Mutagenesis of the VP16 protein of the Herpes Simplex Virus

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Abstract

The herpes simplex virus (HSV-1) encodes a *trans*-activator protein VP16. VP16 activates the immediate early genes (IE) of the viral genome during lytic infection. VP16 has been heavily studied by mutational analysis as a model for transcriptional activation. The goal of this project was to examine two specific aspects of VP16. First, to compare the properties of a previously constructed insertion mutant, in1814, to other mutant strains constructed in our laboratory; this insertion was moved from strain 17 of HSV-1 into the KOS strain, the strain used in our lab. The second aspect of this examination is a mutational analysis of a possible cryptic N-terminal activation domain. To assess whether this region contributes to transcriptional activation of the IE genes, deletion mutants were constructed in the N terminus of VP16, with or without the deletion of the C-terminal activation domain. The constructed viruses will be verified for the proper mutations, before the properties of these viruses are characterized.

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Introduction

Herpesvirus is a rather large and complex family of viruses. They are widespread and prevalent animal pathogens. There are 8 major human *herpesviruses.* Five of the most prevalent forms include Herpes Simplex Virus 1 (HSV-1), varicella zoster virus (VZV), cytomegalovirus (CMV), Herpes Simplex Virus 2 (HSV-2), and Epstein-Barr virus (EBV). The rate of infection by these viruses varies, but it is almost certain that every American will contact one of these viruses during their lifetime. For example, HSV-2 is the cause of genital herpes and is the most prevalent sexually transmitted disease, occurring in around 22% of Americans over the age of 12 ("GOOD" VIRUS / "BAD" VIRUS). In addition, it is estimated that 4 out of 5 people over the age of 35 have been infected with CMV (AHF Physician Information). The pervasiveness of these viruses becomes even more apparent when VZV and HSV-1 are considered. VZV is the cause of chicken pox and can reemerge again later in one's lifetime as shingles. Chickenpox is widespread and is an accepted part of one's grade school experience. Estimates for HSV-1 are even more staggering. Because it is not a sexually transmitted disease or a potentially deadly pathogen, rates of HSV-1 infection are not reported through any agencies, but studies indicate that 80 to 90% of the adult population have been exposed to and possess antibodies against HSV-1 ("GOOD" VIRUS / "BAD" VIRUS). Thus, it is reasonable to say that all Americans will be infected with at least one of the *herpesviruses*.

The prevalence of this family of viruses has led to their extensive study. HSV-1 has been a target of many of these studies because its mild symptoms

make it reasonably safe to work with. HSV-1 infects the oral cavity producing cold sores and fever blisters. Following the initial lytic infection of the epithelium, this virus lives latently in the nervous tissue, typically in the trigeminal ganglion. HSV-1 is an enveloped virus that has an icosahedral nucleocapsid containing double stranded DNA and possesses a region between the capsid and the lipid envelope, termed the tegument. The tegument region is a non-specific, largely undefined, region that contains proteins critical to the virus's pathogenicity. This complex virus has a large genome that consists of 152,260 base pairs (bp) and encodes at least 84 separate proteins (Madigan 2000).

Research continues to explore many of the properties of this HSV-1. The aims of these studies vary greatly, from studies that attempt to find means of treating infection, to others that study its latency. A third area of study uses HSV-1 as a model system in which to study transcriptional regulation and DNA replication. HSV-1 has double stranded DNA and a genome that is relatively small compared to that of a eukaryotic cell, making it a model system in which to study transcriptional regulation. HSV-1 replicates in the nucleus of its eukaryotic host and takes advantage of the cellular machinery for transcription. In doing this HSV-1 gives us a fairly simple (in comparison to the cell itself) system in which to study transcriptional regulation.

This regulation begins when the virus initially penetrates and infects a human epithelial cell. This infection generates the expression of three temporally ordered sets of genes (Honess and Roizman 1974). These sets have been designated as the immediate early (IE or α), delayed early (DE or β) and late

genes (L or γ) (Clements et al. 1977). The gene products of the IE genes activate the DE genes, which in turn are largely responsible for viral DNA replication and activation of the L genes (Roizman and Sears 1996). This whole process is initiated when the IE genes are activated at the time of infection by a virion protein, VP16.

VP16 is a 65 kD protein possessing 490 amino acids, and it is packaged in the tegument of the virion (Batterson and Roizman 1983). VP16 activates of the IE genes through the IE promoters. These promoters have the sequences TAATGARAT (R is a purine) and GCGGAA (Mackem and Roizman 1982, Gaffney et al. 1985, Triezenberg et al. 1988b).

