METHODOLOGY

General Methodology

1. Identify motifs using Minimotif program and select most conserved residues by aligning UL9 protein sequences from different herpes viruses.

2. Design primers to generate mutations within selected conserved residues of UL9 gene using Two Step PCR.

3. Introduce mutations into pcDNA3 mammalian expression vector under CMV promoter.

4. Perform transient transfection to check protein expression of the cell lysates by Western Blot Analysis.

5. Evaluate which of these mutations are functionally important based on Complementation Assay.

6. Carry out Co-Immunoprecipitation assay to verify protein-protein interactions.

7. Perform Plaque Reduction Assay to assess inhibitory activity of the mutant proteins during replication.

Materials and Methods

Cells, Antibodies and Viruses:

African green monkey kidney fibroblasts "Vero cells" were purchased from the American Type Culture Collection (Manassas, Va.) and maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, Calif.) supplemented with 5% Fetal Bovine Serum (FBS), 100 μg/μL penicillin and 100μg/μL streptomycin (Invitrogen). Three anti UL9 antibodies were used in this study: 17B is a monoclonal antibody directed against the N-terminal 33 amino acids of UL9 ; RH-7, (a gift from D. Tenney, Bristol Meyers Squib) the polyclonal antibody was raised against a glutathione S-transferase fusion with the C-terminal domain of UL9 (535-851) and KST-1 polyclonal anti rabbit directed against full length UL9 .

HSV-1 KOS Strain was used as a wild type virus and Hr94, a UL9 LacZ insertion mutant, as a UL9 null virus.

Plasmids:

Mammalian expressing pcDNA3 (Invitrogen) vector was used as a backbone in construction of UL9 mutant plasmids and pcDNA3-UL9-WT encoding UL9 wild type gene served as a PCR template. pcDNA3-UL9-WT plasmid expressing full length UL9 protein under cytomegalovirus (CMV) promoter along with pcDNA3-UL9-C and pcDNA3-UL9-1-394 expressing C-terminal part of UL9 protein (aa 535-851), and N-terminal part (aa 1-394) respectively were used as a controls in must of the assays.

Construction of mutants:

The strategy used for creating the mutants was Two Step PCR. The primers were designed changing the most conserved residues to Alanines, these primers that were designed around the point mutation were overlapping, so in the first step of the PCR two different fragments were amplified. In the second step they were annealed together and the product amplified. Finally the mutated gene was ligated into pcDNA3 vector. Mini, midi and maxi Qiagen Prep Kits were used for DNA isolation, as well for gel extraction.

Table. 1 Generation of mutants for the identification of putative functional motifs of the UL9 protein, using "Mini-motif" program.

Motif	Mutation
WD40-1	SS304/305 AA
SH2	Y 368 A
WD40-2	SS 380/384 AA
MDM2	F 732 A
LC8-1	KST 746/747/748 AAA
LC8-2	QT 749/750 AA
LC8-3	Δ-KSTQT (d746-750)
Nuclear Receptors-1	L 785 A
Nuclear Receptors-2	LL 788/789 AA

1.- Mutations in SH2 and WD40 motifs:

a) First Step PCR for mutations:

Two fragments of UL9 gene were created with the point mutation, the primers used in First Step PCR are listed in Appendix 2.

b) Second Step PCR:

The two fragments created in the first step PCR were annealed in this Second Step (The Tm of each primer was considered for the annealing temperature used in PCR).

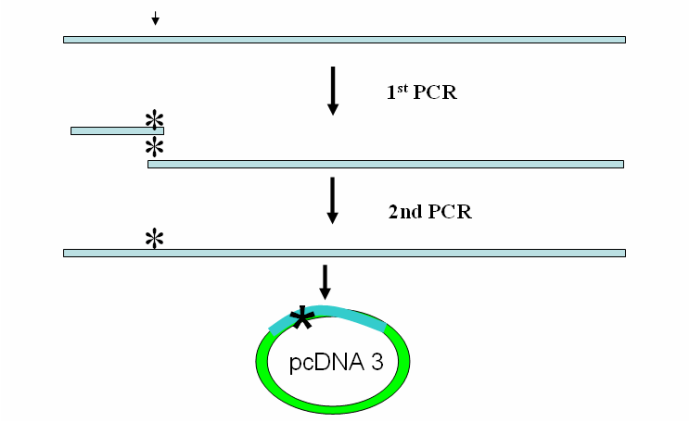


Fig.5 The strategy of mutation we used was Two Step PCR . In this strategy the gene was amplified in two steps. Two fragments were generated in each one overlapping the point mutation and afterwards they were annealed together and cloned into a pcDNA3 vector.

c) Digestion: The gene with point mutation was digested with EcoR1 and BamH1 using EcoR1 Buffer and BSA for the reaction. It was incubated one hour at 37ºC. We ran it in a low melting gel.

