were propagated in Dulbecco's MEM (supplemented with 10% FBS, 1% penicillin/ streptomycin and 1% L-glutamine).

2.2.3. Mammalian Virus Maintenance and Manipulation

2.2.3.1. Virus Stocks

Stocks of each virus were obtained in the following manner: 10 large (15 cm) diameter plates of cells were infected with virus at an MOI of 0.1. Cells were infected in a minimum volume of Dulbecco's MEM without serum and were

overlayed with Dulbecco's MEM supplemented with 10% FBS, after a suitable period of infection (1-2hrs). In certain cases, virus stocks were grown in the presence of 3mM hexamethylene bisacetamide to increase the yield of virus (McFarlane et al., 1992). Infected cells were incubated at 37° C until total cytopathic effects (cpe) were visible (2-4 days). Cells were then harvested and recovered by centrifugation (6 minutes; 4°C; 2000 RPM; IEC rotor 216, IEC Centra-8R Centrifuge (USA)). The cell pellet was resuspended in 10 mLs of supplemented medium and subjected to 3 cycles of freeze-thaw, in liquid nitrogen. The cell debris was spun down (6 minutes, 3000 RPM) and the supernatant was aliquoted 1mL into 10 cryovials and stored at -75° C.

2.2.3.2. Plaque Assays

To determine the titre of a given viral stock, plaque assays were performed. Serial dilutions of virus stock were made in Dulbecco's MEM without serum. The cell line of interest was infected with serial dilutions of the virus, in a minimal volume, for a period of 2 hours. Dulbecco's MEM supplemented with 10% FBS and human immune serum globulin

(to 0.05%) was added to the monolayers of cells. Following 40-48 hrs incubation, the medium was removed and the monolayers were stained with crystal violet (0.1g crystal violet/100mLs of water: absolute ethanol, 3:1). The viral plaques were counted using a microscope to determine the virus titre in pfu/mL.

2.2.3.3. Preparation of Viral DNA

To prepare infectious 8MA and 8MA Δ Sma viral DNA for use in marker transfer experiments, 10 large (15 cm diameter) plates of 16-8 cells were infected with virus at an m.o.i of 10 pfu/cell. Plates were incubated at 37° C until complete cytopathogenic effects were visible (1-2 days). The cells were harvested and collected by centrifugation (6 min; 3000 RPM; 4°C; IEC rotor 216). The cell pellet was washed once with PBS and resuspended in 10 mL of 0.2M EDTA (pH 8.0), containing 0.5% SDS and 100 μ g/mL proteinase K, and then incubated overnight at 37°C. The resuspended pellet was then carefully phenol/chloroform extracted four times and then dialyzed against 0.5 L volumes of 0.1% SSC for a period of 3-4 days. The viral DNA was collected and stored at 4°C. To ensure that the viral DNA was able to

produce virus, a range of volumes of viral DNA (eg. 10 μ l - 120 μ L) was transfected into QL10 cells. The monolayer was analyzed 40-48 hrs post-transfection for the appearance of plaques.

2.2.4. Transfection of DNA into Mammalian Cells

DNA was transfected into mammalian cells utilizing the calcium phosphate co-precipitation method (Graham and van der Eb, 1973).

2.2.5. Transfection of HSV-1 DNA

Transfection of infectious 8MA and 8MA Δ Sma viral DNA for the purpose of generating recombinant virus was done in QL10 cells. To determine the amount of infectious viral DNA to use, a range of volumes was transfected in QL10 cells to determine which gave the desired number of plaques. In general, 50-100 plaques in one well of a 6 well dish was considered optimal. To make a precipitate, the infectious DNA was combined with 31 μ L of 2M CaCl₂ and 10 μ L of salmon testes DNA (1 μ g/ μ L) to a final volume of 250 μ L with dH₂O. This was added dropwise to 250 μ L of 2X HBS, pH 7.05 (0.28M NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄.7H₂O) while vortexing

slowly. The calcium phosphate/ DNA precipitate was allowed to form during a 20 minute incubation at room temperature. Cell monolayers (at 80-90% confluency) were washed once with PBS and overlaid with a fresh volume of Dulbecco's MEM, supplemented with 10% FBS. The precipitate was added dropwise to the cells which were then incubated overnight at 37°C. After overnight incubation, the monolayers were washed 3X with Dulbecco's MEM, supplemented with 10% FBS, and overlaid with a fresh volume of the same medium. Monolayers were monitored for the appearance of cpe (4-5 days post-transfection).

2.2.6. Construction and Identification of Viral Recombinants

2.2.6.1. Marker Transfer Experiments

Recombination of derivatives of VP16 into either 8MA or 8MA Δ Sma viral DNA was performed by marker transfer, targeting into either the viral thymidine kinase (TK) or VP16 loci. The infectious viral DNA was co-transfected with 1-4 μ g of the construct containing the sequence of interest (linearized plasmid DNA) onto QL10 cells, as described in

the section on transfecting viral DNA. When complete cpe could be observed, the cells were harvested and stocks were made. The stocks were then assayed for titre on QL10 cells and Vero cells.

2.2.6.2. Selection for Marker Transfer into the TK Locus

In targeting marker transfer to the viral TK locus, recombinant viruses were assayed for growth in the presence of 100 μ g/mL 5-Bromo-2'-deoxycytidine (BrdC). Approximately 1000 pfu of the virus burst stock was used to infect onto 10 large (15 cm diameter) plates of QL10 cells in a minimum volume of Dulbecco's MEM, without serum. Infection was allowed to progress for 1-2 hrs after which 20 mLs of Dulbecco's MEM supplemented with 10% FBS, 100 μ g/mL BrdC and 0.05% human immune serum globulin, was added to each plate. After 3-4 days incubation at 37°C, plaques were picked into sub-confluent 24 well dishes of QL10 cells in the continued presence of BrdC selection. The 24 well dishes of infected QL10 cells were incubated at 37°C until complete cpe was evident. At this point, wells containing infected cells were harvested into two aliquots: 400 μ L stored at -80°C (for the purposes of growing a stock) and 600 μ L for use in

preparing the viral DNA for Southern blot analysis.

2.2.6.3. Selecting for Marker Transfer into the VP16 Locus

Targeting marker transfer into the VP16 locus was facilitated by the fact that the viruses 8MA and 8MA Δ Sma are severely reduced in their ability to form plaques on Vero cells. These viruses are propagated in cell lines which supply VP16 *in trans*, such as 16-8 or QL10 cells. By virtue of the fact that the markers being targeted into the VP16 locus were derivatives of VP16, it was possible to select for recombinant viruses that were able to form plaques on Vero cells. The procedure followed in this case was quite similar to that for selection of TK minus viruses, except that the selection is based solely on the cell line. Briefly, 1000 pfu's from the virus burst stocks were infected onto 10 large (15 cm) plates of Vero cells in a minimum volume of Dulbecco's MEM, without serum. After 1-2 hours, 20 mL of Dulbecco's MEM, supplemented with 10% FBS and 0.05% human immune serum globulin was added to each plate. After 3-4 days incubation, plaques were picked into sub-confluent 24 well dishes of Vero cells. After the appearance of complete cpe in the 24 well dishes, the wells of infected cells were harvested and aliquotted as described

above.

2.2.7. Analysis of Viral DNA by Southern Blot Hybridization

2.2.7.1. Preparation of DNA from Harvested Virus

Infected cells were harvested from a 24 well dish, after complete cpe was observed. The 600 μ l of cell suspension designated for viral DNA extraction was pelleted by centrifugation at 14,000 RPM for 3 min at 4°C. The media was removed by aspiration and the pellet was resuspended in 500 μ L of Urea-SDS buffer, pH 7.5 (7M urea, 350 mM NaCl, 10mM Tris, 20 mM EDTA, 10% SDS) and vortexed vigorously. 500 μ L of phenol:chloroform (1:1) was added and the tube was again vortexed. The cellular debris was removed by centrifugation at 14,000 RPM for 15 min at 4°C. The supernatant was extracted with 500 μ L of chloroform: isoamyl alcohol (24:1). Viral DNA was precipitated with ethanol and recovered by centrifugation. The pelleted DNA was dissolved in 33 μ L of dH₂O.

2.2.7.2. Southern Blot Analysis

Recombinant viruses were characterized by Southern blot analysis of viral DNA preparations (Brown, 1993). DNA

probes were generated by ^{32}P -labeling of plasmid DNA by a random-primed hexanucleotide procedure and purified by elution through a Sephadex G-50 spin column (Sambrook *et al*, 1989).

2.2.8. Metabolic Labeling of Infected Cells and Preparation of Cell Extracts

2.2.8.1. Discontinuous Metabolic Labeling of HSV-1 Infected Cells

Cells (in either 6 cm plates or 6 well dishes) were infected with virus at a defined MOI in a minimum volume of Dulbecco's MEM, without serum for 1 hr. At times 1, 5, 11 and 23 hours post-infection, the cells were washed 3X with PBS and overlaid with 1-2 mLs of Dulbecco's MEM minus methionine (supplemented with 10% Dulbecco's MEM minus serum, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 1% sodium pyruvate) with 50 μCi of ^{35}S -Met. The pulse-label was allowed to persist for 1 hr and at times 2, 6, 12 and 24 hrs post infection, the labeling-media was removed by aspiration.

2.2.8.2. Preparation of HSV-1 Infected Cell Extracts

Cells from metabolic labeling experiments were harvested at 2, 6, 12 and 24 hours post-infection. After the labeling media was removed, cells were washed 3X with phosphate buffered saline and 0.5 mLs of chilled RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1 % NP40, 0.5% DOC, 0.1% SDS, 1mM PMSF and 100u/mL traysylol) was added. Cells were incubated on ice for 30 min and then scraped into microfuge tubes. Cell debris was removed by centrifugation for 5 min at 14,000 RPM using an Eppendorf microfuge at 4°C. Lysates were then removed into fresh Eppendorf tubes and stored at -20°C.

2.2.9. Immunoprecipitation of HSV-1 Polypeptides and Analysis of Infected Cell Extracts

2.2.9.1. Immunoprecipitation of HSV-1 Polypeptides from Infected Cell Extracts

For the purposes of immunoprecipitation, a volume comprising approximately 10^6 cpm from each infected cell extract was used. The extract was mixed with the appropriate titre of a given antibody at 4°C, for a period

of 1 hour. Rabbit α -Sal antibody is a polyclonal serum which recognizes the *SalI* fragment of VP16. Rabbit anti-HSV-1 glycoprotein B (gB), gD, gH and TK polyclonal antibodies were provided by Dr. D.C. Johnson. A polyclonal rabbit antibody, R1, which recognizes both subunits of HSV-1 ribonucleotide reductase, was provided by Dr. S. Bachetti. 100 μ L of swelled Protein A-Sepharose beads (prepared in RIPA 'minus' buffer, without PMSF and trasylol) was added to the extract/antibody mixture at 4°C and mixed for 1 hr. The beads were washed 5X with chilled RIPA 'minus' containing 0.3% SDS and once with a final wash buffer (50 mM Tris, 2 mM EDTA, pH 7.5 at 25°C). Proteins were eluted off the beads by boiling for 5 minutes in 20 μ L of 2X sodium dodecyl sulfate (SDS) sample buffer (Sambrook *et al*, 1989) with recovery of the supernatant by centrifugation for 5 min at 14,000 RPM at 4°C.

2.2.9.2. Analysis of Infected Cell Extracts and Immunoprecipitated Polypeptides

Infected cell extracts and immunoprecipitated HSV-1 polypeptides were analyzed on 10% SDS-polyacrylamide gels (Sambrook *et al*, 1989) and visualized by autoradiography.

Polypeptide relative molecular mass was determined through the use of ^{14}C - labeled molecular weight standards.

2.2.10. Single Step Replication Assay

Sub-confluent 6 cm dishes of Vero and 16-8 cells were infected in tandem with virus at an MOI of 3. Cells were infected with virus in 0.5 mL of Dulbecco's MEM, without serum, for a period of 1 hour. After 1 hour, the cells were washed twice with PBS to remove any residual virus in the media. The cells were then overlaid with 4 mLs of Dulbecco's MEM, supplemented with 10% FBS and the plates were left to incubate at 37°C. At times 3, 12, and 24 hours post-infection the infected cells were harvested by scraping off the plate and extracts were made. The cell pellet was resuspended in Dulbecco's MEM, supplemented with 10% FBS and following freeze-thaw, was removed by centrifugation. The 200 μL supernatant containing the virus was aliquoted and stored at -80°C. Extracts of virus from each cell line and each time point were then assayed for titre on QL10 cells (as previously described).

Results

HSV-1 Mutants Encoding Tail-Deleted VP16 Derivatives

3.1. Construction of an HSV-1 Mutant Encoding a Truncated 369 amino acid VP16.

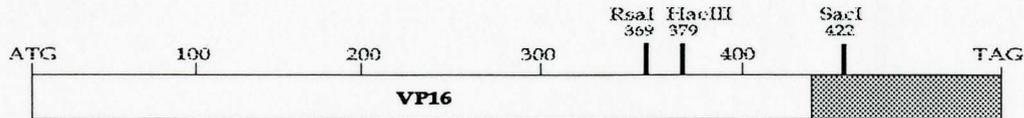
HSV-1 mutants which encode tail-deleted derivatives of VP16 truncated to 422 (Lam et al., 1996) and 379 (Lam, Q., 1995) amino acids, have been isolated. These mutants have been studied for their ability to rescue viral protein synthesis during infection compared to 8MA virus. While the 8MA virus (which is deleted for VP16) displays complete arrest in its protein synthesis between 2-5 hours post-infection, the transactivation incompetent VP16 derivatives 379 and 422 are able to sustain viral translation to 9 and 12 hours, respectively (Lam, Q, 1995; Lam et al., 1996). The growth characteristics of these mutants have not been

studied in detail. In other studies, using the two-hybrid activator system to study the specific interaction between VP16 and vhs, VP16-369 was found capable of binding to vhs (Smibert *et al.*, 1994). At least one purpose of the interaction between VP16 and vhs is understood, that VP16 is required to attenuate the destructive activity of vhs and rescue viral gene expression (Lam *et al.*, 1996). Though it is clear that the acidic tail of VP16 is dispensable for this function, it was of interest to determine if small regions of VP16 were sufficient, as a means to identify further regions in VP16 that are required for this function.

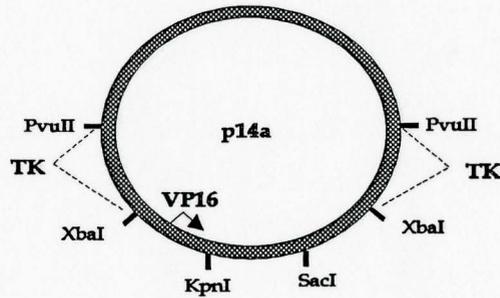
In studies of reporter gene activation in a yeast system, tail-deleted derivatives of VP16 were capable of activating transcription (Popova *et al.*, 1995). The greatest C-terminal deletion in VP16 that could activate transcription comprised the N-terminal 369 amino acids. It has not been determined if these derivatives can activate viral gene transcription *in vivo*, nor if they are necessary to do so.