Initial work was done to determine the crucial regions of VP16. This was done through co-transfectional studies in which a plasmid carrying VP16 was introduced into a strain of mammalian cells with a reporter gene bearing an IE promoter. The plasmids introduced carried only portions of the VP16 gene and through this deletional analysis it was found that the crucial domain for VP16's activational ability was the 80 C-terminal amino acids (AA 410-490). This region was subdivided into two activation domains (Cousens et al.1989, Triezenberg et al. 1988a, Triezenberg 1995). Further analysis of this region also lead to the surprising discovery that hydrophobic amino acids are the key to transcriptional activation, in contrast to the widely held opinion that the highly conserved acidic amino acids conferred this ability (Cress and Triezenberg 1991).

As knowledge about VP16 increased it was found, as hypothesized, that this protein did not work alone. Studies soon began to indicate that VP16

activated transcription through protein--protein interactions (Triezenberg et al. 1988b). The recruitment of VP16 to DNA is mediated by the Oct-1 protein (O'Hare and Goding 1988, Preston et al. 1988, Prujin et al. 1988, Kristie et al. 1989, Stern et al.1989). The Oct-1 protein binds to the conserved TAATGARAT sequence previously mentioned. This binding is aided by the HCF protein (Katan et al. 1990, Xiao and Capone 1990, Kristie and Sharp 1993, Wilson et al. 1993).

This information generates a picture of VP16's ability to activate transcription. Oct-1 through its interaction with VP16 is able to recognize and bind to the IE gene promoter, this interaction is facilitated by the HCF protein, generating a hetero-trimeric complex in which VP16 contributes the activation domain, and Oct-1 and HCF secure this activation domain to the DNA. The placement of this activation domain allows interaction with the basal transcription machinery, resulting in IE gene transcription.

The study of VP16 continues with mutational analysis of the protein. One such mutant, named in1814, was constructed in strain 17 of the virus. This mutant possesses a four amino acid insertion at codon [illegible]. Interestingly this mutation lacks the ability to activate the IE genes (A[illegible]al. 1989). The cause of this seems to be that the insertion sequence is in the middle of the proposed Oct-1 interaction domain. The phenotype of this mutant, including its growth rate, plaque size and morphology, is used as a reference point in the description of new VP16 mutants.

The use and intrinsic value of in1814 as a phenotypic descriptor (meaning that its phenotype is well characterized) make this mutant a key virus to possess

for the sake of comparison. Our use of this virus as a phenotypic descriptor has been limited because we utilize a different strain of virus, the KOS strain. For this reason it was deemed necessary to construct this insertion mutant in the KOS strain of the virus.

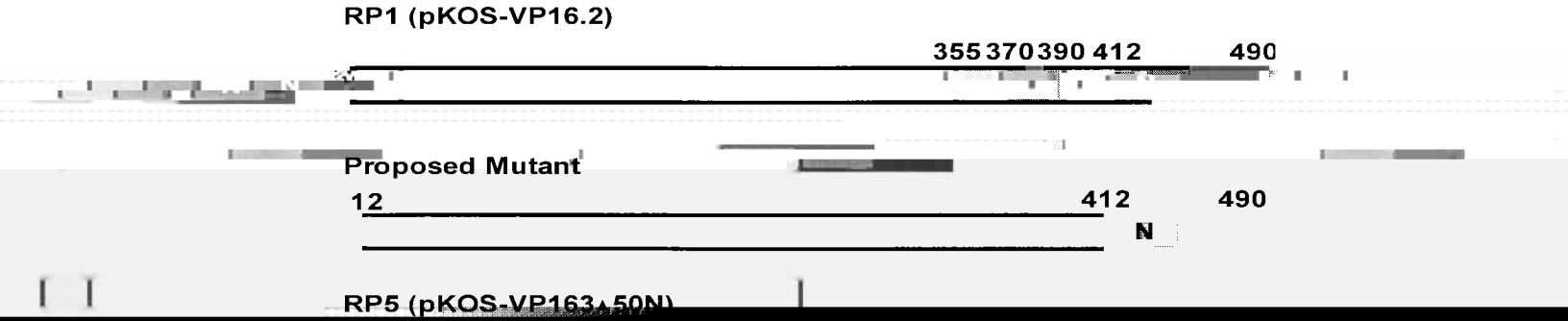
A second goal of this study examines the possibility of a putative, weak N-terminal activation domain. Cohen and colleagues (Moriuchi et al. 1995) have described a sequence of hydrophobic amino acids in the N-terminus of VP16 that is similar to sequences observed in VP16 homologs in other herpesviruses. One such sequence in VP-16's counterpart, ORF10, in VZV, has the properties of a typical activation domain.