d) Gel extraction was used to purify DNA using the Invitrogen Gel Extraction kit.

e) Ligation: Mutated gene was ligated into pcDNA3 vector (appendix 4) using T4 ligase and T4 ligase buffer for one hour at room temperature. 50-100 ng DNA was used according to the manufacturer´s instruction.

f) *E.coli* DH5α Transformation: 2 µL Cloning reaction was added into Competent *E. coli* cells, then incubated on ice for 30 minutes, heat-shocked the cells for 30 seconds at 42°C without shaking, and immediately transfered the tubes to ice. Then, 250 µl of room temperature LB medium was added and shaked (200 rpm) at 37°C for 1 hour. 10-50 µl from each transformation was spreaded on a prewarmed selective plate and ncubated plates at 37°C.

g) Analizing Transformants: 4 colonies were picked for each mutation and cultured them overnight in LB medium containing Ampicillin. DNA was purified using Miniprep Qiagen Kit .The plasmids were analysed by restriction analysis to confirm the presence and correct orientation of the insert. BamH1 an EcoR1 restriction enzymes were used.

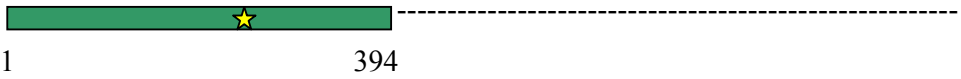
g) Sequencing: DNA samples were verified by sequencing.

Construction of N-terminal truncations (SH2 and WD40 motif mutants):

Truncations from 1 to 394 residues of full length mutants of SH2 and WD40 motifs was created as shown in Figure 6 using UL9 start BamH1 and 394His reverse primers, digested with EcoRI and BamHI enzimes and finally introduced into pcDNA3 vector. The presence of the mutations was confirmed by sequencing.

SH2 mutant (Y 368 A):

SH2 truncation:



WD40-1 mutant (SS304/305 AA)

WD40 truncation:

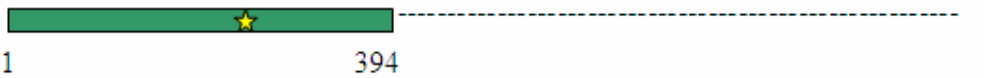


Fig. 6 SH2 and WD40 truncations. Truncations containing the N terminal domain of the protein were generated for the co-Immunoprecipitation assay. UL9 when it forms dimmers it is shown that either interacts with its N and C terminal domain (Chattopadhyay, 2006), so to measure if the point mutation was disrupting dimmer formation it was necessary to make them.

2.- Mutations in MDM2, LC8 Motifs and Motif for Interaction with Nuclear Receptor:

LC8, MDM2 and Motif for Interaction with Nuclear Receptors mutants were created by Two Step PCR and subcloning techiques and verified by sequencing.

The second step PCR after trying with different conditions (changing annealing temperatures, designing new primers with more homology with the template, changing DMSO and Magnesium concentrations, etc) did not work. Several possible problems may have contributed to this failure: the high GC content and the fact that the fragments were very different in size, so they did not anneal, or they annealed to non-specific sites of the gene.

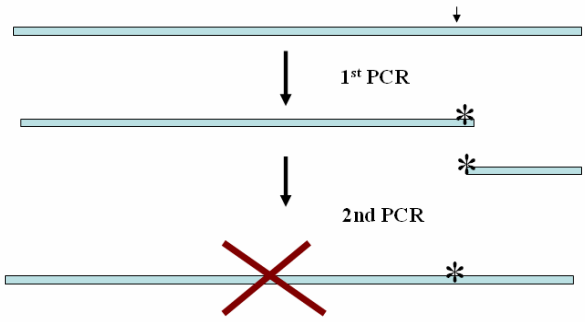


Fig. 7 Second Step of Two Step PCR Strategy did not work for the creation of these mutants. The two fragments generated in the first step did not anneal because of its different sizes. Conditions such as Magnesium concentrations, annealing temperatures, buffers, enhancers were changed, but the result remained the same: the second step of the PCR didn´t work.

So subcloning was performed cutting the longer fragments with one one-cut enzyme (MacVector software was used and New England Biolabs software, see restriction map in appendix 5) and perform the second step PCR with a shorter fragment. ApoI enzyme was used to create a smaller fragment by digesting the 2.2 kb fragment (figure 14), then these two fragments were used in the second step PCR to introduce the mutation and finally these two pieces were ligated with pcDNA3 vector.