In recombining tail-deleted VP16 derivatives into 8MA, Lam *et al.*, generated two types of mutants. The first had the mutant gene recombined into the VP16 locus and were

a)

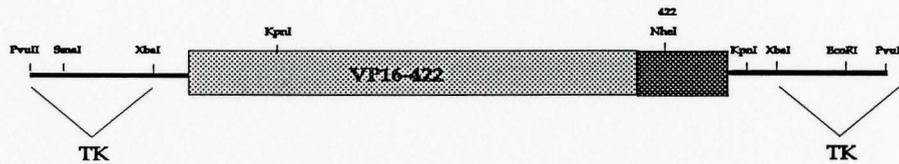


b)

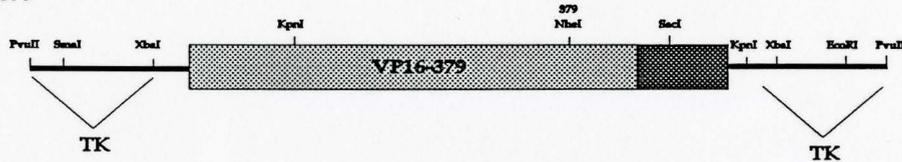


c)

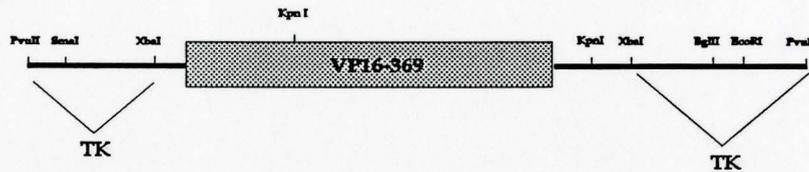
14a/422



14a/379



14a/369

**Figure 3:**

- a) HSV-1 VP16 showing the restriction sites at 369, 379 and 422.
- b) Plasmid 14a which encodes the full length VP16 gene flanked by HSV-1 thymidine kinase (TK) sequence.
- c) Plasmids 14a/422, 14a/379 and 14a/369: The VP16-422 and -379 genes are full length and contain linker inserted stop codons (introducing unique NheI sites) yielding truncated proteins. VP16-369 contains a deletion in VP16 which yields the truncated product. Each mutant gene is flanked by HSV-1 TK sequence as in 14a.

designated V379 and V422. The second set maintained the β -galactosidase gene in the VP16 locus (as in 8MA), but recombined the mutant gene into the thymidine kinase (TK) locus under selection for TK minus viral recombinants. These mutants were designated E379 and E422. The truncated VP16 encoded by the mutant genes VP16-379 and VP16-422 results from the insertion of a linker which has termination codons in all 3 reading frames, at amino acids 379 and 422, respectively (Figure 3a). These genes are encoded by the plasmids 14a/379 and 14a/422 (Figure 3b,c). No apparent difference is derived from having VP16 recombined into the either the TK or the VP16 locus in the mutant viruses (Dr. J. Smiley, personal communication).

The VP16-369 gene contains a deletion in VP16 rather than a linker inserted termination codon as found in the VP16-379 and VP16-422 genes (Figure 3a). To recombine the VP16-369 gene into 8MA, we employed the method that had been used in constructing E379 and E422. Initially, infectious 8MA DNA was co-transfected with plasmid 14a/369 (Figure 3b) into QL10 cells. QL10 cells were constructed by transforming Vero cells with a plasmid encoding a histidinol dehydrogenase resistance gene and the VP16 gene driven by

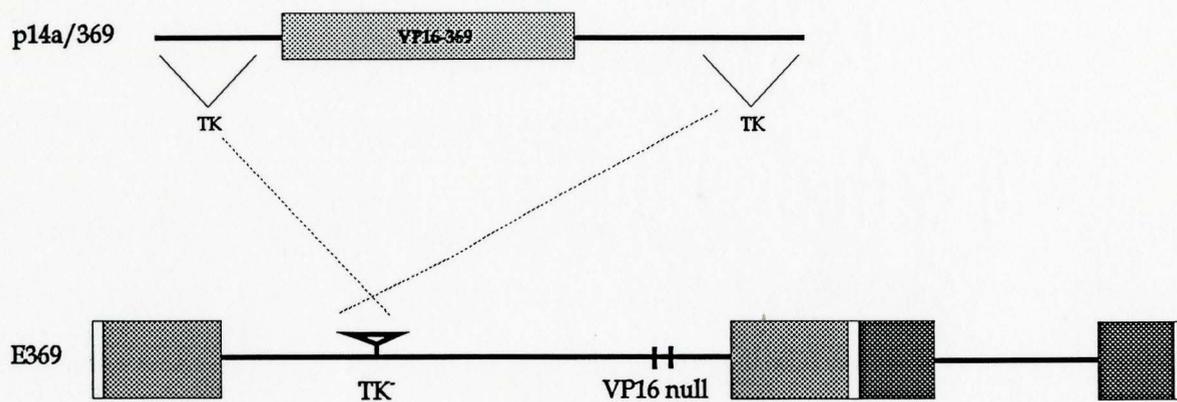


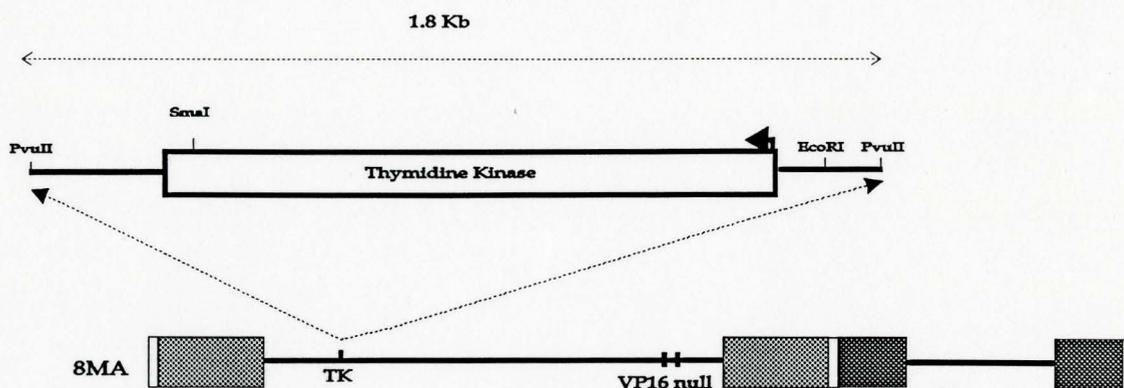
Figure 4:

Construction of E369. VP16-369 flanked between HSV-1 TK sequence in p14a/369 recombines into the TK locus of 8MA, creating E369. The null mutation in the VP16 locus of 8MA remains in E369.

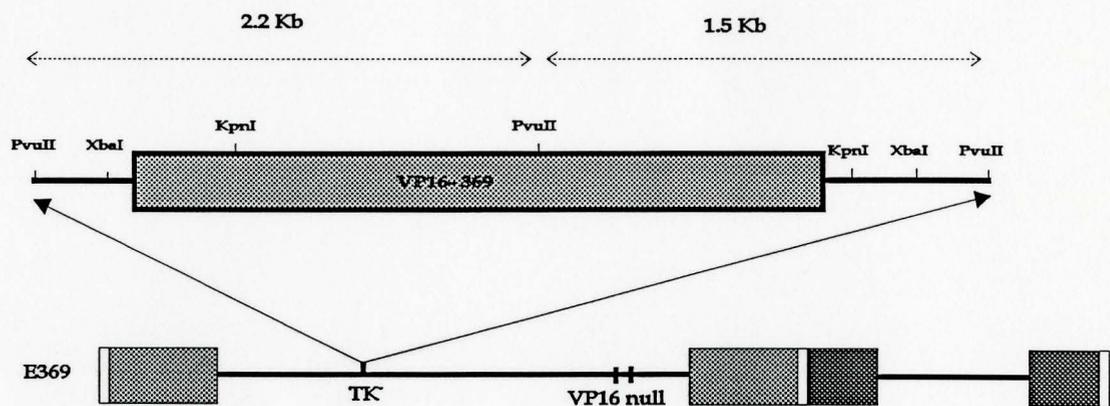
the HSV-1 gD promoter (Lam *et al.*, 1996). The QL10 cells support more efficient plaque formation by the 8MA null mutant and were thus employed to assist in plaque formation by any recombinant viruses generated. From the initial virus burst, one thousand plaque forming units (PFUs) were harvested and plated onto 10 large (15 cm) plates of QL10 cells, in the presence of 100 μ g/mL BrdC (a cytosine analog which prevents HSV-1 DNA replication). Viruses which recombined the VP16-369 gene into their TK locus became TK minus, and were able to grow in the presence of BrdC (Figure 4). In the continued presence of selection, plaques were picked onto 24 well dishes of QL10 cells. Any plaques that developed were harvested for Southern blot analysis.

In screening for recombinant virus, the 8MA thymidine kinase locus was probed to ensure that it had been disrupted by the recombination of the VP16-369 gene into it. To observe the disruption, viral DNA and plasmid controls were probed with a plasmid ptkSB-x, which encodes HSV-1 TK (Figure 5). The expected disruption in TK was evident in the viruses which had taken up the VP16-369 gene, thus they were named E369.

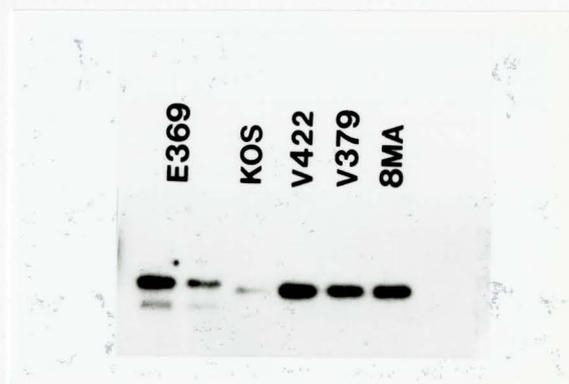
a)



b)



c)

**Figure 5:**

Disruption of the TK locus by VP16-369 and Southern blot analysis of E369. The recombination of VP16-369 into the TK locus of 8MA introduces a new PvuII site, which facilitates the screening of recombinant viruses. The expected fragment sizes are indicated above each map.

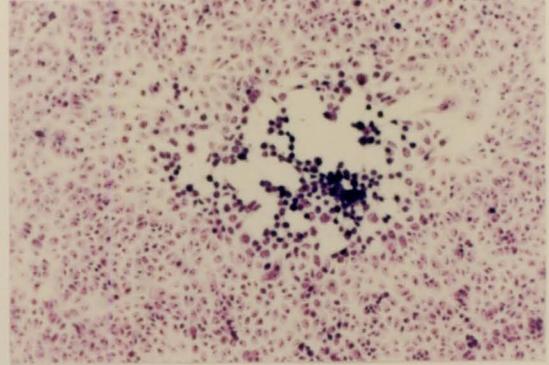
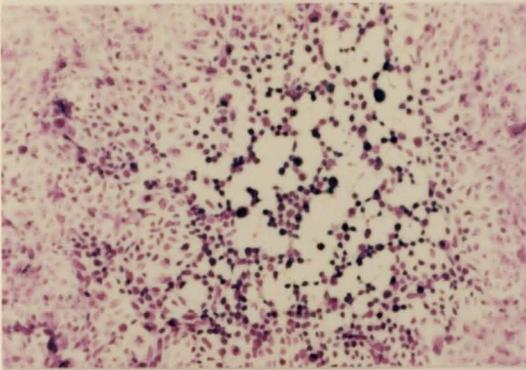
3.2. Characterization of E369

To determine the effect of the progressive VP16 truncation on the viability of the mutant virus, E369 was assayed for growth on a cell line which supplies VP16 in *trans* and on one which does not. The tail-deleted VP16 mutants are generally propagated in 16-8 cells, which express VP16, to improve virus yield. E369 stocks derived from 16-8 cells were boosted one-hundred fold in titre by growth in the presence of 3mM Hexamethylene bisacetamide (HMBA), which enhances the lytic cycle of HSV-1 by stimulating the transcription of certain HSV-1 genes (McFarlane *et al.*, 1992). 16-8 cells were created by transforming Vero cells with the gene encoding aminoglycoside phosphotransferase and the VP16 gene driven by an Moloney murine sarcoma virus LTR (Weinheimer *et al.*, 1992). The presence of the endogenous VP16 enhances the yield of virus when growing stocks (results not shown).

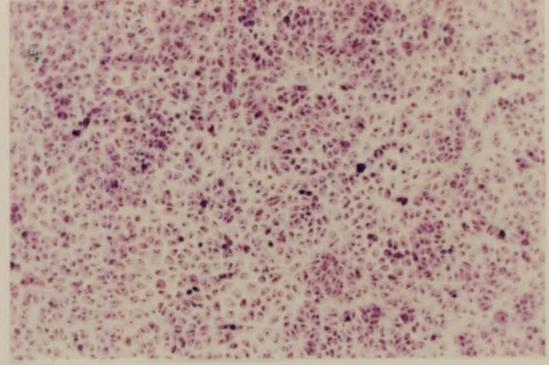
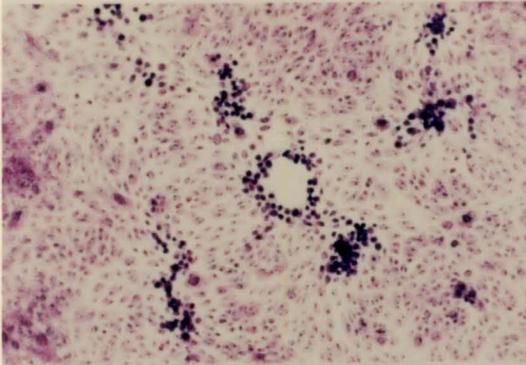
To compare the plaque morphology of the mutant viruses on cell lines which do and do not supply VP16 in *trans*, we used QL10 cells and Vero cells, respectively. A stock of E369 propagated on 16-8 cells was titred in tandem on QL10 cells and Vero cells and plaques were stained with crystal

QL10

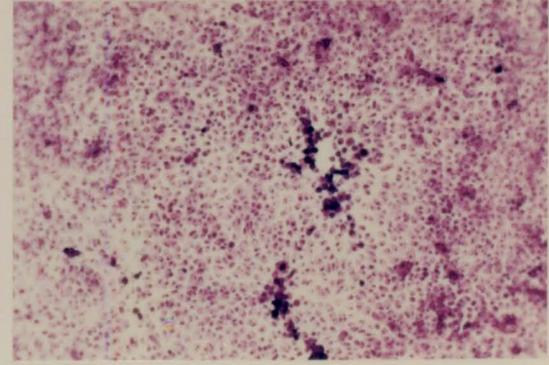
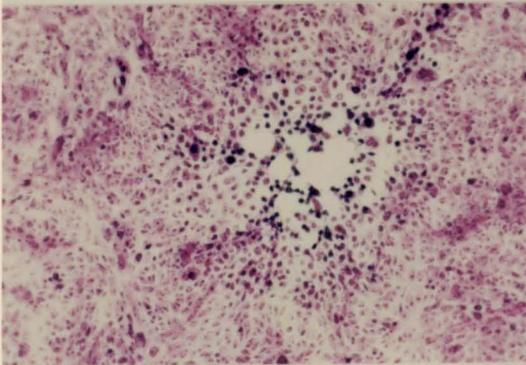
Vero



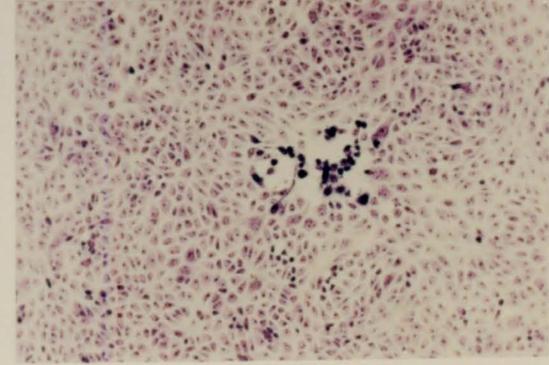
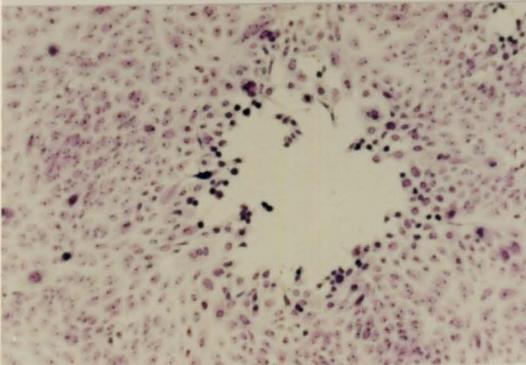
i) KOS



ii) 8MA



iii) V379



iv) E369

violet. The plaque morphology for E369 on these cell lines parallels what was observed for V379 and V422. On QL10 cells (which supply VP16 in *trans*), E369 plaques are large clearings in the monolayer, comparable in size to the wild type virus KOS (Plate 1). On Vero cells, the plaques were significantly smaller in size and produced very little clearing of the monolayer. The KOS virus, on the other hand, displayed the same morphology on Vero cells as it did on the complementing cell line. 8MA virus forms plaques similar to KOS on QL10 cells, yet it does not form any plaques on Vero cells (Plate 1).