Cohen's analysis of the N-terminal of ORF10 was done with Gal-4 fusion proteins. These fusion proteins are the result of combining the Gal-4 DNA binding domain with regions of the ORF10 protein and analyzing what regions best activate transcription of the Gal-4 driven reporter gene in mammalian cells. This technique demonstrated that amino acids 5-79 held ORF10's activation domain. This region was sequenced and through database analysis was compared to similar activator proteins within the herpes family of viruses. The sequence was found to be highly conserved especially in some key hydrophobic amino acids. These alignments generated consensus sequences in the N-terminus of two VP16 homologs, UL48 of the bovine herpes viruses and GENE 12 of the equine herpes viruses, along with the N-terminal half of VP16's C-terminal activation domain and in the first twelve amino acids of VP16. The prevalence of this N-terminal activation domain and the presence of some of the

key amino acids in VP16's N-terminus led to the question of whether VP16 might possess an N-terminal activation domain.

To test this hypothesis two new viruses carrying mutations in the VP16 protein will be constructed. The initial start codon of VP16 will be eliminated, shifting the start of translation to the 12th codon. This will eliminate the aforementioned conserved sequence. This mutation will be constructed in wild type HSV-1 and in an HSV-1 mutant that lacks the powerful C-terminal activation domain (see Figure 1 for proposed constructs).

A.



[redacted]

C-terminal activation domain. To asses the activation ability of these mutant viruses they will be compared to viruses with wild type VP16 and to that of a previously constructed mutant, RP5 that lacks the entire VP16 C-terminal activation domain (Tal-Singer et al. 1999).

Materials and Methods

Construction of pBRS-1

The plasmid, pBRS-1 was constructed to incorporate the in1814 mutation into the KOS VP16 gene. The parental plasmid from which it was made is pKOS-VP16ΔkpnI. This plasmid, previously constructed in the Triezenberg

[illegible]

Containing this plasmid were grown from a frozen glycerol stock onto ampicillin plates (100 mg/mL). A single colony was picked and a 1 liter liquid culture in LB medium was grown at 37° C. The resulting cells were collected by centrifugation, and the supernatant discarded. The pellet was resuspended in 10 mL of GET (50mM glucose, 25mM Tris Cl, pH 8.0, 10mM EDTA, autoclaved and stored at 0° C) and 4 mL of 25 mg/mL lysozyme was added. This solution was incubated for 10 mins at room temperature. Forty mL of 0.2 N NaOH, 1% SDS was added and the mixture was incubated for 10 mins on ice. Next 30 mL of 7.5 M ammonium acetate was added. The solution was incubated 10 mins on ice and then spun at 10000 rpm in the Sorvall centrifuge, using the Sorvall GSA rotor for 10 mins. The supernatant was transferred to fresh tubes pouring through glass wool. Fifty-four mL of 2-propanol was added and a precipitate was allowed to form (10 mins). The resulting solution was spun for 10 mins at 10000 rpm in GSA rotor. The supernatant was then discarded and the pellet was washed with ice cold 70% ethanol. Finally, the pellet was re-dissolved in 4 mL of distilled water.

This resuspended DNA was then purified using a cesium chloride (CsCl) gradient. Cesium chloride was added to the DNA solution giving a final

measurement between 35 and 38% on the Brix handheld refractometer. The

dialyzed in a 1x TE solution (pH 8.0).

The in1814 DNA was isolated from our viral stock. The virions were lysed by adding 50μL of a 10% SDS solution to 500μL of viral stock. This solution was allowed to incubate for 10 mins. The viral DNA was then isolated through a series of washes. The first wash consisted of 500 μL of a 50/50 phenol/chloroform solution. From the resulting solution the upper hydrophilic layer was removed and placed in a fresh microfuge tube. The hydrophobic solution was then washed with 500μL distilled water and the hydrophilic layer removed and combined with the previous layer. The hydrophobic layer was discarded and 500 μL of chloroform was added to the aqueous solution. The aqueous layer was again removed and placed in a fresh vial. Six hundred μL isopropanol and 100 μL of 7.5M ammonium acetate was then added to the aqueous phase causing the DNA to precipitate. This was centrifuged for 10 mins. The supernatant was discarded and the pellet washed with ice-cold 70% ethanol. The DNA was re-dissolved in water.