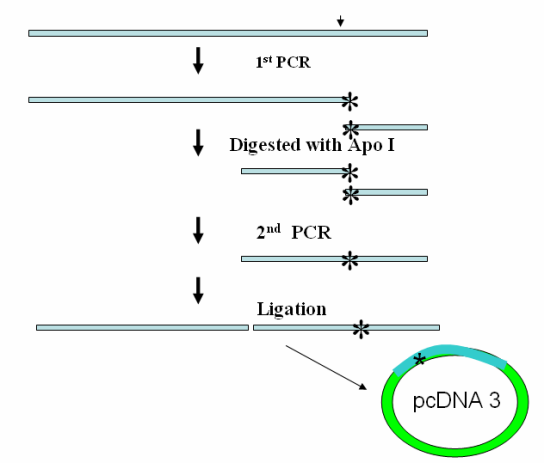


Fig. 8 Subcloning Strategy for creating LC8, MDM2 and Motif for Interaction with Nuclear Receptor mutants. Because the second step of the PCR didn´t work, subcloning was performed cutting the long fragment with a one-cut-enzime and afterwards performing with that one the second step PCR. This time the fragments were similar in size. After the second step everything was ligated into a pcDNA3 vector.

The screening showed positive clones, but the sequencing results showed that the correct fragments had not in fact been obtained. There was a problem in the ligation step. The orientation of the insert was incorrect, because the restriction sites for *ApoI* and *EcoRI* were similar:

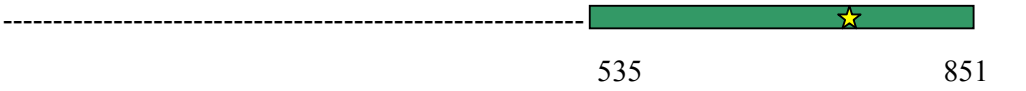
Apo I	Eco RI
5´ R`AATT´Y 3´	5´ G`AATTC 3´
3´ Y`TTAA´R 5´	3´ C TTAA´G 5´

To overcome this problem the fragments were only digested with Apo I enzime (without digesting with Eco RI or BamH1 so they cannot anneal at these sites) and ligated first the two fragments, amplified by PCR, digested them with EcoRI and BamH1 and they were ligated into the vector.
Clones for Nuclear Receptor Motif were not obtained.

Construction of C-terminal truncations (in LC8 and Mdm2 motif mutants):

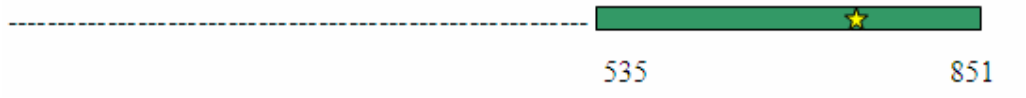
C-terminal fragments of this mutations were generated from aminoacid 535 to 851 using a His Tag 535 forward primer and the EcoR1 end primer like Fig. 15 shows.

LC8-1 fragment (535-851: KST 746/747/748 AAA):



LC8-2 fragment (535-851:QT 749/750 AA):

LC8-3 fragment (535-851: d-KSTQT (d746-750):



MDM2 fragment (535-851: F 732 A):

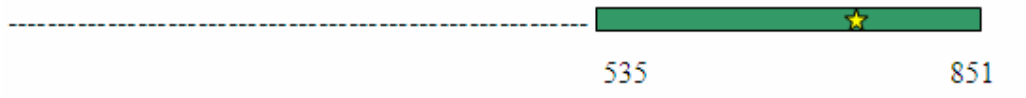


Fig. 9 LC8 and MDM2 C-Terminal Fragments. Fragments containing the C terminal domain of the protein were generated for the co-Immunoprecipitation assay. UL9 when it forms dimmers it is shown that either interacts with its N and C terminal domain (Chattopadhyay,2006) so to measure if the point mutation was disrupting dimmer formation it was necessary to make them.

Transient transfection and Western Blot Analysis:

Subconfluent monolayers of Vero cells in 60 mm^2 dishes were transfected with 2 μg of plasmid DNA containing wild type or mutant versions of the UL9 gene with Lipofectamine Plus reagent (Invitrogen, Calif.) according to the manufacturer's instructions. After 24h concentrated cell lysates were prepared in SDS-PAGE loading buffer, subjected to electrophoresis in 10% SDS-Polyacrylamide gels and transferred onto enhanced chemiluminescence membranes. We loaded samples (previously boiled for 10 minutes) and marker, ran in gel at 100-200 volts. Then we gathered tank, cassettes, sponges, filter paper and cold SDS transfer buffer for Western Blot. We cut two pieces filter paper and one piece of membrane per gel, we soaked membrane and gel in transfer buffer for 15 minutes. We opened transfer cassette and arranged the "sandwich" putting in the black side the sponge, one filter paper, the gel, then the membrane, another filter paper and a sponge. We closed it with red against white, black against black and filled tank with transfer buffer. We ran nitrocellulose transfer in 4ºC cold room at 75-90 volts for 1 ¼ hours - 2 hours. We incubate membrane in 5% milk block (made in TBST) for 1-2 hors in room temperature (30 minutes – 1 hour) or leaved in 4ºC cold room overnight. We incubated in 1º antibody (diluted in TBST) 1-2 hours in room temperature or leave in 4ºC cold room overnight. We washed three times with TBST, 10 minutes each wash with rocking. Then we incubated in 2º antibody (diluted in TBST) 30 minutes-1 hour in room temperature, washed 3x in TBST, 10 minutes each wash with rocking, we developed via Alkaline phosphate and incubated in AP developing buffer (in 10 mL AP developing buffer, add 66 μL of NBT (Promega), mix well, then add 33 μL BCIP (Promega)). We poured developing solution onto membrane and rocked solution back and forth until developed.