In accordance with its plaque morphology, E369 displays a marked difference in titre between the cell line which complements for wild type VP16 and one which does not. For the purposes of comparison, virus stocks were titred in tandem on 16-8 and Vero cells and the ratio of the difference in titre between the cell lines was determined.

The dependance of 8MA on the presence of VP16 is evident by virtue of the almost 10^5 - fold difference in titre between the cell line (Table 1). In the viruses encoding tail-deleted VP16, a discrepancy in titre also exists between 16-8 and Vero cells, though not of same

Virus	16-8 Titre	Vero Titre	16-8/ Vero
KOS	6.8×10^8	9.0×10^8	0.75
8MA	5.1×10^7	1.0×10^3	5.1×10^4
V422	2.2×10^7	3.2×10^6	6.9
V379	8.3×10^8	5.0×10^6	1.7×10^2
E369	3.8×10^8	1.0×10^6	3.8×10^2

Table 1: HSV-1 Virus Titres on 16-8 and Vero Cells.

16-8 derived stocks of each virus were titred by serial dilution in tandem on 16-8 and Vero cells. 16-8 cells provide full length VP16 in *trans*. Plaques were counted and virus titres expressed in PFU/mL (Plaque forming units). The titres of each virus on 16-8 cells and on Vero cells were then expressed as a ratio.

magnitude as seen for the 8MA virus. It is evident that as VP16 is progressively truncated from the C-terminus, the difference in titre between the complementing and non-complementing cell line increases. In Table 1, the ratio shown increases from approximately 7-fold to almost 400-fold moving from V422 to E369. Thus, it is evident that progressive truncation of VP16 from its carboxy-terminus correlates with an increasingly apparent preference for growth on a cell line which supplies full length VP16 in *trans*. This is further evidenced by the fact that attempts recombine VP16-335 into 8MA, using this same protocol have been unsuccessful (Dr. J. Smiley, personal communication). The VP16-335 mutation carries a lethal phenotype which might result from a dominant interference with the transactivation or assembly functions of the endogenous VP16, or eradication of the viral messages due to an inability of VP16-335 to completely attenuate vhs activity. Clearly, 335 amino acids of VP16 are beyond the limit of truncation to VP16 which will permit virus growth. Furthermore, the VP16-369 must retain an essential function which is lost in truncation of VP16 to 335 amino acids. The endogenous VP16 present during infection of a complementing cell line serves a function(s)

upon which the mutant virus depends increasingly as its copy of VP16 is progressively truncated from the carboxyl-terminus. It is conceivable that the further truncations in VP16 render it increasingly unable to perform its role in virus assembly or to attenuate vhs activity. The trend may occur because of the inability of tail-deleted VP16 proteins to drive immediate-early gene expression. However, if the tail-less VP16 derivatives are having such an effect *in vivo*, this idea would seem unlikely because VP16-369 was among the strongest tail-less activators of reporter gene expression in yeast systems.

3.3. E369 Host Shutoff Phenotype and VP16 Production

The VP16 null mutant 8MA displays complete attenuation of viral protein synthesis early in infection. The tail-less VP16 derivatives VP16-422 and VP16-379 are sufficient to partially rescue this defect (Lam, 1995; Lam *et al.*, 1996). To show this, infected Vero cell extracts from 2, 6 and 12 hours post-infection were analyzed by SDS-PAGE and visualized by autoradiography (Figure 6). With the absence

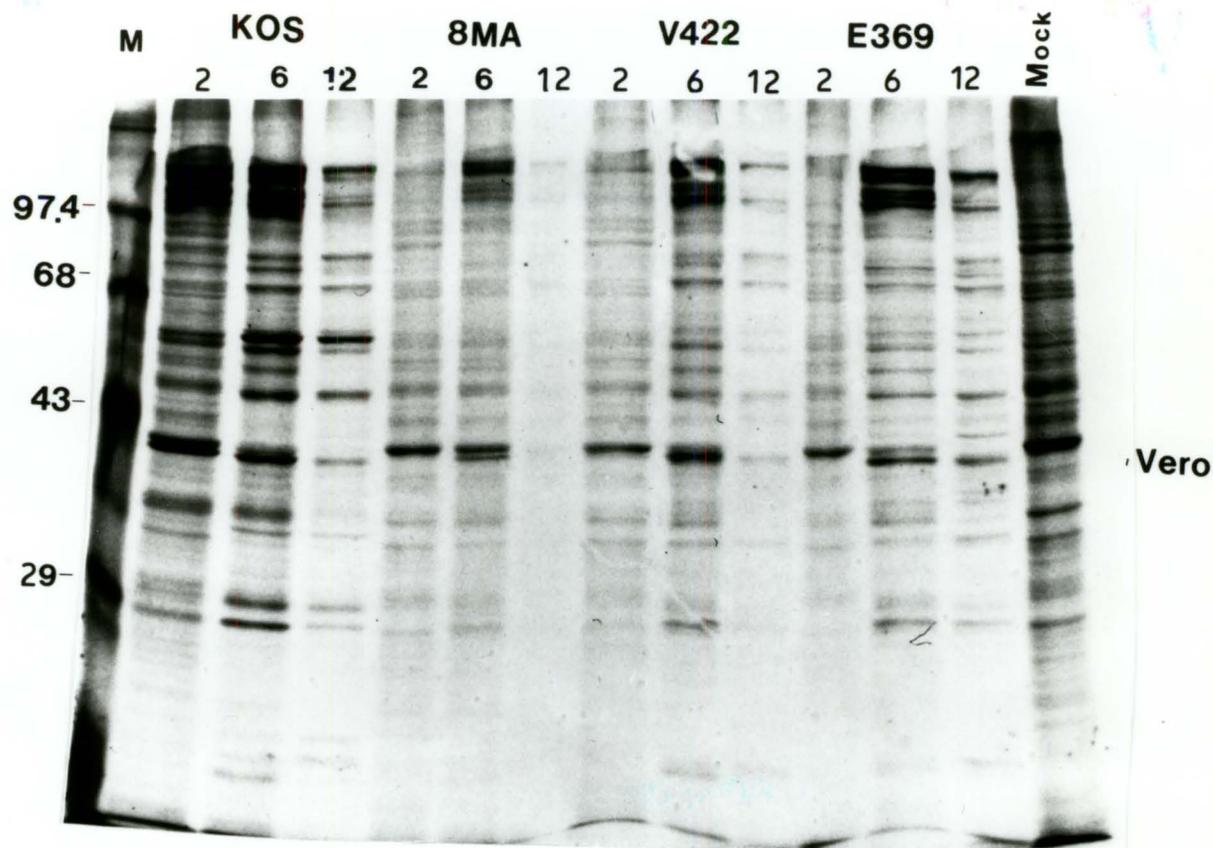


Figure 6: Host Shutoff Phenotype of HSV-1 Mutants.

Vero cells were infected with 16-8 derived stocks of each virus at an MOI of 10, except for E369 (MOI of 5).

³⁵S-labelled infected cell extracts from various time points were analyzed by SDS-PAGE and autoradiography. 8MA displays an early arrest to protein production, while the mutants V422 and E369 are able to sustain protein synthesis to later times in infection.

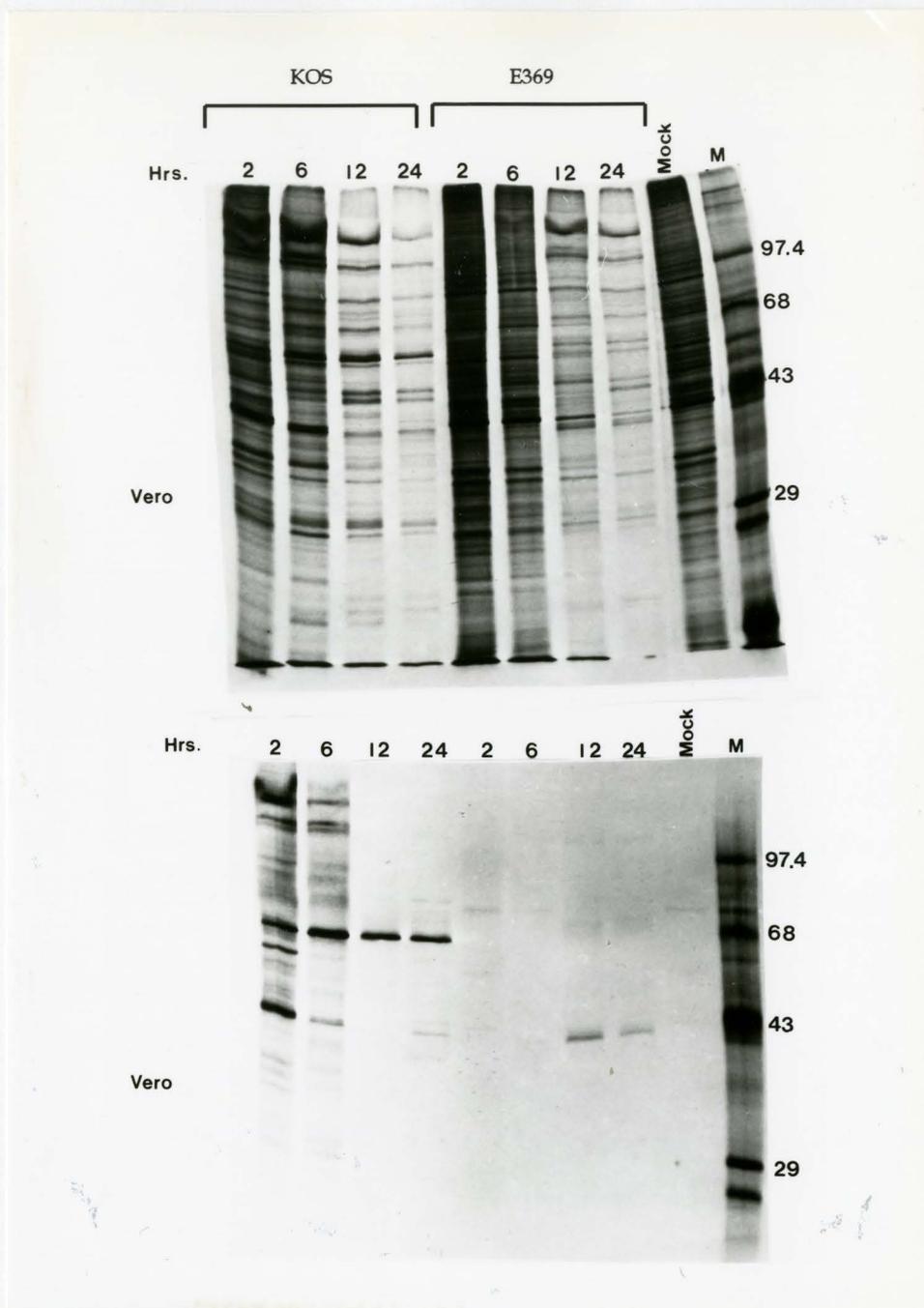


Figure 7: E369 Host Shutoff Phenotype and VP16-369 Production During Infection.

a) Host shutoff phenotype: Vero cells were infected with each virus at an MOI of 5. E369 stock is derived from 16-8 cells, Kos from Vero cells. ^{35}S -Met labelled infected cell extracts from various times were analyzed by SDS-PAGE and autoradiography.

b) Immunoprecipitation of VP16-369 from infected Vero cell extracts using a polyclonal rabbit antiserum to VP16. Proteins were resolved on a 10% SDS-PAGE, followed by autoradiography. Full length VP16 in KOS runs close to 65 kDa and in the E369 mutant, at approximately 37 kDa.

of VP16, 8MA viral protein synthesis is rapidly shut down because the activity of vhs cannot be downregulated. V422 shows viral protein bands evident at late times in infection and when compared to 8MA it is clear that they are capable of attenuating vhs activity to some degree.

The ability of tail-deleted VP16 derivatives 379 (not shown) and 422 to sustain viral gene expression to levels albeit below wild-type, but far above 8MA, raises the question of how far VP16 can be truncated and still perform this function. From *in vitro* studies of the specific interaction between VP16 and vhs, it might be expected that VP16-369 represents this limit. VP16-369 forms a strong interaction with the virion host shutoff protein in the two-hybrid activator system, but a further truncation in VP16 to 335 amino acids eliminates the binding (Smibert *et al.*, 1994).

Vero cells were infected with E369 and at selected times, infected cell extracts were analyzed by SDS-PAGE (Figure 6 and Figure 7a). Distinct bands which correspond to E369 viral proteins can be seen at late times in infection. The E369 virus is able to produce viral proteins, though at lower levels than KOS (wild-type).

Thus, the 369 N-terminal amino acids of VP16 are sufficient to prevent complete destruction of the viral messages by vhs as evidenced by the presence of these viral protein bands late in infection. It is difficult to determine if this is a direct result of the binding of VP16-369 to vhs, however, it is a plausible hypothesis considering that the only relevant difference between 8MA and E369, or in sustaining viral protein synthesis during infection, is the 369 amino acids of VP16 in E369.