This isolation gave a very small amount of DNA that [illegible] quently

[illegible]1R (5' CCCGACGTTCCGGCCA 3') and ST286 (3' CGCCATCGCCACGTCCT 5'). PCR was performed with 25pM of each primer, 1mM dNTP's, 2.5 units of Pfu polymerase, 5% DMSO and 1x PCR buffer. The [illegible]

fragment (4900 bp) was isolated and the DNA purified using the Gene Clean DNA isolation kit. Similarly a 700 bp fragment was isolated from the gel that contained the in1814 digestion. Next, the parental plasmid was treated with a phosphatase to remove the termi[illegible] groups to prevent self-ligation. The insertion (in1814) was then combined with the vector in a ligation reaction using T4 ligase. The reaction was run overnight at 16° C. The ligation mixture was then transformed into DH5α cells via electroporation (at 200Ω, 200μF and 2.5 V). The cells were rocked for one hr at 37° C and plated onto ampicillin plates. Colonies were subsequently picked and tested via restriction digestion for in1814 insertion. Candidate plasmids were cut with KpnI and BamHI to test for the presence of the in1814 mutation, which added a secondary BamHI site. Colonies that appeared to possess a plasmid with this insertion

CGCCATCGCCACGTCCCT 5'), ST287 (3' CGCCGCAATCAACACC 5') and

the VP16 gene. Once isolated a colony containing pBRS-1 was picked and grown in liquid culture. From this liquid culture, 1 mL of broth with bacteria was added to 1 mL of glycerol to yield a 50% glycerol stock, which was subsequently frozen.

Construction of pBRS-2 and pBRS-3

abolishing that codon, forcing the start of translation down 11 codons and introducing a novel BglII site. pBRS-2 carries a WT VP16 gene and pBRS-3 carries a copy of the VP16 gene that lacks the C-terminal activation domain. This was done through a technique known as QuikChange™ Mutagenesis was used to introduce specific point mutations into the parental plasmid. Mutagenic primers were ordered that would anneal to the parental plasmid at the desired

ACCCAACAGATCTCTTGGTCGACGAGCTGTTTGCCGACATG GAC GCG 3') and ST391, the complement of ST390. The parental plasmids used were pKOS VP16.2 (complete VP16 gene and amp resistance) and pKOS-VP163Δ50N (VP16 without C-terminal activation domain and amp resistance). These plasmids had both been previously constructed and their DNA was harvested using a Alkaline Lysis, CsCl prep as described earlier.

The parental plasmids were then taken through a PCR reaction with the mutagenic primers. The conditions of the PCR reaction are as follows: 125 ng of each primer while varying the amount of DNA template (from 20-500 ng), 1 mM dNTP's, 2.5 units Pfu polymerase, and 1x PCR buffer in a 50 pi reaction. This reaction was cycled 16 times under the following conditions: at 95°C for 30 seconds, followed by one min at 55°C and finally 12 mins at 68°C. Once the cycles were complete the sample was cooled at 1°C per min to 4°C. The gradually cooling of the samples was to allow for maximum annealing of the newly created mutated plasmids, which are linear as the reaction lacked a ligase. This reaction changed 3 bp (bold), inserts a Bglll site (underlined) and causes the translational start site to shift downstream 12 codons italics) as it change the sequence from ACCCAA***TG*** GAC CTC TTG GTC GAC GAG CTG TTT GCC GAC ATG GAC GCG to ACCCAACAGATCTCTTGGTCGACGAGCTGTTT GCCGACA***TG*** GAC GCG.

Once this was completed a Dpnl digestion was run on the product digesting away all parental DNA. Dpnl digests methylated DNA so it is imperative that during the production of the parental plasmid a methylation positive strain of ,)-./ is used. Remaining DNA was subsequently transformed into DH5a cells via electroporation (as previously described). Colonies were then screened via a Bglll, Pstl restriction digestion. Colonies containing the correct newly mutated plasmid showed two fragments when run on a 1% agarose gel. Once the colonies were screened, plasmids isolated from colonies carrying the mutation were sequenced to ensure that no additional mutations

were introduced during PCR. Again 50% glycerol stocks were made of the bacteria carrying the desired plasmids.

Co-Transfection Experiments

The co-transfections were done in cultured vero cells (a cell line derived from the kidney of an African green monkey). These cells were grown in Dulbecco Modified Eagle Medium (DMEM), buffered with bicarbonate and adjusted to a pH of 7.2. The media was supplemented with 10% fetal calf serum (FCS).

