RESULTS

1.-Minimotif Analysis

Minimotif program identified several motifs throughout UL9 (appendix 8), but we have selected a few including SH2, WD40, MDM2 and Nuclear Receptor Motif to study. The positions of these motifs are shown in Figure 6. We will mention some general information about each one and how they might be related to our protein of interest.

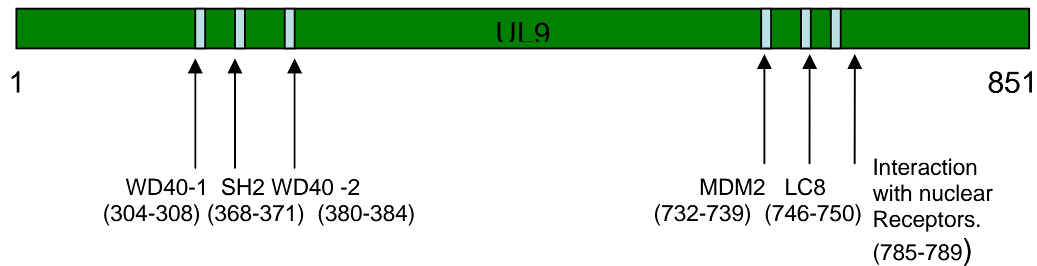


Fig. 10 Minimotif Showed putative important functional motifs for UL9 protein. Using theMinimotif Miner tool for analyzing the UL9 protein, many motifs were displayed (appendix 8), some of them were chosen because their localization in the protein were the region for dimmerization was reported previously (Chatopadhyay, 2006), and for the probable important functionality in the protein.

Significance of these motifs for UL9 protein:

1. SH2 Domains (Y??P): These motifs for SH2 domains are found in signaling molecules, in tyrosine kinases and in adaptor proteins involved in association or phosphorylation (Pawson, 2001, Nakamoto, 1996). In position 368 there is a YVKP Motif for SH2 Domain in UL9. As mentioned before, UL9 is phosphorylated during infection (Isler and Schaffer, 2001). It is possible that this motif may be involved in regulation of UL9 by phosphorylation or might be involved in UL9-UL9 interaction or association with other viral or cellular proteins .

2. WD40 domain(S???S): Many regulatory proteins have WD40 domains that mediate protein-protein interactions (Denisenko,1998). In position 304 and 380 there are SSTVS and SVYQS Motifs for WD40 domains in UL9. As it was mentioned before, UL9 is involved in interactions with itself and with several other proteins (Marintchneva and Weller, 2001;Chattopadhyay et al, 2006, Tanguy Le Gac, 2002). Therefore this motif was selected to mutate in order to understand the importance of this motif in UL9-UL9 interaction or its interaction with other proteins.

3. MDM2 (F???W??-I/L/V): Mdm2 is an oncoprotein required for supression of apoptosis. It is a regulator of p53 activity, it is an E3 ubiquitin ligase. P53 is related to apoptosis and when its bound to Mdm2, p53 is ubiquitinad and degraded by the proteosome (Limesand, 2006). In position 732 there is a FKIIWGEL Motif for MDM2 domain in UL9. As it was mentioned previously that UL9 is phosphorylated and ubiquitinated in neural cells (Lehman, 2003) , this may lead us to think that maybe this residues are related with ubiquitination and degradation of this protein at the later stage of infection. Alternatively UL9 might be involved in delaying apoptosis though Mdm2.

4. LC8 (K/R-STQT): Light chain 8 of the dynein machinery (LC8) has been reported to interact with a large variety of proteins permitting their transport. LC8 forms a dimer and has been proposed to stabilize dimer formation in other proteins to which it binds. In position 746 there is a KSTQT Motif for LC8 domain in UL9. It has been found by a pepscan technique (*in vitro*) that UL9 interacts with LC8 (Martinez-Moreno et al., 2003). Perhaps LC8 promotes UL9 dimer formation, it might help in UL9 dimerization.

5. Motif for interaction with nuclear receptors (L??LL): This motif it is a very conserved motif among herpes viruses and lies in the region which has been identified as UL9-UL9 dimerization mapped region, we are not sure about the relation to nuclear receptor in UL9, but we have selected this domain to narrow down this already mapped region. In position 785 there is a LYALL Motif for interaction with nuclear receptors in UL9.

6. 2.- Alignment

UL9 protein sequences of different viruses were aligned using Clustal W Software .Figure 7 shows the selected portions of the protein which are relevant for our study. As shown in the alignment, these motifs are mostly conserved in these viruses and most conserved residues were changed to Alanines or deleted (in the case of LC8 motif).For complete sequence see appendix 3.