The truncated VP16 derivatives produced by the viral mutants V379 and V422 are located at approximately 43 and 48 kDa, respectively, when analyzed by SDS-PAGE (results not shown). To determine if E369 was producing its truncated protein, VP16-369 was immunoprecipitated from infected Vero cell extracts with a polyclonal rabbit anti-serum (Figure 7b). The 37 kDa protein was visualized by SDS-PAGE and autoradiography. It was produced at much lower levels and at later times than the 65 kDa wild-type VP16 immunoprecipitated from KOS infected Vero cell extracts. This may reflect a delay in the kinetics of E369 gene expression which could result from a number of circumstances (inability to completely attenuate vhs activity, lower

levels of transactivation with the tail-deleted VP16 or perhaps a defect in viral entry associated with the mutant virus).

Although its titre on Vero cells is almost one-thousand times lower than KOS, the viral mutant E369, which encodes a truncated 369 amino acid VP16 derivative, is viable. Clearly, the 369 N-terminal amino acids of VP16 are sufficient to carry out all the functions of wild-type VP16 that are essential to virus survival. The answer may lie in its ability to perform the assembly function(s) of wild-type VP16, to downregulate vhs activity or to stimulate transcription to levels high enough that the cascade of viral gene expression needed for lytic infection can occur.

HSV-1 Double Mutants Which Encode Tail-Less VP16s and a Deletion (Δ Sma) in Vhs.

3.4. Construction of V422 Δ Sma, V379 Δ Sma and E369 Δ Sma.

While deletion of the entire VP16 open reading frame from HSV-1 correlates with a lethal defect in assembly (Weinheimer *et al.*, 1992), it is evident that the acidic tail of VP16 is not an absolute requirement for HSV-1 growth

in tissue culture. The mutants V422, V379 and E369 display a preference for growth on a cell line which complements with wild-type VP16, however, they can replicate (albeit at significantly lower titres), on a non-complementing cell line.

HSV-1 mutants with defects in the virion host shutoff gene are not significantly altered in their growth characteristics. They lack the characteristic HSV-1 host shutoff phenotype and produce slightly smaller plaques than wild-type virus. However, in analysis of single rounds of replication, they grow at rates only slightly below wild-type virus (Read *et al.*, 1993, Read and Frenkel, 1983). Two well studied viral mutants of vhs, Δ Sma and vhs-1, display the characteristic defect in host shutoff and are quite similar in both plaque morphology and growth rates. The key difference between the two mutants is that vhs- Δ Sma is not packaged into new virus, either because it lacks a region required for its transport, or it is conformationally altered to such an extent that it cannot be assembled (Read *et al.*, 1993). It is interesting that the deletion in vhs- Δ Sma also eliminates binding to VP16, which may indicate why it is not packaged (Smibert *et al.*, 1994).

It is plausible that the preference for growth on a complementing cell line by V422, V379 and E369 is a result of the inability of tail-deleted VP16 to completely attenuate vhs activity during infection. If so, inactivating vhs within these mutants should relieve the dependence on the presence of wild-type VP16. It was of interest, therefore, to determine the effects of eliminating the interaction between vhs and the tail-deleted VP16 derivatives on replication and plaque morphology by constructing double mutant viruses.

In constructing the double mutant viruses, a similar procedure to that used in making E369 was employed. The plasmids 14a/422, 14a/379, 14a/369 (Figure 3c) were each co-transfected with infectious 8MA Δ Sma DNA into QL10 cells. 8MA Δ Sma is deleted for VP16 and contains an inactivating mutation in its vhs locus (Lam et al., 1996). It also contains a spontaneous mutation within its TK locus and therefore, displays a TK-minus phenotype (Dr. J. Smiley, personal communication). Thus, the selection procedure which was used in constructing E369, could not be used in creating the double mutant viruses. Viral recombinants were selected by virtue of their ability to replicate on Vero

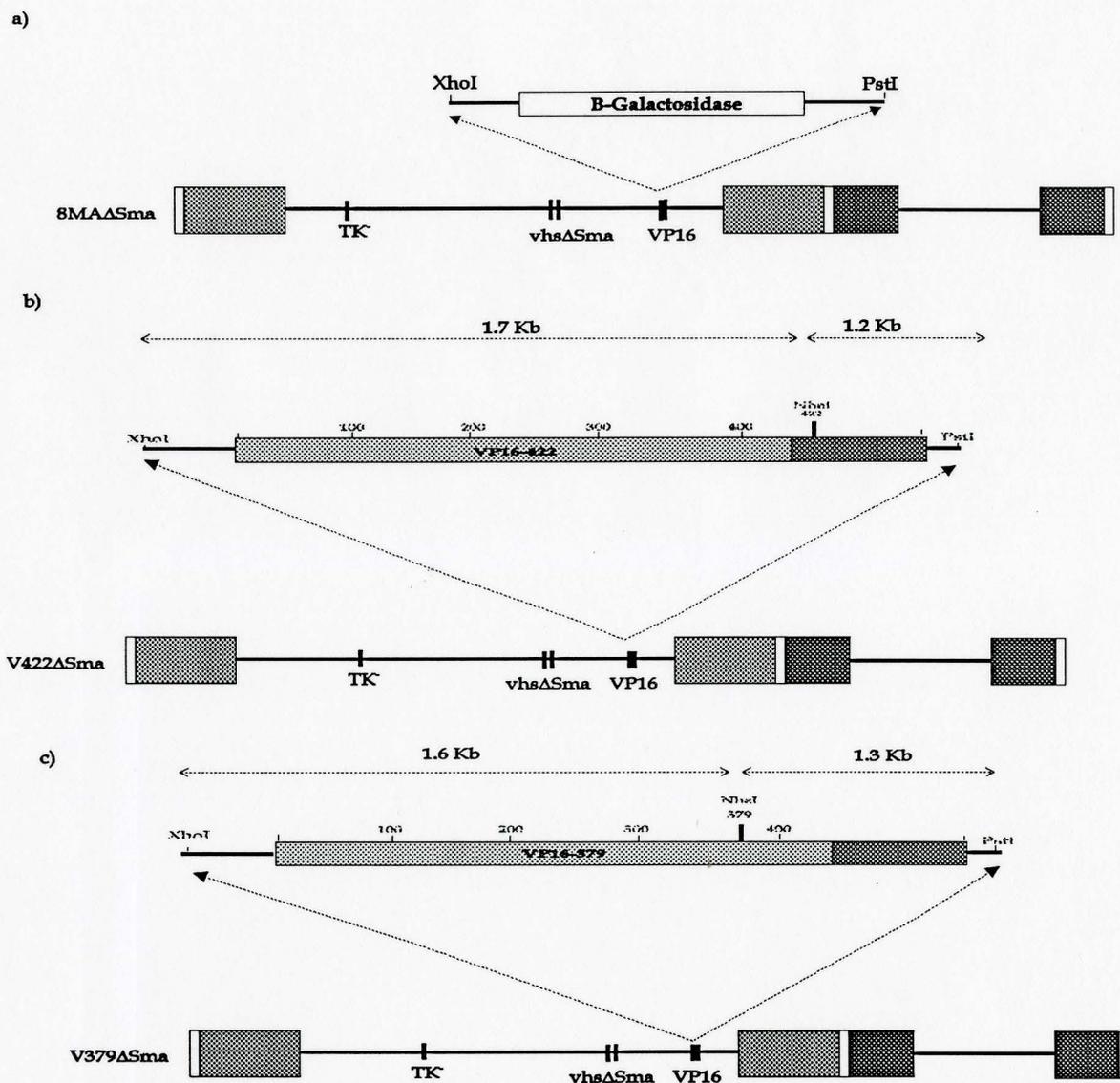


Figure 8:

Identification of recombinants into the VP16 locus of 8MA Δ Sma. a) 8MA Δ Sma contains a deletion (Δ Sma) in its vhs locus and a β -Galactosidase gene in its VP16 locus. A spontaneous mutation in the thymidine kinase locus renders the virus TK minus. Screening for the presence of the VP16-422 (b) and VP16-379 (c) genes recombined into the VP16 locus of 8MA Δ Sma was facilitated by the presence of the unique NheI sites within each mutant gene.

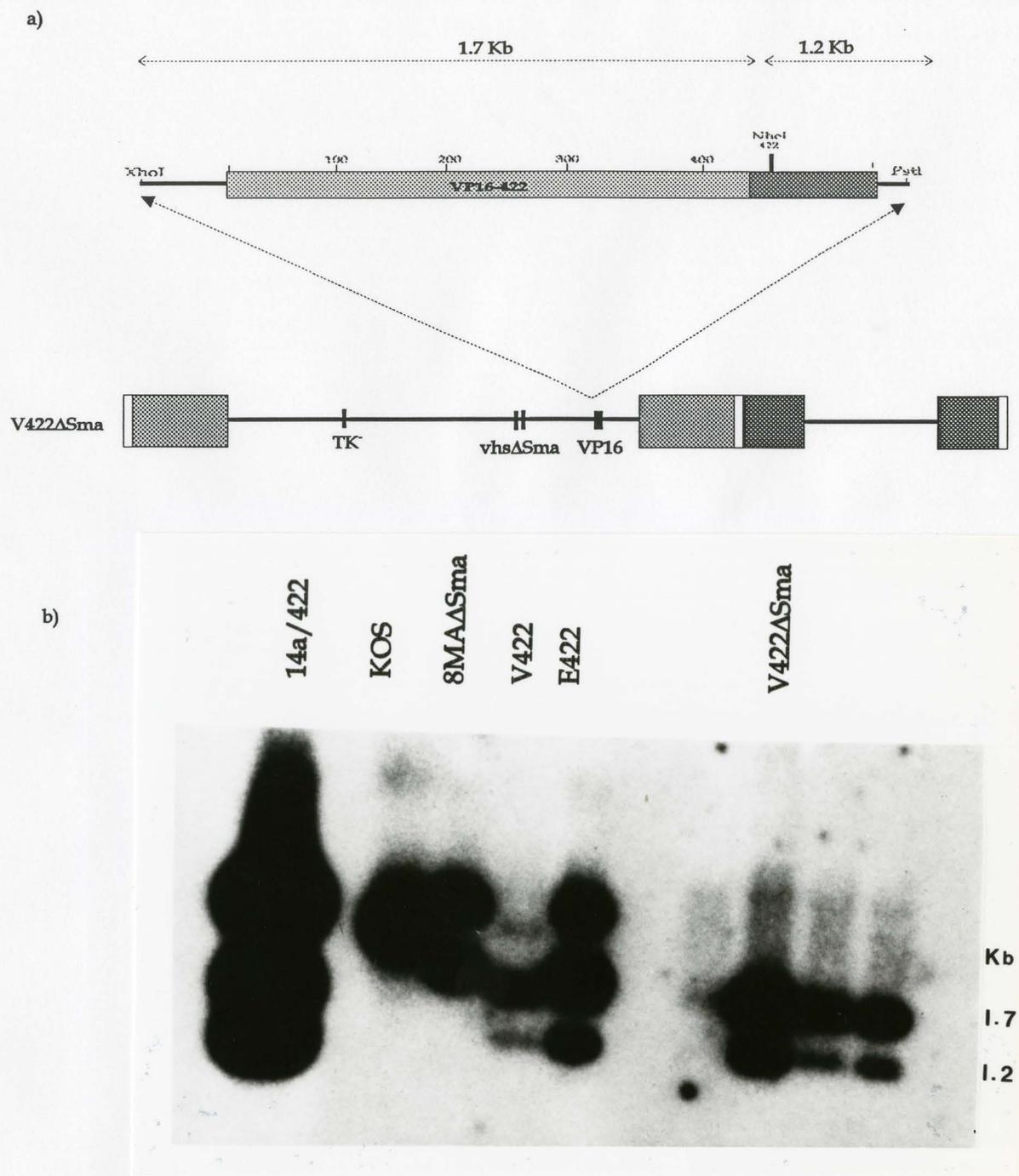


Figure 9:

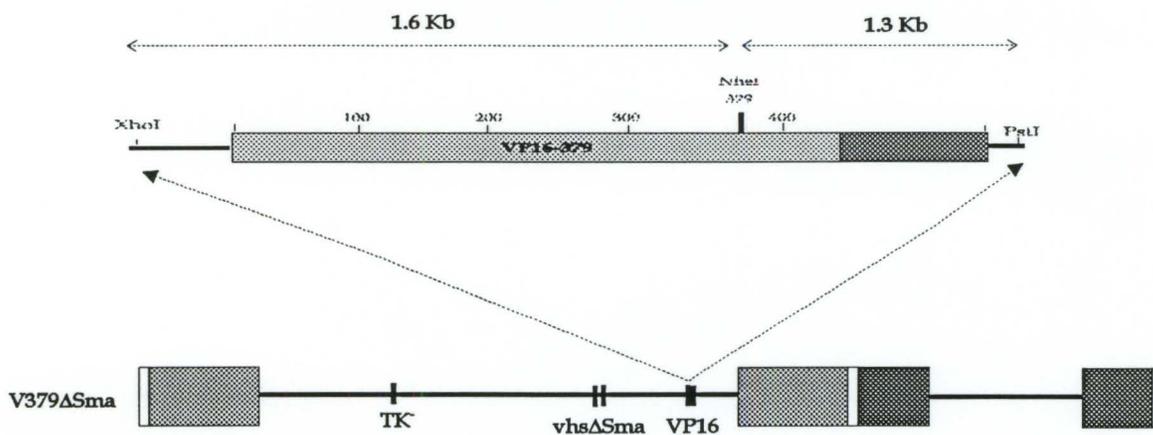
Recombination of VP16-422 into the VP16 locus of 8MAΔSma and Southern blot analysis of V422ΔSma. Viral DNA was digested with XhoI/PstI/NheI and plasmid DNA with XbaI/NheI. The blot was probed with pKOSVP16. The bands corresponding to VP16-422 are seen in the plasmid control, V422 and V422ΔSma lanes.

cells (the non-complementing cell line) because 8MA Δ Sma itself requires VP16 supplied in *trans* to replicate. One thousand PFUs from the initial virus burst (on QL10 cells) were plated onto 10 large plates of Vero cells. Plaques were picked onto 24 well dishes of Vero cells and then harvested for Southern blot analysis.