The day before the transfections, p60 plates (60 x 15 mm dishes) were seeded with vero cells. Approximately 5x10^5 cells were seeded to ensure that the plates would be confluent for the co-transfection. The co-transfections were done with 8MA DNA and the mutated VP16 gene excised from pBRS-1,2 or 3. 8MA is an HSV-1 mutant derived from the KOS strain of HSV-1 by replacing the VP16 gene with the ,)-./% LacZ gene. This mutant is unable to propagate in standard vero cells. In order to overcome this problem a cell line derived form vero cells was constructed that stably expresses VP16, termed the 16-8 cell line (Weinheimer et al., 1992). The 8MA virus, originally a gift from Weinheimer and colleagues, was taken from our viral stocks and the DNA was isolated as previously described. The isolated 8MA DNA and the modified VP16 genes, excised from the mutated plasmids via a PstI/EcoRI digestion, were co-precipitated via a calcium-phosphate co-precipitation.

Before beginning the co-precipitation, fresh DMEM with FCS was placed on the cell culture. The co-precipitation began by adding 5 pg of the 8MA DNA to

5 pg of the excised VP16 gene in a vial. This was mixed and added to a solution of 250 pL 2x HBS buffer (pH 7.05), 25 pg of sheared salmon sperm DNA and brought to a total volume of 500 pL with sterilized distilled water. This mixture was vortexed and 31.25 pL of 2M $CaCl_2$ was added to begin the precipitation. After 20 mins this mixture was added to the vero cells.

The vero cells were then placed in the incubator (maintained at 37°C and 8-10% CO_2) for 5 hrs. After five hrs a grainy precipitation was observed in the media. The media was removed from the cells and they were washed with PBSa solution. Subsequently, the cells were shocked with a 10% glycerol in PBSa for 3 mins. The cells were then quickly washed with PBSa to remove all glycerol and overlaid with DMEM containing 5% FCS and 0.9% Sea Plaque™ agarose. Next the cells were incubated for 4-7 days waiting for viral plaques to form. Once plaques appeared they were picked and subsequently plaque purified. Plaque purification is a simple process in which the agarose surrounding the plaques is taken up in a pasteur pipette and placed in a vial with 1 mL of DMEM. This vial was then frozen for use in further purifications and infections.

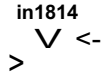
The transfections for pBRS-1 and pBRS-3 were performed in 16-8 cells (cells derived from vero cells, but that stably produce the VP16 gene product, so viruses lacking a functional VP16 can grow). An identical co-precipitation reaction was performed. The only difference was the use of DMEM without phenol red. This was done so plaques formed by the 8MA virus and plaques formed by recombinant viruses could be distinguished using through the LacZ gene of the 8MA virus. As soon as plaques are observed plates were treated

with 1 mg/mL of 5-Bromo-4-chloro-3-indolyl-p-D-galactopyranoside (X-gal) in DMEM without phenol red. Plates were incubated 24-36 hrs and the white plaques were picked. These plaques were then plaque purified.

Results

Construction of the Plasmids

pBRS-1 was successfully formed through the insertion of the in1814 mutation into the KOS VP16 gene of pKOS-VP16Akpnl. The pKOS-VP16Akpnl plasmid is a 5600 bp plasmid that contains the entire KOS VP16 open reading frame (ORF) and ampicillan resistance. The in1814 VP16 gene was isolated from viral DNA and amplified via PCR. The amplified product of the PCR reaction was then cut and ligated with the pKOS-VP16Akpnl plasmid, yielding a new plasmid, pBRS-1 (a schematic diagram of the procedure is shown is see Fig 2). The identity of the new plasmids was assessed by restriction endonuclease digestion, taking advantage of the new BamHI site present in the in 1814 insertion as shown in the plasmid map (Fig. 3A) These digestions were done with



Strain 17

Fig 2. Schematic diagram of the procedure used to move the in1814 mutation into KOS VP16. The in1814 region was amplified via PCR, primers are symbolized by horizontal arrows. Vertical arrows denote the cut sites on the plasmid and on the PCR product for ligation. The horizontal arrows on the plasmid represent the ends of the VP16 gene

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Kpnl and BamHI to verify the insertions presence. If the in1814 mutation is not present the plasmid will be cut into 2 fragments, a 4600 bp piece and a 1000 bp piece; if the mutation is present (thus a new BamHI site) three fragments (a 4600 bp, 600bp and a 400 bp) should be observed, when the digestion is run on an

A.