Complementation Assay:

Vero cells transiently transfected with plasmids expressing UL9 mutant proteins were superinfected with UL9 deficient virus (Hr94). The viral progeny was collected and virus titers were established in plaque assay on a permissive cell line (Hel28).

a)Transfection: Vero Cells were plated a day before in 60 mm dishes 80-90% confluent plates of cells were transfected acoording to the manufacturers protocol (Lipofectamine technique) with 2 μg of each DNA. Transfection was carried out in serum free environment. Transfection media was enriched with serum after 3 hours and plates incubated at 37ºC for 20-22 hours.

b)Superinfection with null virus: Transfection media was removed and cells were infected with Hr94 (Ul9 null virus) at multiplicity of infection (MOI) of 3 (3 infection particles per cell). Plates were incubated for one hour with shaking every 15 minutes. After one hour the inoculum was removed null and dishes were washed twice with PBS. Cells were overlayed with growth medium (DMEM + 5% FBS) and incubated for 24 hours at 37ºC.

c)Collection of the virus: Cells were scraped and moved to the growth medium into the tubes. Tubes were freezed and thawed twice to release the virus and spinned down the cell bebrys at 1500rpm for 10 minutes at 4ºC. Supernatants were moved into the new tubes.

d)Plaque Assay: 35 mm dishes of Hel28 cells 90-100% confluent were prepared one day before. Plates were infected with multiple dilution of each viral stock, incubated for one hour with shaking every 15 minutes. Inoculum was removed and cells were overlaid with 2% methylcellulose (prepared in DMEM). Plates were incubated for 3-4 days at37ºC. Cells were fixed by overlaying plates containing the methylcellulose with 4% formaldehyde. After 2 hours plates were washed with water to remove the fixing solution and the methylcellulose and stained with 2% of crystal violet. After 30 minutes

plates were washed with water to remove the dye. Plaques were counted and viral titers calculated.

Immunoprecipitation:

Confluent Vero cells grown in 60 mm^2 dishes were transfected with plasmids of interest and allowed to incubate at 37^oC for 24 hrs. The medium was aspirated and cells were washed twice with cold 1X PBS. Cells were scraped into the PBS and moved to eppendorf tubes. Cells were peletted by low-speed centrifugation and the PBS was removed. The cells were lysed on ice in 500 µl RIPA buffer (300 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) for 30 min, for complete lysing cells were passed through 21 G needle and incubated on ice for additional 30 mins.

The insoluble materials were removed by centrifugation at 10,000 rpm at 4^oC for 10 min. Each cell lysate was pre-cleared with normal rabbit IgG. Each immunoprecipitation reaction of 500 µl (approximately 500 ng of total protein) of transfected cell lysate was incubated with 7 µl of primary antibody RH-7 for 2 h at 4^oC. 40 µL of a protein A- agarose beads slurry (Santa Cruz Biotech, Santa Barbara, Calif.) was added to each reaction and incubated over night at 4^oC. The beads were collected by centrifugation at 2500 rpm for 5 min followed by four 10 min washes with RIPA buffer. Finally, 45µl of PBS and 15 µl of 4X SDS-PAGE buffer was added and samples were boiled for 5 min. Immunoprecipitated proteins were analyzed in Western Blot analysis probing with 17B antibody.

Plaque Reduction Assay:

The plaque reduction assay was performed by cotransfecting nearly confluent 60 mm plates of Vero Cells with KOS infectious DNA and 10 fold molar excess of UL9 wild type expressing plasmid, UL9 mutants expressing plasmids or empty plasmids not expressing UL9. Transfection was carried out using Lipofectamine Plus Reagents (Invitrogen) following manufacturers recommendations. Three hours postransfection, the medium was removed and cells were overlaid with 2% methylcellulose prepared in DMEM. Plates were incubated for 4-5 days, fixed with 4% formaldehyde for at least two hours. The fixation solution along with methyl cellulose was washed off with water and stained with 2% of solution of crystal violet for 30 minutes to visualize the plaques. Staining solution was washed off with water and the plaques were counted. The results are presented as normalized plaque number where plaque number obtained for infectious DNA alone (with empty plasmid) was considered to be equal as one hundred.