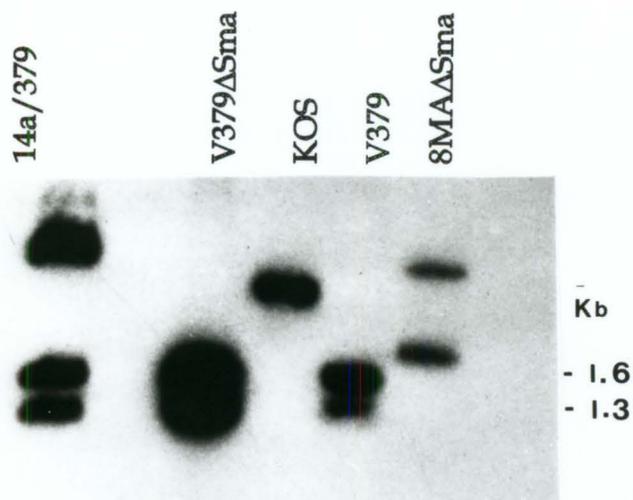
a) V422 Δ Sma

To confirm that the recombinant virus contained the appropriate mutation in its VP16 locus, isolates were compared against known viruses and other plasmid DNA controls in a Southern blot analysis. The VP16-422 gene contains an unique NheI site at amino acid codon 422 which facilitated the screening process (Figure 8) The viral DNA and plasmid controls were probed with pKOSVP16, which encodes the wild type VP16 gene. The four potential recombinant virus isolates displayed the same pattern as seen in the virus V422 and in the plasmid 14a/422 when probed with pKOSVP16, indicating that the appropriate VP16 mutation had been incorporated into the virus V422 Δ Sma (Figure 9). The double mutant virus was then analyzed to ensure the presence of the Δ Sma mutation in the vhs locus. Again, samples were run against known viruses and other

a)



b)

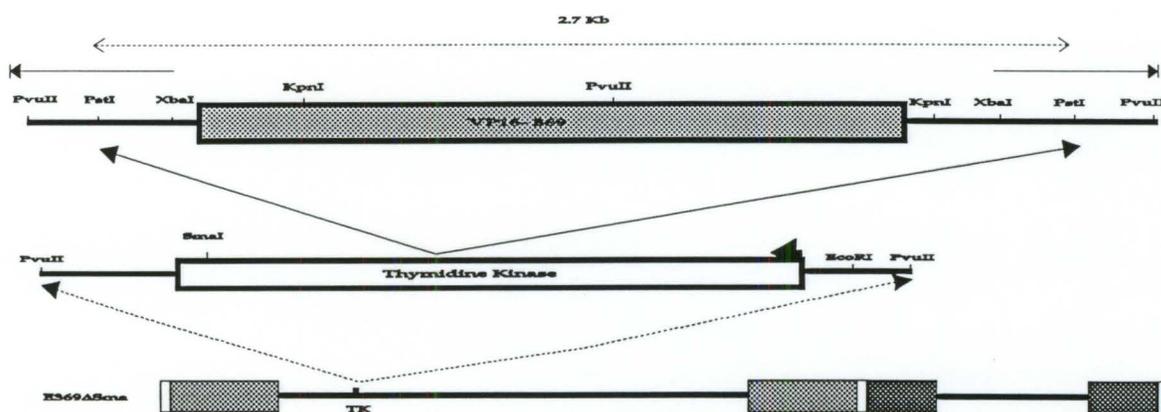
**Figure 10:**

Recombination of VP16-379 into the VP16 locus of 8MAΔSma and Southern blot analysis of V379ΔSma. Viral DNA was digested with XhoI/PstI/NheI and plasmid DNA with XbaI/NheI. The blot was probed with pKOSVP16. The bands corresponding to VP16-379 are seen in the plasmid control, V379 and V379ΔSma lanes.

plasmid controls. The DNA samples were cut with ApaI and EcoRV. Viruses containing the Δ Sma mutation can be distinguished because the deletion in vhs eliminates one of the two ApaI sites (Figure 12). The Δ Sma deletion is evident in V422 Δ Sma when compared to the patterns for the KOS and 8MA Δ Sma viruses (Figure 12).

b) V379 Δ Sma

The mutation in the VP16-379 gene was also created by linker insertion, which introduces stop codons in all three reading frames and a unique NheI site at the codon specifying amino acid 379 (Figure 8). In screening for the presence of the mutant VP16 gene in the potential recombinant viruses, isolates were compared against known viruses and a plasmid control. The pattern of the V379 Δ Sma double mutant virus matches that of the V379 virus and the 14a/379 plasmid control when probed with pKOSVP16 in a Southern blot (Figure 10). To confirm that the V379 Δ Sma virus contains the Δ Sma deletion in its vhs locus, the virus was compared in a Southern blot to other known viruses and plasmid controls. The pattern for V379 Δ Sma matches that of 8MA Δ Sma when probed with pCMVvhs, indicating the presence of the correct mutation in this virus (Figure 12).



a)

b)

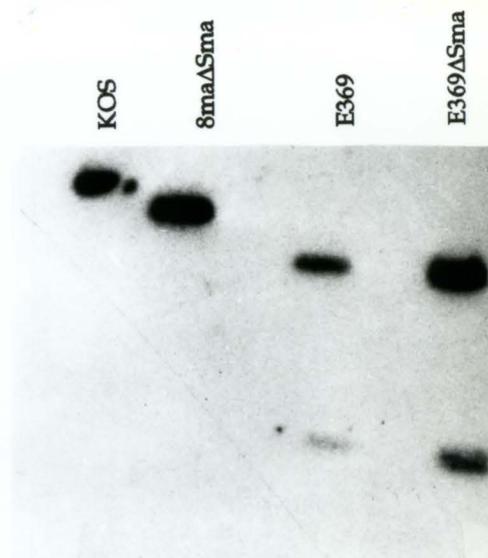
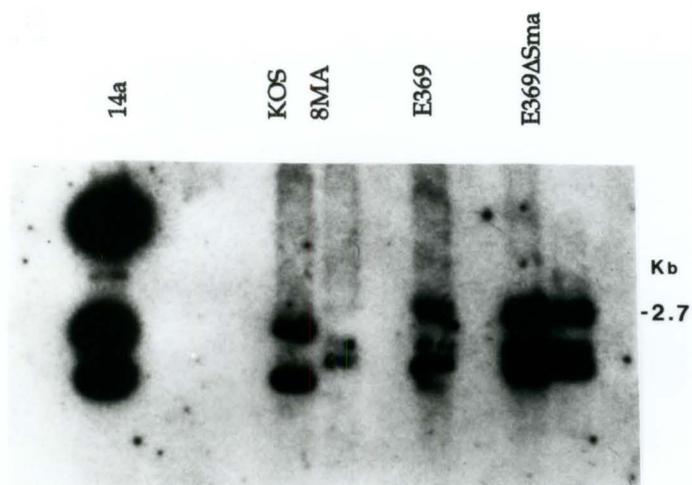


Figure 11:

Recombination of VP16-369 into the tk locus of 8MAΔSma and Southern blot analysis of E369ΔSma.

a) Viral DNA was digested with XhoI/PstI/SacI and plasmid DNA with XbaI/SacI. The 2.7 Kb band is the largest seen in E369 and E369ΔSma. The remaining bands are also seen in 8MAΔSma, and are the result of probing with pKOSVP16 which has sequences homologous to those surrounding the VP16 locus in 8MAΔSma.

b) Screening for the disruption of the tk locus in E369ΔSma. DNA was digested with XbaI/KpnI. Recombination into the tk locus introduces the XbaI sites flanking the VP16-369 gene and facilitates the identification of E369 and E369ΔSma when probing with the HSV-1 thymidine kinase gene encoded on ptkSB-x.

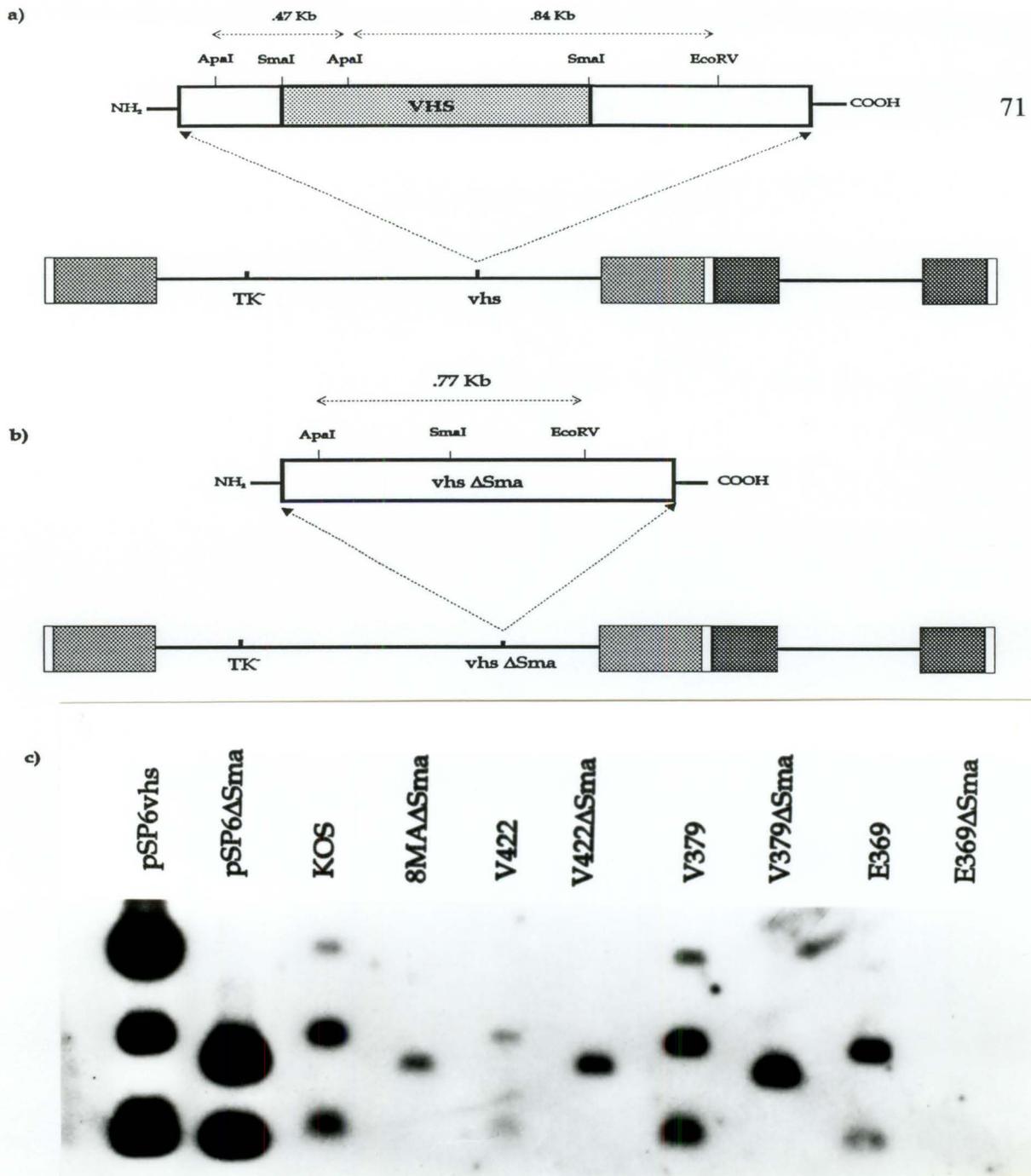


Figure 12:

The double mutant viruses contain the Δ Sma deletion.

a) Schematic structure of the vhs gene with expected fragment sizes indicated.

b) Schematic structure of the vhs gene containing the Δ Sma deletion with expected fragment sizes indicated.

c) All DNA samples were digested with ApaI and EcoRV to differentiate between viruses containing wild type vhs and those containing the deletion in Δ Sma; the blot was probed with pCMVvhs. The lowest band in the plasmid control lanes is a sequence common to the plasmids.

c) E369 Δ Sma

Two screening procedures were used in confirming the presence of the truncated VP16-369 gene in the virus E369 Δ Sma. The truncated derivative of VP16 lacks a SacI site that is present in the wild type gene, thus it was possible to distinguish between the two. The potential recombinants were compared against known viruses and a plasmid control in a Southern blot analysis. The E369 Δ Sma virus shows a pattern similar to E369 but which differs from the wild type KOS and the plasmid 14a (which contains the wild type VP16 gene) when probed with pKOSVP16 (Figure 11a). This indicated that the VP16-369 gene had likely been recombined into the thymidine kinase locus, as in E369. To confirm the disruption of this locus the E369 Δ Sma isolates were compared against known virus controls. In a Southern blot probed with ptkSB-x (which encodes HSV-1 Thymidine Kinase), the E369 Δ Sma virus pattern matches that of E369, confirming that this locus has been disrupted by the insertion of the mutant VP16 gene (Figure 11b) (See Figure 5 for map). The presence of the Δ Sma mutation in E369 Δ Sma was confirmed by Southern blot analysis and probing with pCMVvhs. The pattern characteristic of all viruses with the Δ Sma deletion

is seen in E369 Δ Sma (Figure 12).

3.5. Characterization of the Double Mutant Viruses.

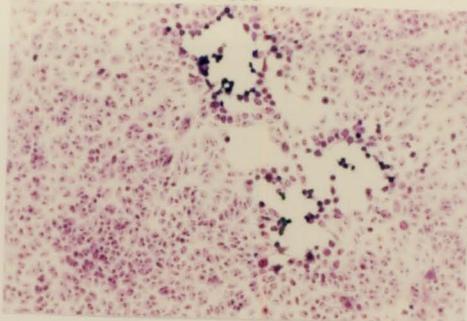
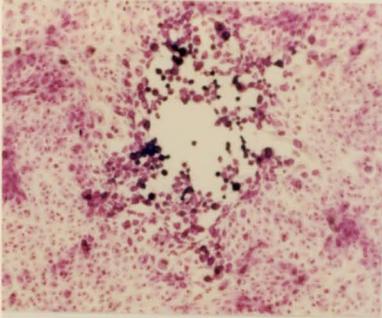
We hypothesized that inactivating vhs within the viruses encoding tail-deleted VP16 would confer a growth advantage. However, they grew poorly even when propagated which provided VP16 in *trans*. This problem was rectified by propagating the double mutants on the complementing cell line in the presence of 3mM Hexamethylene bisacetamide (HMBA), which enhances the transcription of select HSV-1 genes and helps to drive the lytic cycle. It is interesting to note that propagation on the complementing 16-8 cell line is sufficient to generate a good stock of the mutants V422 and V379. With E369 and each of the double mutants, it was necessary to propagate them in the presence of HMBA on the complementing cell line. For V422 Δ Sma and V379 Δ Sma, this need might result from the loss of interaction between vhs and VP16, such that the tail-deleted derivatives are more able to negatively influence the transcription of viral genes by squelching host factors away from the endogenous VP16, in the absence of vhs binding. The amino terminus of

Plate 2: Plaque Morphology of HSV-1 Mutants on QL10 and Vero cells.

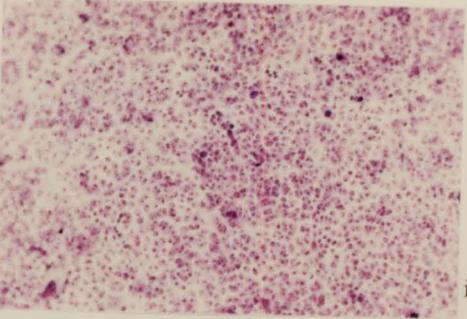
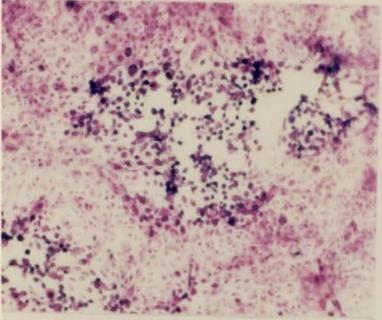
16-8 derived stocks of each virus (except Vero derived Δ sma) were titred in tandem on QL10 and Vero cells. Monolayers were stained with crystal violet 48 hours post-infection and plaques were viewed under 100X magnification.