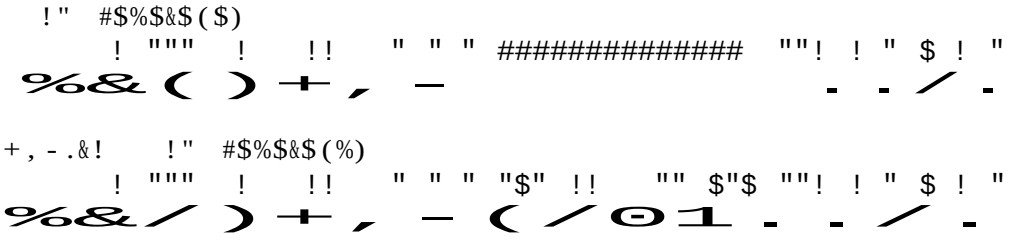

Figure 3. Restriction digestion and sequence verification of the pBRS-1 plasmid. (A) plasmid map of pBRS-1 (B) The parental and constructed plasmids were digested with BamHI and EcoRI to test for the insertion of the in1814 mutation. The parental plasmid is in lane 3, pBRS-1 is in lane 2 and the ladder in lane 1. (C) Sequencing results of the wild type VP16 gene and of pBRS-1. The four amino acid insertion is clearly shown in pBRS-1.

agarose gel. Both results can be clearly seen in figure 3B. This plasmid was sequenced to verify the presence of the mutation and to ensure that no other mutations were introduced through PCR (Fig 3C). The sequencing confirmed that we had produced and isolated pBRS-1, and now had the in1814 insertion in a KOS VP16gene.

Two additional mutant forms of VP16 were generated in the construction of the pBRS-2 and pBRS-3 plasmids. These versions of the VP16 gene both lack the first 11 codons when translated and pBRS-3 also lacks the entire C-terminal activation domain. This procedure entails the production of primers carrying point mutations. The mutagenized primers are then carried through a PCR reaction with the parental plasmids. Once the PCR reaction is completed a Dpnl digestion was performed on the product digesting away all parental DNA (see Figure 4 for a schematic diagram).

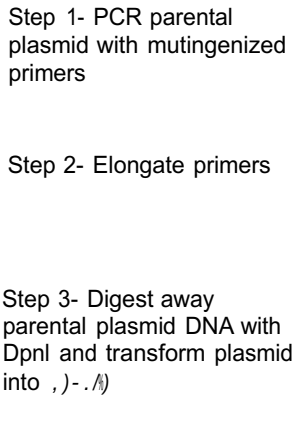

Fig 4. QuikChange™ Mutagenesis. The arrows denote the primers and the asterisks the mutation in the primers. The black circles are the parental plasmids and gray represents the newly mutagenized plasmids.

Dpnl digests methylated DNA so it is imperative that a methylation positive strain of ,)-./ is used to produce the parental plasmids. This procedure, called QuikChange™ Mutagenesis was performed on both the pKOS-VP16.2 (plasmid complete VP16 gene and amp resistance) and pKOS-VP163A50N plasmid (VP16 without C-terminal activation domain and amp resistance).

0 used this method to introduced 3 point mutations into the initial start codon for the transcription of VP16. This change forces the start of translation to shift downstream 12 amino acids, by mutating the first AUG codon, forcing the use of the second AUG 36 bp downstream. This shift eliminates the aforementioned N-terminal activation sequence. It also introduced a novel BglII site. This site was introduced to make verification of the mutation simple and can be seen in Figure 5A in the restriction maps of the constructed plasmids.

The products of the QuikChange PCR reactions were transformed into DH5a cells and colonies were tested by restriction digestion, taking advantage of the novel BglII site. If the mutation is present the digestion with BglII and BamHI, yields a 5000 bp fragment and a 1500 bp fragment. This result is shown in Figure 5B. One of each mutant plasmid was isolated. Plasmids that were identified by restriction digestion were then sequenced to confirm the mutations and the accuracy of the PCR. This sequencing data clearly showed the modifications of the DNA sequence (Fig 5C). At this point, we had mutated the VP16 gene three different times. The first mutation is housed in pBRS-1 and is simply the insertion of the in1814 mutation into the KOS VP16 gene. pBRS-2 houses a N-terminal deletion mutant, in which the initial start codon is modified, generating a deletion of the first 12 amino acids upon translation. pBRS-3 is another N-terminal deletion mutant, identical to pBRS-2 only its VP16 gene lacks the C-terminal activation domain.

A.