QL10

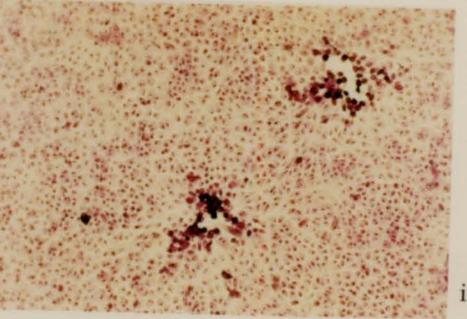
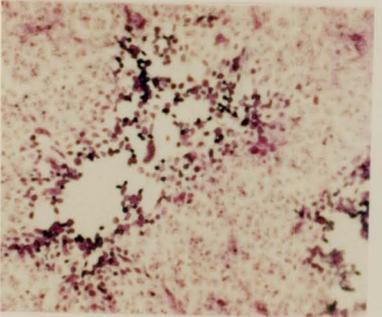
Vero



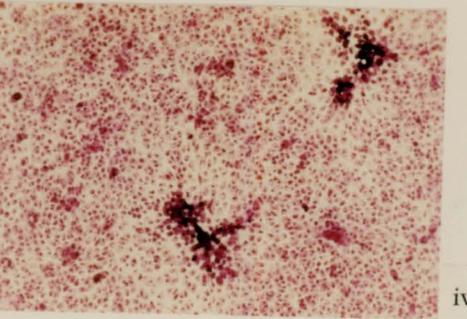
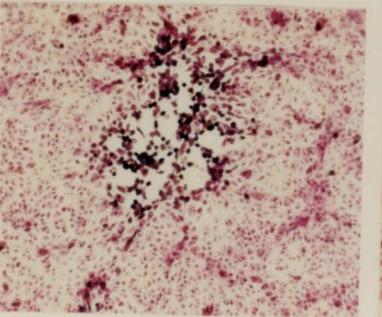
i) Δ Sma



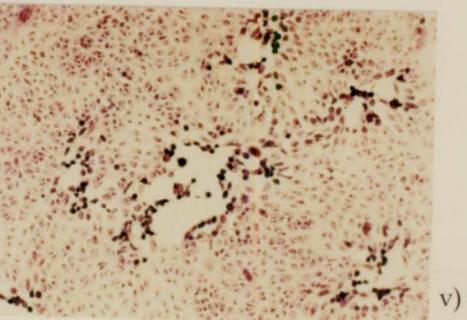
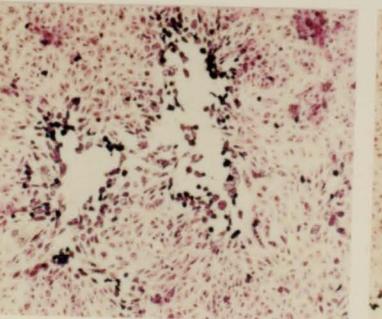
ii) 8MA Δ Sma



iii) V422 Δ Sma



iv) V379 Δ Sma



v) E369 Δ Sma

VP16 is the region responsible for promoter recognition and also interaction with host factors such as Oct-1 and HCF (Xiao and Capone, 1990; Werstuck and Capone, 1989) which associate with VP16 as a prerequisite to its binding to DNA. With E369 and E369 Δ Sma, the dependance on HMBA might result from the same effect. With regards to E369, it is conceivable that *in vivo*, VP16-369 is more easily dissociated from vhs and thus, can interfere with viral gene transcription in the same fashion.

Stocks of each double mutant virus propagated on 16-8 cells (which provides VP16 *in trans*), were assayed for their plaque morphology. The stocks were titred in tandem on QL10 cells (complementing) and Vero cells (non-complementing) and the plaques were stained with crystal violet. QL10 cells were used as the complementing cell line because they support better plaque formation by VP16 mutant viruses (Plate 2).

a) V422 Δ Sma

The plaque morphology of V422 Δ Sma on QL10 cells resembles that of the wild type virus, KOS. Plaques are large clearings in the monolayer with many surrounding rounded cells. On Vero cells a pattern similar to that for

V422 is seen, the plaques are much smaller, with only a slight clearing of the monolayer. The plaque morphology for the virus Δ Sma is quite similar on Vero and QL10 cells, the plaques being slightly smaller on the non-complementing cell line. 8MA Δ Sma produces no plaques on Vero cells, but in the presence of wild type VP16, on QL10 cells, its plaque morphology resembles wild type virus (Plate 2).

b) V379 Δ Sma

On the complementing cell line, QL10 cells, the V379 Δ Sma double mutant virus produces plaques which are almost as large as wild type virus. On Vero cells, the plaques are smaller with very little or no clearing of the monolayer. The plaques appear as aggregates of rounded cells (Plate 2).

c) E369 Δ Sma

The E369 Δ Sma mutant plaque formation is characteristic of the other two double mutants on the complementing cell line, QL10 cells. On Vero cells it differs slightly from V379 Δ Sma and V422 Δ Sma. The plaques on the non-complementing cell line are largely heterogenous in size and consist of

Virus	16-8 Titre	Vero Titre	16-8/ Vero
KOS	6.8×10^8	9.0×10^8	0.75
8MA	5.1×10^7	1.0×10^3	5.1×10^4
8MA Δ Sma	1.0×10^8	1.0×10^3	1.0×10^5
Δ Sma	9.6×10^7	2.5×10^8	0.38
V422	2.2×10^7	3.2×10^6	6.9
V379	8.3×10^8	5.0×10^6	1.7×10^2
E369	3.8×10^8	1.0×10^6	3.8×10^2
V422 Δ Sma	5.1×10^7	2.4×10^7	2.1
V379 Δ Sma	1.6×10^7	5.0×10^6	3.2
E369 Δ Sma	1.0×10^6	1.2×10^6	0.83

Table 2: HSV-1 Virus Titres on 16-8 and Vero Cells.

16-8 derived stocks of each virus were titred by serial dilution in tandem on 16-8 and Vero cells. The 16-8 cell line provides full length VP16 in *trans*. Plaques were counted and virus titre was expressed in PFU/mL (Plaque forming units). The titres of each virus on 16-8 cells and on Vero cells were then expressed as a ratio.

clearings in the monolayer surrounded by rounded cells (Plate 2).

It was difficult to generate a stock of this virus which would permit significant analysis of its characteristics. The virus grows very slowly but produces reasonable cytopathogenic effects (cpe) during infection. However, the stock titres were consistently low, indicating that the virus likely has a very high particle to plaque forming unit ratio.

The plaque morphology of the single and double mutants are similar but this doesn't necessarily show that the double mutant viruses displayed the same preference for growth on a complementing cell line as seen with the single mutants. Stocks of each virus propagated on 16-8 cells were diluted serially in tandem on 16-8 cells and Vero cells and the resulting plaques were counted to determine if a disparity in titre is apparent for the double mutants (Table 2).

The single mutants V422, V379 and E369 titre at significantly higher levels on the complementing 16-8 cells than Vero cells. It appears that as VP16 is progressively truncated from 422 to 369 amino acids, the single mutant

virus relies increasingly upon the wild-type VP16 supplied by the complementing cell line. By inactivating vhs within these same virus mutants, the preference for growth on the complementing cell line is no longer apparent. The ratio of titre between 16-8/Vero cells is quite small for each of the new double mutant viruses. In fact, this ratio declines over 300 times from the E369 mutant to the E369 Δ Sma double mutant. Furthermore, the trend that showed an increased preference for *trans*-VP16 correlating to progressive truncation of VP16 within the single mutant viruses, is gone. It is apparent that by inactivating vhs in the viruses encoding the tail-deleted VP16, the requirement for some function of wild-type VP16 has been alleviated. Perhaps the tail-deleted VP16 derivatives are not capable of fully arresting vhs activity and this limits or hinders the expression of viral genes in those mutants. Through the aid of the endogenous VP16 from 16-8 cells, the single mutants V422, V379 and E369 are better able to contend with vhs activity and thus, are able to replicate like wild-type virus on these cells. Alternatively, the acid tail of VP16 may assist the tail-less derivatives in fulfilling a role in virus assembly. In the absence of vhs-VP16 interaction in

the double mutant viruses, the tail-less mutants may be better able to effect this function in the presence or absence of endogenous VP16.

3.6. HSV-1 Polypeptides Produced by the Double Mutant Viruses.

VP16 was immunoprecipitated from ³⁵S-Met labelled infected 16-8 cell extracts with a polyclonal rabbit antiserum to VP16. The full length VP16 protein found in KOS and ΔSma runs at approximately 65 kDa. The KOS and ΔSma viruses are derived from Vero cells, while the remaining mutant virus stocks are derived, by necessity, from 16-8 cells. The endogenous VP16 which is incorporated into the mutant viruses is not visualized in these experiments, the 65 kDa band observed in addition to the truncated VP16 bands is made by the infected cell line, 16-8. The truncated VP16-422 runs at about 48 kDa and VP16-379 is seen at approximately 43 kDa (Figure 13). There is a clear delay in the appearance of both viral and endogenous VP16 by the double mutant viruses. Although interesting, this is not

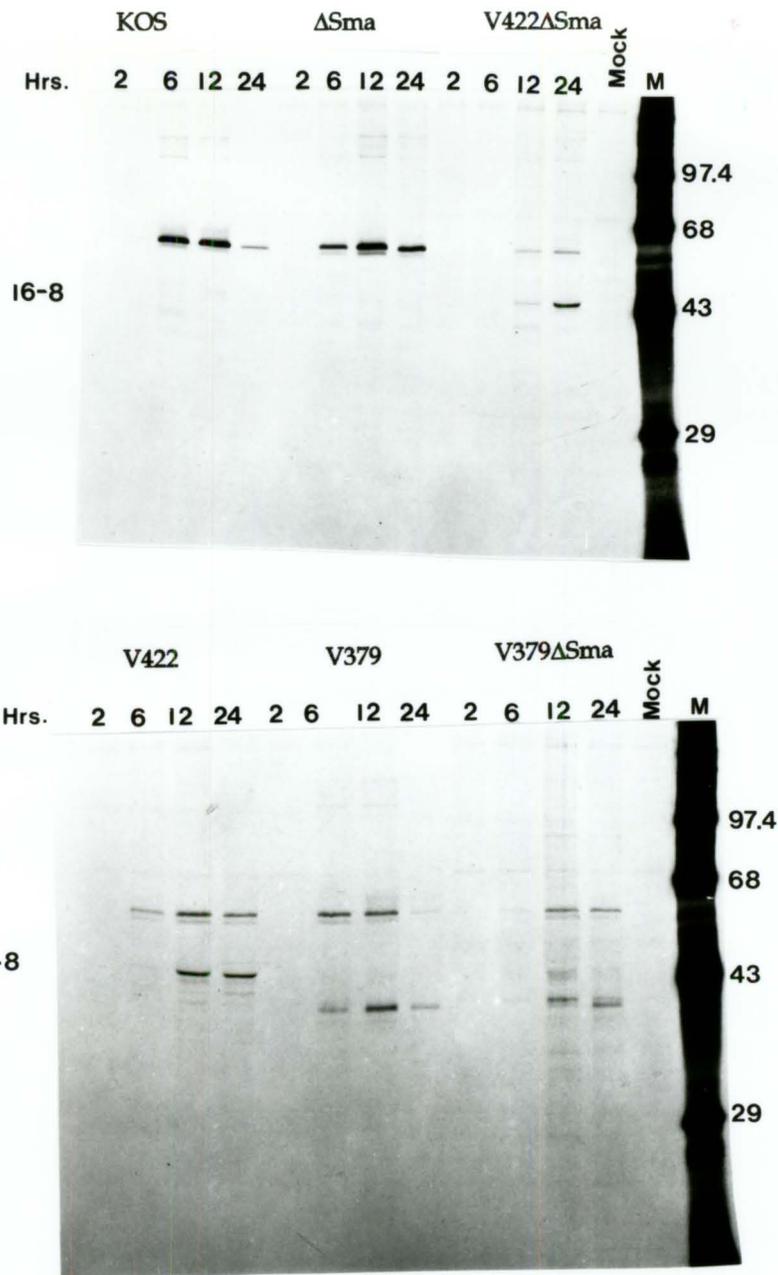


Figure 13: VP16 Production by V422 Δ Sma and V379 Δ Sma. 16-8 cells were infected with 16-8 derived stocks of each virus (except KOS and Δ Sma, which are Vero derived) and pulse-labelled for 1 hour with 35 S-Met. VP16 was immunoprecipitated from infected cell extracts at various times with a polyclonal antiserum to VP16. Immunoprecipitated proteins were resolved on a 10% SDS-PAGE and visualized by autoradiography.

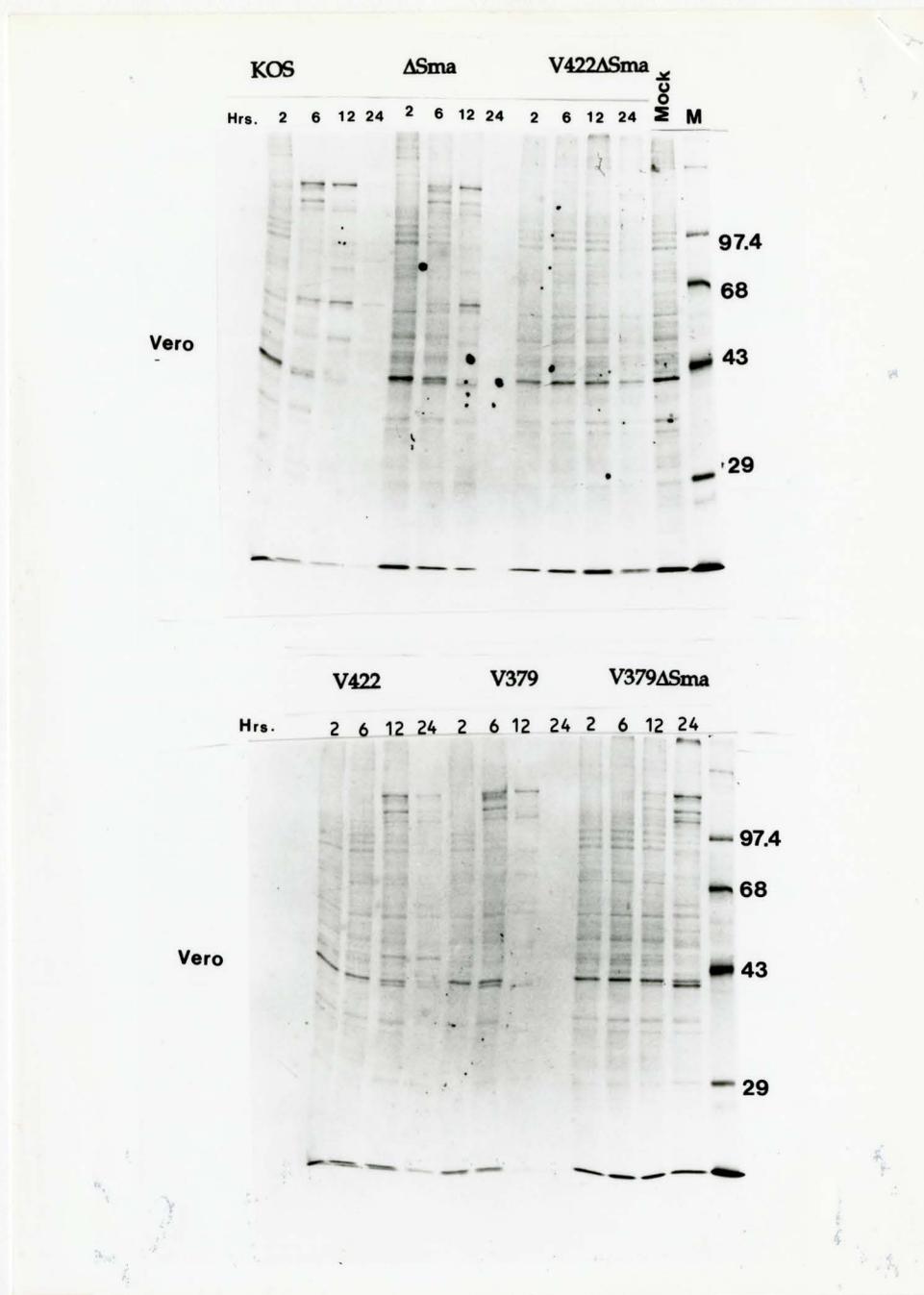


Figure 14: Host Shutoff Phenotype of the Double Mutant Viruses. Vero cells were infected with 16-8 derived stocks of each virus (except for KOS and Δ Sma, which are Vero derived). 35 S-Met labelled infected cell extracts from various times were analyzed by SDS-PAGE and autoradiography. As expected, the host shutoff phenotype is absent in both V422 Δ Sma and V379 Δ Sma. Δ Sma at the 24 hour point was incorrectly prepared and thus shows no bands.

necessarily a result of the disruption of the interaction between the tail-deleted VP16 and vhs affecting protein expression, because the restrictions that the double mutation imparts on other aspects of virus growth are undetermined. For instance, the effects of the double mutation on viral entry are undetermined and any delay in the kinetics of viral entry would likely result in delayed expression of viral proteins.

Infected cell extracts from the same labelling experiment on Vero cells were analyzed by SDS-PAGE and autoradiography (Figure 14). As might be expected, there is no evidence of the host shutoff phenomenon in the lanes for V422 Δ Sma and V379 Δ Sma.

To further characterize the double mutant virus and to study the ability of the tail-deleted mutants to produce HSV-1 polypeptides, the production of proteins representative of each kinetic class were studied. To ensure that only the tail-less VP16 could influence the expression of viral genes early in infection, the mutant viruses were derived from Vero cells (to eliminate the presence of wild-type VP16 in the virus). Vero cells were infected with each virus at an MOI of 3 and pulse-labelled

with ^{35}S -Met for 1 hour prior to harvest. The infected cells were harvested at 2, 6, 12 and 24 hours post-infection and extracts were made.

Ribonucleotide reductase consists of 38 and 144 kDa subunits, which comprise the immediate-early and early components of the protein (Bachetti *et al.*, 1984). ΔSma and KOS (only 6 hour point shown) produce similar levels of the large subunit (Figure 15). V379 and V379 ΔSma do not produce ribonucleotide reductase to the same level. It is difficult, however, to draw direct comparison between these viruses. For example, it is conceivable that the mutant viruses will need a greater virus load to initiate a productive infection in a given cell due to the fact that the number of 'infectious' particles on average is lower in a mutant than in wild-type virus. Thus, while each virus infects with the same MOI, the mutant viruses may actually require 100 times more virus than wild-type entering the cell in order to express its genes. Again it is interesting to observe the characteristic delay in the appearance of the protein produced by V379 ΔSma . However, as mentioned, this cannot be ascribed specifically to the double mutation in V379 ΔSma and its hindering of viral gene expression because

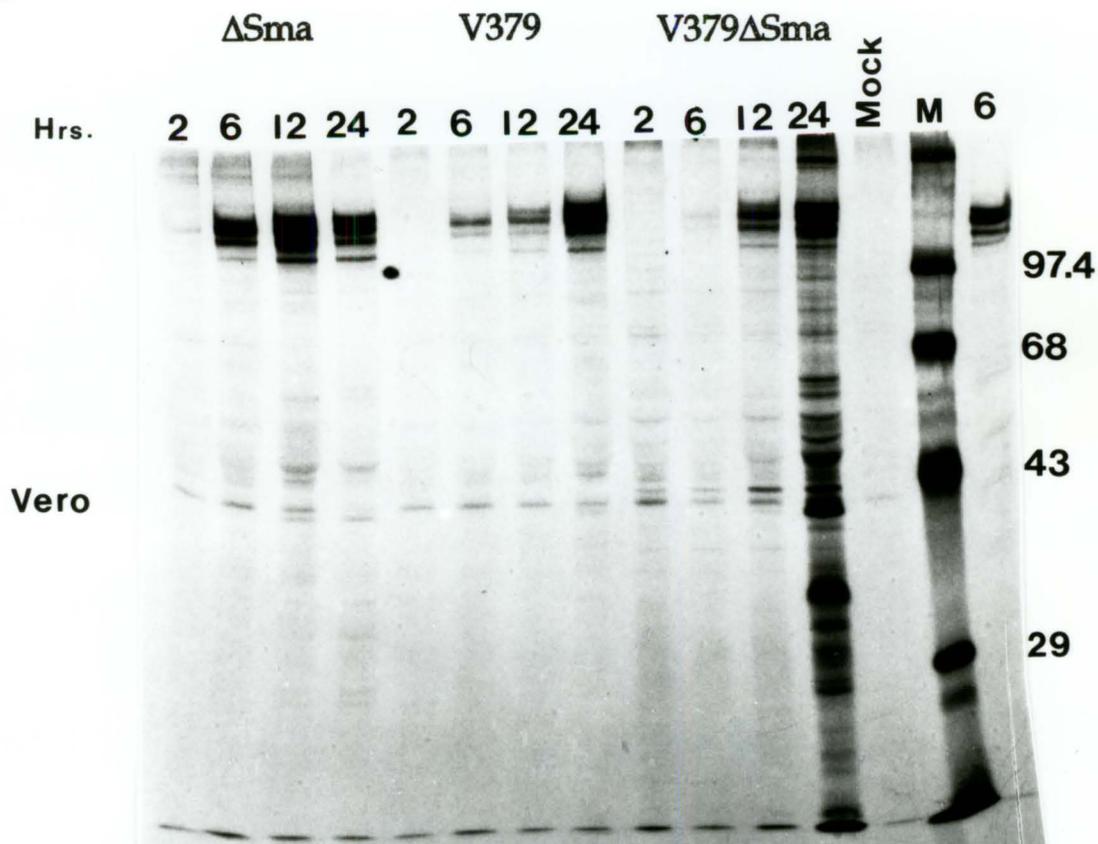


Figure 15: Production of Ribonucleotide Reductase by HSV-1 Mutants.

Vero cells were infected with each Vero-derived virus at an MOI of 3. Cells were pulse-labelled with ^{35}S -Met for 1 hour prior to harvesting at 2, 6, 12 and 24 hours. Ribonucleotide reductase was immunoprecipitated from infected cell extracts at various times with a polyclonal rabbit antiserum (R1) and the products were resolved on a 10% SDS-PAGE followed by autoradiography. The KOS control at far right is from the 6 hour time point.

other effects that these mutations might have on the virus are undetermined. The HSV-1 late protein glycoprotein B (gB) runs at approximately 108 kDa and the characteristic delay in expression by V379 Δ Sma is again evident (Figure 16). It is clear that there is a correlation between the double mutation and a delay in the kinetics of HSV-1 protein expression, whether this is actually due to the absence of vhs activity, the inability of tail-less VP16 to drive transcription or both, is unclear.

The tail-less VP16 derivatives encoded by V379 and V379 Δ Sma appear sufficient to effect expression of select HSV-1 proteins, in the absence of wild-type VP16. This would seem to indicate that some residual transcriptional activity is present within the 379 N-terminal amino acids of VP16 or that the virus can utilize other means (perhaps host transcription factors) to initiate its gene expression. The immediate-early promoters are known to contain numerous binding sites for the host transcription factor SP1 (Jones and Tijan, 1985).

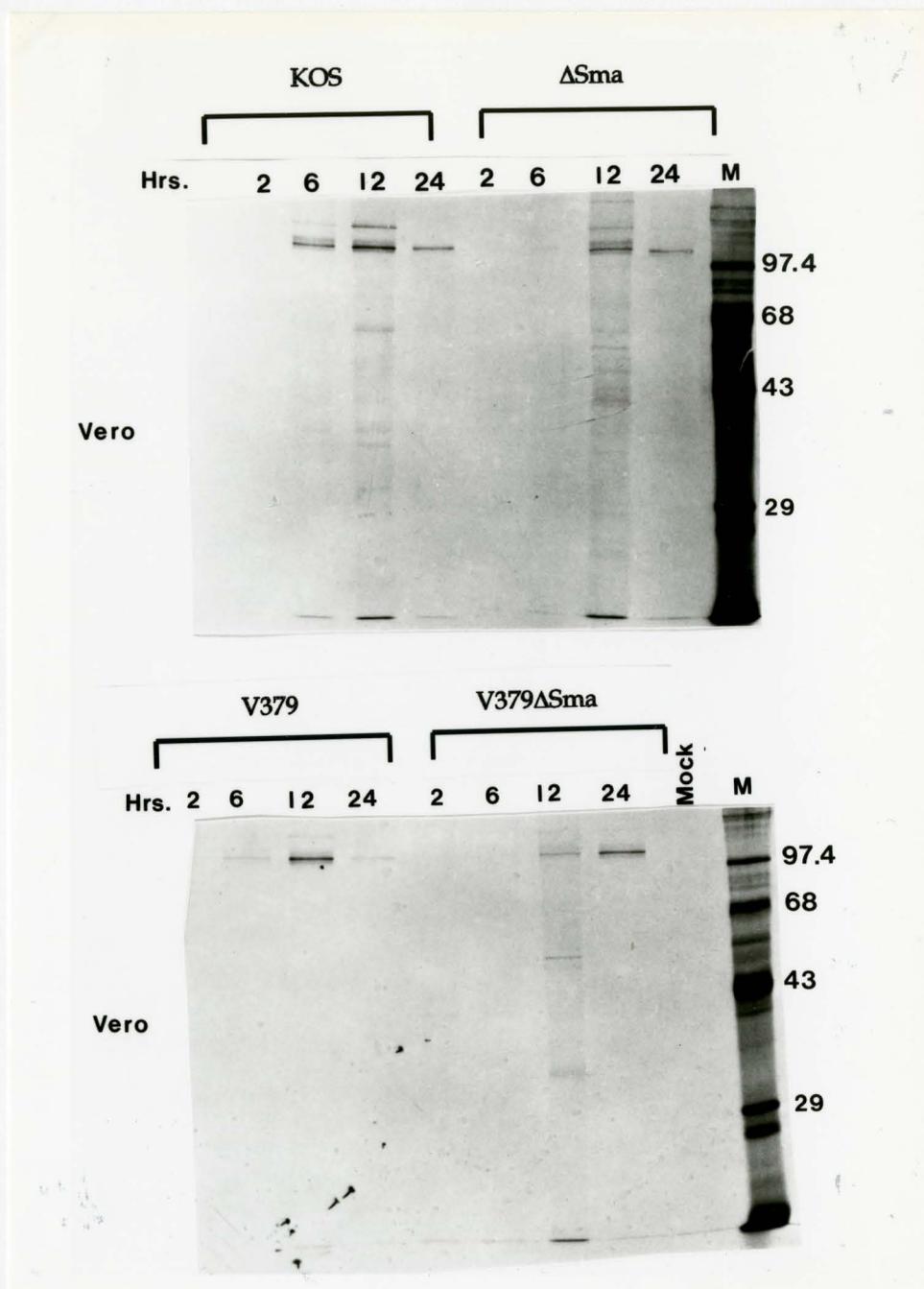


Figure 16: Production of Glycoprotein B by HSV-1 Mutants. Vero cells were infected with each Vero-derived virus at an MOI of 3. Cells were pulse-labelled for 1 hour with ^{35}S -Met and harvested at 2, 6, 12 and 24 hours. Glycoprotein B was immunoprecipitated with a polyclonal antiserum and the proteins were resolved on a 10% SDS-PAGE followed by autoradiography.

3.7. Single Step Replication Assay

To examine the effect of the mutations in VP16 and vhs on virus growth, single step growth curves for KOS, V379, V379 Δ Sma and 8MA Δ Sma were constructed. 16-8 and Vero cells were infected with 16-8 derived viruses at an MOI of 3 for 1 hour. Following infection, the cells were washed twice with PBS to eliminate the presence of residual virus particles in the media. The cells were harvested at 3, 12 and 24 hours post-infection and virus stocks were made. Each virus stock was assayed for titre on QL10 cells. QL10 cells were chosen because the viral plaques formed on this cell line are easier to distinguish than on 16-8 cells.

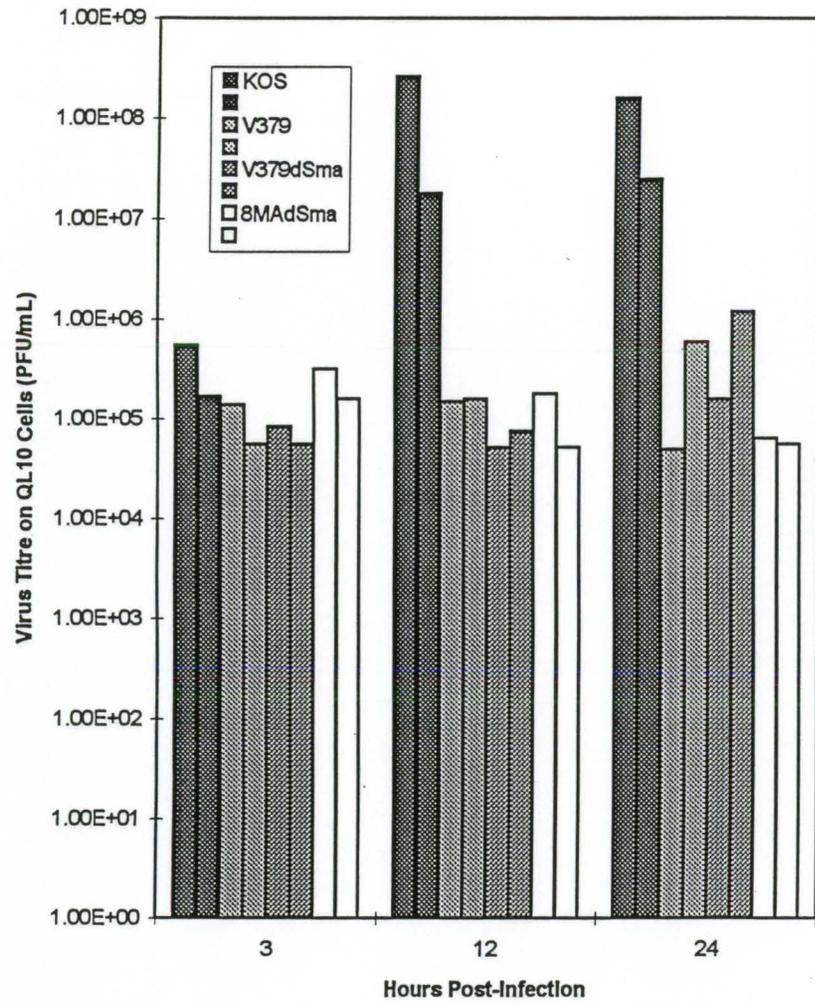
The viruses display a similar pattern of growth on the 16-8 cells, which supply VP16 in *trans* (Figure 17a). V379 has a slightly higher final titre than V379 Δ Sma on 16-8 cells, approximately 10-fold lower than the wild-type virus, KOS. 8MA Δ Sma yields a 50-fold lower level of plaque forming units than does KOS at 24 hours. On Vero cells, V379 Δ Sma produces about 2-fold more virus than V379, which is still more than 100 times less than KOS (Figure 17b). 8MA Δ Sma falls to about 1500-fold less virus production at 24 hours post-infection

compared to the wild-type virus. Through analysis of the virus growth patterns between cell lines, it is evident that the presence of VP16 supplied by the 16-8 cells has a greater effect on the V379 virus than V379 Δ Sma. On Vero cells, in the absence of endogenous VP16, V379 Δ Sma actually produces a higher virus titre. This implies that the removal of vhs is beneficial to V379 Δ Sma on Vero cells and that the VP16 in 16-8 cells is assisting V379 in attenuating vhs.