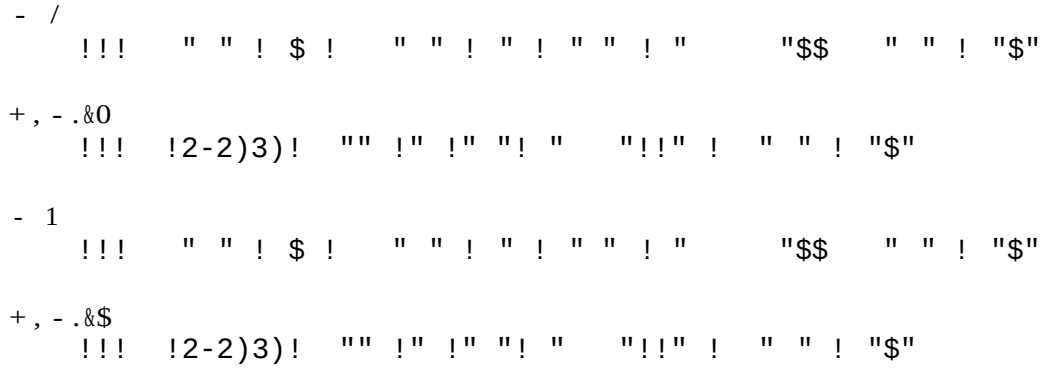

Figure 5. Restriction digestion and sequence verification of the pBRS-2/3 plasmids. (A) plasmid maps of pBRS-2 and 3 (B) One of the parental plasmids (VP16.2) and both constructed plasmids (BRS-2 + 3) were digested with BglII and BamHI to test for the modification of the original start codon. (C) Sequencing results of the parental plasmids and of pBRS-2 and 3 clearly show the point mutations (start codons are underlined, point mutations are in bold and new BglII site is in all caps).

Co-Transfection Experiments

The goal of the co-transfection was the recombination of the mutated VP16 genes into the viral genome of HSV-1. To do this each mutant gene was excised from its respective plasmid and transfected with viral DNA in a calcium phosphate co-precipitation (schematic diagram shown in Fig. 6). The viral DNA used was from the 8MA virus and upon recombination should result in a virus able to grow in vero cells. In order to understand this process it is imperative to understand the 8MA virus. The HSV-1 mutant 8MA was derived from the KOS strain of HSV-1 by replacing the VP16 gene with the E.coli LacZ gene, while maintaining the same promoters. This mutant is unable to propagate in standard vero cells. To bypass this problem a type of vero cells was made that stably expresses the VP16 gene product. These cells were termed 16-8 cells (Weinheimer et al. 1992).

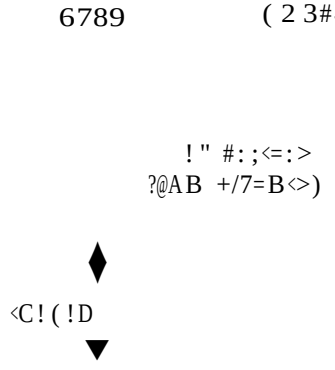

Figure 6. Schematic of the Co-transfection. Both 8MA DNA and the mutated VP16 genes DNA were transfected. The 'x's symbolize the desired recombination generating a active KOS HSV-1 with a VP16 gene.

The inability of 8MA to grow in vero cells ensures that viral plaques will form only if the 8MA virus recombines with an active VP16 gene. This strategy assumes that the introduced mutation does not itself severely handicap the recombinant viruses ability to propagate. If the mutation is too detrimental to the

VP16 gene, the recombinant virus would also be unable to survive, not allowing the use of the standard vero cell. In the case of pBRS-2, it seems safe to assume that the mutation will not be severe enough to prevent propagation, since the gene still possess the entire dominant activation domain. For this reason the transfection with pBRS-2 was performed in standard vero cells and recombinant plaques appeared in 7 days. Two controls were also carried out. The first, a negative control designed to guarantee that the 8MA virus couldn't propagate used only 8MA DNA. As a positive control, the transfection was performed with 8MA DNA and a wild type VP16 gene. This control would demonstrate that the transfection had worked even if plaques were not seen in the true transfection. The transfection attempts were successful and currently this new virus, named BS-2, is being plaque purified. Once complete the entire VP16 gene will be verified by sequencing.

The isolation of pBRS-1 and pBRS-3 required a different protocol. These co-transfections were performed in 16-8 cells in anticipation that the introduced mutations would produce an unhealthy virus that could not propagate well. This could lead to a number of undesirable results if the reaction was carried out in vero cells. The first major undesirable result is no result, namely that no plaques would appear. Secondly, if plaques did appear we would have to carefully guard against revertant mutants. Unfortunately, 16-8 cells introduce an additional complication, namely they support the growth of the 8MA virus. This being the case we cannot assume that all plaques formed carry the desired mutation.