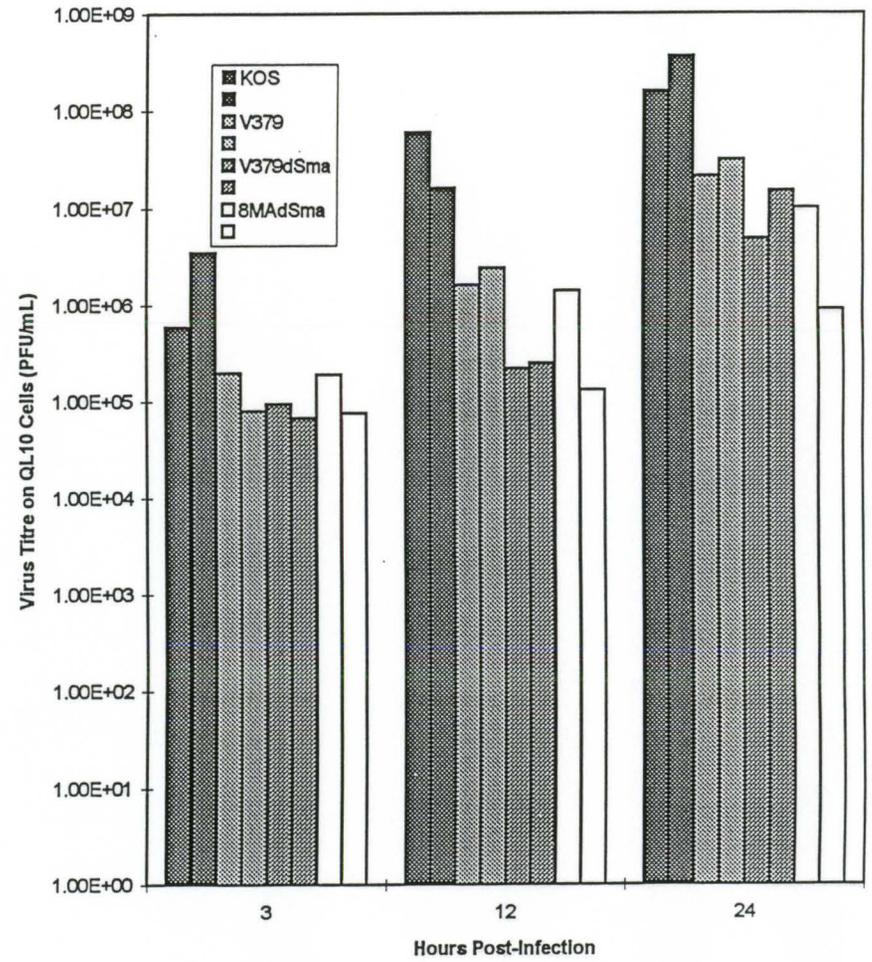
V379 Δ Sma shows a characteristic lag in its rate of growth early in infection on 16-8s and Vero cells. By 24 hours post-infection on both cell lines, it has either caught or surpassed V379 in titre. Thus, on Vero cells, the absence of vhs in V379 Δ Sma appears to initially impede its growth relative to V379, yet at late times in infection, enables it to yield significantly higher titres of virus. Underlying such characteristics on Vero cells may be the inability of V379 to fully downregulate vhs activity. Viral protein synthesis may be restricted at later times in infection in V379 on Vero cells, consequently affecting virus yield. Again, it is conceivable that in the presence of vhs, the tail-less VP16 derivatives rely on the

Figure 17: Single Step Growth Curves for HSV-1 Mutants.
16-8 (a) and Vero (b) cells were infected in tandem with each 16-8 derived virus (except for Vero derived KOS) at an MOI of 3. After 1 hour of infection, the cells were washed twice with PBS to remove residual virus from the media. Cells were harvested at 3, 12 and 24 hours post-infection and virus stocks were made. The virus stocks were titred on QL10 cells.

Single Step Growth Curve - Vero cells



Single Step Growth Curve - 16-8 Cells



endogenous VP16 to perform a role in virus assembly which they are unable to fulfill. In the absence of vhs, the tail-less derivative of VP16 is free to partially compensate for this requirement.

Discussion

The viable HSV-1 mutants V422 and V379 revealed that the acid tail of VP16 is not essential to virus replication in tissue culture (Lam, 1995, Lam et al., 1996). While these tail-deleted mutants display a clear preference for growth on a cell line which provides full length VP16 in *trans*, they are able to replicate on the non-complementing Vero cells.

The mutant E369 constructed here furthers the extent by which tail-less VP16 can be truncated and still perform necessary functions during infection. In vitro, VP16-369 is the tested limit for two functions of VP16. First, it is capable of enhancing transcription of a reporter gene in yeast, while the next truncation mutant VP16-335 cannot (Popova et al., 1995). VP16-369 binds specifically to the virion host shutoff protein of HSV-1, and again the next

truncation VP16-335, does not. It is not surprising then, that attempts to create a virus mutant encoding a 335 amino acid VP16 derivative have been unsuccessful (Dr. J. Smiley, personal communication).

In characterizing E369, it appeared that VP16-369 was able to attenuate vhs activity as the E369 mutant showed evidence of viral protein synthesis later in infection. The other tail-less VP16 proteins 379 and 422 helped sustain protein synthesis in the viral mutants to 9 and 12 hours, respectively (Lam, 1995).

A characteristic of the tail-deleted VP16 mutant viruses became apparent in analysis of their plaque morphology and titres on different cell lines. Each of these viruses produced morphologically wild-type plaques on a cell line (QL10 cells) which supplied full length VP16 in *trans*. On Vero cells, which do not provide VP16, the plaques were consistently smaller in size, often appearing as clumps of rounded up cells. When a 16-8 derived stock of these mutant viruses was titred in tandem on 16-8 and Vero cells, a trend was observed. With progressive truncation to the VP16 protein in these viruses, came an increasingly apparent requirement for full length VP16

during replication. This was evidenced by the difference in titre of a given stock of the mutant viruses on each cell line. In fact, E369 produced nearly a 400-fold higher titre on 16-8 cells than on Vero cells. Clearly, the endogenous VP16 was assuming some requirement that the tail-deleted VP16 molecule could not fully provide.

It is difficult to determine where the need for full length VP16 lies in the mutant viruses. The viruses can use the full length VP16 acquired from propagation on the 16-8 cell line, to transactivate their genes during the initial round of infection. Whether the truncated VP16 protein incorporated into newly made virus is able to do the same, is undetermined. If not, the full length VP16 provided by the 16-8 cells would account for the higher titres by each of these mutants on this cell line.

Each of the tail-deleted VP16 derivatives used here have been shown to bind to vhs *in vitro*. However, the situation during infection might be completely different. It is conceivable that as VP16 is progressively truncated from the C-terminus, it requires the assistance of full-length VP16 to completely attenuate vhs activity during infection. If it were saving the viral transcripts from

vhs-induced degradation, the preference for growth on a cell line which provides full length VP16 in *trans*, would be clear.

The defect in the 8MA virus (which is deleted for VP16) lies in the assembly of the virus (Weinheimer *et al.*, 1992). Each of the mutants V422, V379 and E369 are capable of growth on Vero cells (in the absence of wild-type VP16), albeit to lower levels than on 16-8 cells. Thus, the tail-less VP16 derivatives in these viruses must be able to at least partially compensate for the defect in 8MA and complete the assembly function required from wild-type VP16. However, it is possible that as VP16 is progressively truncated from its carboxy-terminus, it becomes increasingly unable to fulfill this role and requires the endogenous VP16 from 16-8 cells to produce wild-type levels of virus.

We predicted that inactivating the vhs gene within these virus mutants would be beneficial. By recombining each of the tail-deleted VP16 genes (422, 379 and 369) into the 8MA Δ Sma background, we generated three double mutant viruses. The plaque morphology of the double mutants was not significantly different from the single mutants. As with the single mutants, the plaques formed on the

complementing QL10 cells were similar to wild-type. On Vero cells, the plaques were small, generally clumps of rounded cells. When the titres of the double mutant viruses on 16-8 and Vero cells were compared, an interesting difference became apparent. The single mutants V422, V379 and E369 displayed a preference for growth on the complementing 16-8 cells and the difference in titre for a given stock of each virus between 16-8 and Vero cells increased as the viral VP16 protein was truncated from the C-terminus. The double mutant viruses V422 Δ Sma, V379 Δ Sma and E369 Δ Sma, however, did not display this trend. In fact, the preference for growth on the 16-8 cell line was no longer observed.

The double mutant viruses were not healthier than their single mutant counterparts, in fact, they had to be propagated on the 16-8 cell line in the presence of 3mM HMBA (to enhance virus growth). This may result from a dominant negative interference with viral gene transcription by the tail-less VP16 derivatives, whereby host factors are sequestered away from full length VP16 in the 16-8 cells. It is interesting that only the double mutant viruses and E369 required the HMBA during virus stock growth. Perhaps the absence of VP16-vhs interaction in the double mutants

leaves the tail-less VP16 free to interact with host factors. As for E369, it is conceivable that VP16-369 is more easily dissociated from vhs *in vivo*, allowing it to sequester host factors from the endogenous VP16. The HMBA compensates by non-specifically stimulating the expression of certain HSV-1 genes, essentially driving the lytic cycle (McFarlane *et al.*, 1992). When titred in tandem on 16-8 and Vero cells, no preference for the 16-8 cell line was seen by the double mutant viruses (Table 2) which seemed to indicate that the apparent requirement for full length VP16 by the single mutant viruses had to do with the presence of vhs in those same viruses. Perhaps the tail-deleted VP16 alone is not sufficient to fully attenuate vhs activity and it is the presence of the full length VP16 which boosts the virus titre by assisting in the downregulation of vhs. However, E369 shows evidence of some viral proteins late in infection, thus, there is no obvious correlation here. It is also possible that the tail-less VP16 derivatives are only partially able to fulfill an important role in virus assembly and that the endogenous VP16 from 16-8 cells assists in this capacity.

Within the double mutant viruses, the disruption of the

interaction between vhs and the tail-less VP16 and the elimination of vhs activity has interesting effects. Firstly, it removed the preference for growth on a complementing cell line. Perhaps in the absence of vhs binding, the tail-less VP16 derivatives are better able to perform a limited structural role. Alternatively, the loss of the preference for full length VP16 during infection by the inactivation of vhs, suggests that these viruses are benefiting from not having to contend with attenuating vhs.

In analyzing the expression of HSV-1 proteins by the double mutant viruses, specifically to determine how they were affected by the tail-deleted VP16 derivatives in the absence of vhs, virus stocks were grown on Vero cells, to eliminate the incorporation of a full-length VP16 into the viruses. Certain HSV-1 polypeptides were immunoprecipitated from infected Vero cell extracts and analyzed by SDS-PAGE and autoradiography. It was evident that both the single mutant V379 and the double mutant V379 Δ Sma were able to produce the proteins studied. Thus, in the absence of wild-type VP16, the tail-deleted VP16 derivatives could either enhance viral gene expression or at least did not prevent it. The possibility that the viral immediate-early genes

can be expressed by host transcription factors is quite real (Jones and Tijan, 1985).

A characteristic delay in the expression of HSV-1 proteins was apparent with the double mutant virus, however, this cannot be specifically ascribed to the double mutation. If there are lower levels of infectious particles generated by the mutant virus, it might require 100 times more virus to be equivalent in infectivity to wild-type virus. Thus, infecting with an equivalent MOI of mutant and wild-type viruses does not necessarily ensure that both viruses will be equally able to establish an infection in a particular cell. Still, there is clearly a delay in the kinetics of protein expression by the double mutant viruses, which might result from the tail-less VP16 derivatives interfering with viral immediate-early gene transcription. In addition, the absence of vhs could result in a delay in viral gene expression when using the host machinery to express its genes. Interestingly, the mutants Δ Sma and V379 Δ Sma show a similar pattern of delay in the production of gB, which suggests that the lack of vhs might be responsible for this occurrence.

Through analysis of single rounds of replication by the

viral mutants, 16-8 derived viruses were infected onto both 16-8 and Vero cells. Again, the preference for the wild-type copy of VP16 from the 16-8 cells is evident in the growth characteristics of V379. V379 produces slightly higher titres of virus than V379 Δ Sma on the complementing cell line, but on Vero cells, the double mutant has an approximately 2-fold higher titre at the 24 hour time point. VP16-379 in V379 Δ Sma is clearly able to perform a function which it cannot fully effect in V379. It appears that in association with vhs, VP16-379 cannot fulfill an important role in assembly or is less able to contend with vhs activity in the absence of full length VP16.

In generating stocks of the E369 Δ Sma virus, plaque formation was seen, however, the titre of the virus produced was consistently low. The viral mutant clearly has a low ratio of infectious particles relative to the total amount of particles produced. This begs the question of how such an uninfected virus is able to spread and form plaques and raises the possibility that it is able to spread between neighbouring cells without infecting them in conventional fashion.

In summary, while it appears that the acid activation

domain of VP16 is not essential to HSV-1 replication in tissue culture, those virus mutants which lack it experience significant increases in titre by growth on a cell line which supplies it in *trans*. With the concomitant inactivation of the *vhs* gene in the tail-deleted VP16 mutants, the preference for wild-type VP16 is no longer observed. This might indicate that the tail-less VP16 derivatives aren't able to fully attenuate *vhs* activity, and that the presence of full length VP16 during infection assists in this function, yielding higher titres of virus. Alternatively, or in addition, the tail-less VP16 derivatives may be hampered in their ability to complete the important structural role that VP16 plays during virus assembly. The preference for full length VP16 is removed in the double mutant viruses, which suggests that in the absence of *vhs* interaction, the tail-less VP16 derivatives are more able to carry out some function that was being compensated for by the endogenous VP16 in the single mutant viruses. Much remains to be discovered about the manner in which VP16 attenuates *vhs* during infection, and the temporal nature of this interaction. The double mutant viruses may prove useful in studying the assembly functions of the tail-

deleted VP16 proteins in the absence of vhs binding and in further understanding the mechanism by which HSV-1 uses a tightly regulated interaction between vhs and VP16 to accomplish many crucial events in lytic infection.

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