This problem can be avoided by taken advantage of the LacZ gene integrated into the 8MA virus. 8MA plaques will express the LacZ gene and thus turn blue in the presence of X-gal, while mutant plaques will remain colorless. To make this distinction, transfected cells were overlaid with 1 mg/mL of X-gal as soon as plaques appeared. Plates were then incubated 24-36 hrs and the white colonies were picked. This time three controls were performed. The negative control consisted of only VP16 DNA. There were two positive controls. The first only possessed 8MA DNA and was a general control to ensure the transfection worked. The second positive control was done with 8MA DNA and a wild type VP16 gene and served as a control for whether or not recombination occurred.

This procedure has its disadvantages. First of all the transfections had to incubate for 7 days before plaques appeared and by this point almost all of the cells in culture are dead. This is further complicated by the X-gal treatment. X-gal is only soluble in a toxic solvent and must develop for 24 hrs, killing the remaining cells. These factors make it difficult to observe the plaques, since a plaque is essentially a ring of dead cells that can no longer adhere to the plate. In addition the differentiation between blue and white plaques was difficult. With this in mind, multiple plaques (about 20) were selected for further study. Efforts are currently underway at the Triezenberg lab to purify and isolate pure mutants without 8MA contamination.

At the present time only one mutant virus is in hand, BS-2 (the N-terminal deletion mutant with VP16's activation domain). The other two virus BS-1 (virus

carrying the in1814 mutation) and BS-3 (the other N-terminal mutant that lacks VP16's activation domain) are in the process of purification and isolation.

Discussion

All three of the desired constructs were successfully made. From these plasmids one of the desired viruses was obtained, BS-2. The other two viruses may have been obtained, but further purification and confirmation is necessary. The constructed plasmids have been verified through sequence analysis.

The analysis of BS-1 should allow a comparison between two different means of disrupting the activation ability of VP16. The in1814 mutation disrupts transcription because it is in the Oct-1 binding domain and interferes with binding. The mutations commonly constructed in our lab disrupt VP16's activation domain. Thus, moving the in1814 mutation in the KOS strain would allow direct comparisons as to the effects of these two different ways of crippling VP16. This greatly contrasts our current situation, in which we are unable to make direct comparisons between any of the mutants we construct and the in1814 mutation. This inability results because it cannot be said, with any degree of certainty that our observations are the result of the mutation or just arise from the differing properties of the strains. This situation couple be further improved if we find that BS-1 is phenotypically similar to the in1814 mutation. This would be useful because it would open up all previous research for comparison.

We have good reason to believe that BS-1 wiil have a phenotype similar to the in1814 phenotype. The sequence of the VP16 genes in strain 17 and the KOS strain are almost identical. This highly conserved nature seems to suggest that the function of VP16 remains constant. What we cannot speak to is whether or not strain 17 may have other genes that affect the phenotype of the in1814

insertion. Despite this uncertainty we anticipate that the same mutation in VP16 of any strain of HSV-1 would have a similar effect on viral replication and viability.

On the other hand there remains a slight possibility that the BS-1 mutant will be quite distinct from the in1814 mutation. If this turns out to be the case, a whole series of new questions will be raised. These questions include: what activity of VP16 does this mutation affect in one strain and not the other? Why does this mutation have a more pronounced effect in one strain? Is there anything inherently different about VP16's function in these two strains? If this result is obtained, a much more thorough and careful analysis of BS-1 would have to be performed. Although this would be more time consuming it would prove useful and still allow the analysis of our mutants in the KOS strain with a mutant that possesses an insertion at codon 379.

The analysis of BS-2 and BS-3 could prove to be even more interesting. Through our analysis we hope to determine if an N-terminal activation does exist. Its existence could paint a picture of HSV-Ts evolution and its relation to other viruses in the herpes family. These two constructs both carry identical N-terminal deletions. Once obtained we can do comparison studies to determine the level of activation ability that these modified VP16's possess. A detailed comparison of BS-2 and BS-3 with wild type VP16 and the previously constructed RP5 mutant, which lacks VP16's entire C-terminal activation domain, could prove very interesting. These studies would include analysis and comparison of plaque morphology, an examination of the viral growth curves and lastly an assay to assess the expression of the lE genes. These assays should generate a general