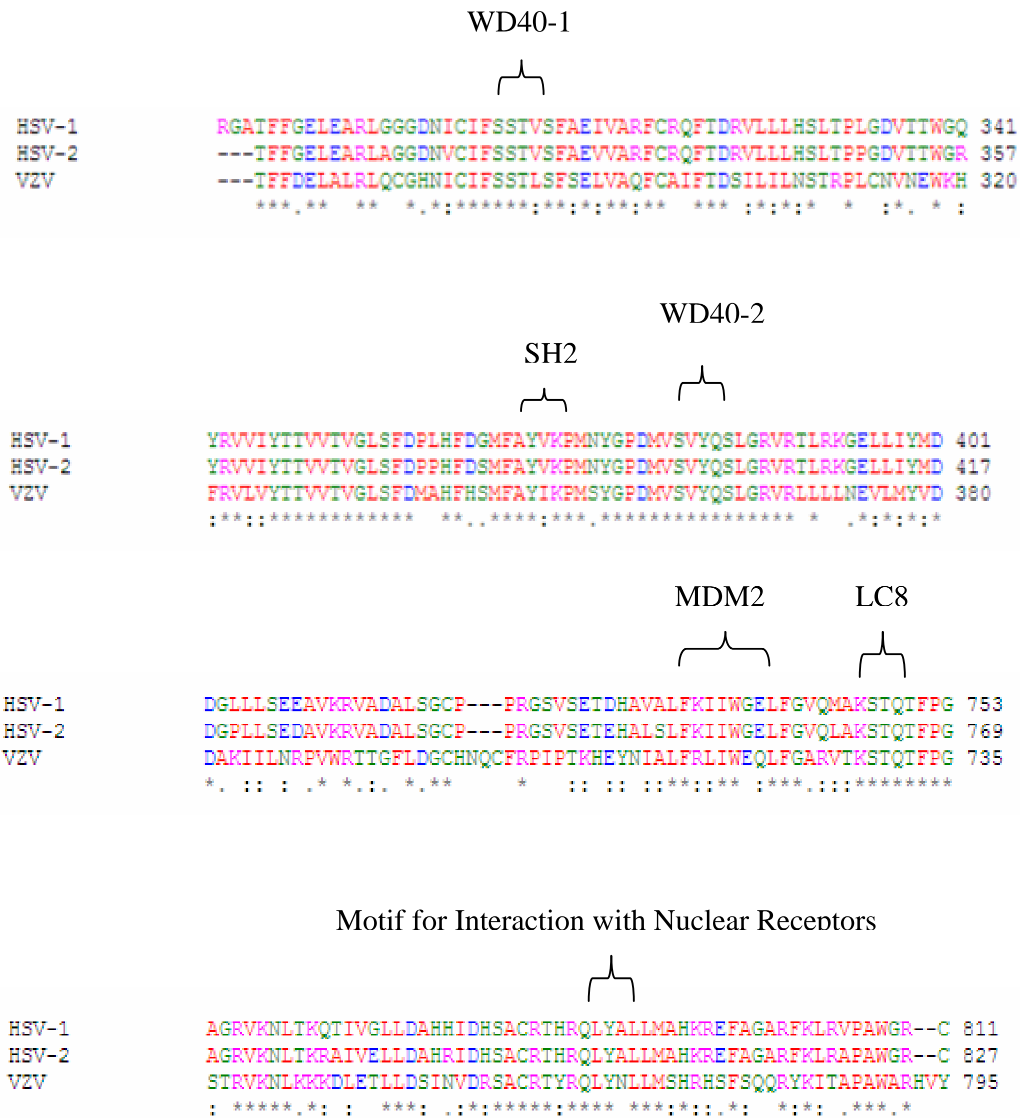


Fig. 11 Alignment of UL9. An aligment of the UL9 protein regions in different Herpes Viruses was made (the rest of the Herpes viruses don´t codify for an origin binding protein, so they don´t have an homolog with UL9), showing the most conserved aminoacids within the motifs that Minimotif Miner displayed.

3.-SH2 and WD40 mutations

SH2, WD40-1 and WD40-2 mutants were created by Two Step PCR and verified by sequencing.

Detection of Mutant Proteins:

Vero cells were transfected with mutant plasmids as described in Materials and Methods. Cell Lysates were prepared using SDS buffer, boiled for 10 minutes, and loaded into 10% SDS-PAGE. As shown in Figure 9, mutants were expressing almost equal amount of protein as wild type UL9

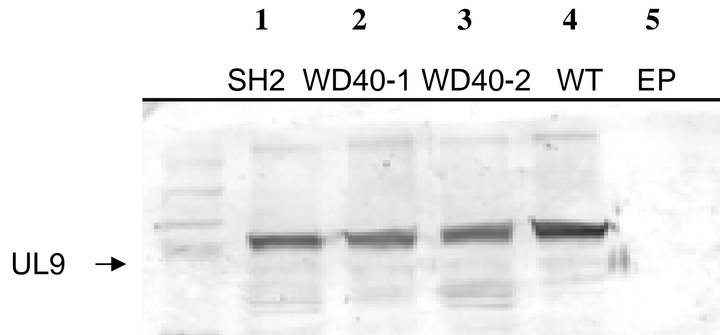


Fig. 12 The three mutants expressed equal levels of protein as the wild type UL9. An analysis by Western Blot showed that the mutants expressed protein in equal levels. Lines one, two and three include the samples for the mutant proteins SH2, WD40-1 and WD40-2, while lines 4 and 5 include samples for the wild type protein and the empty plasmid respectively.

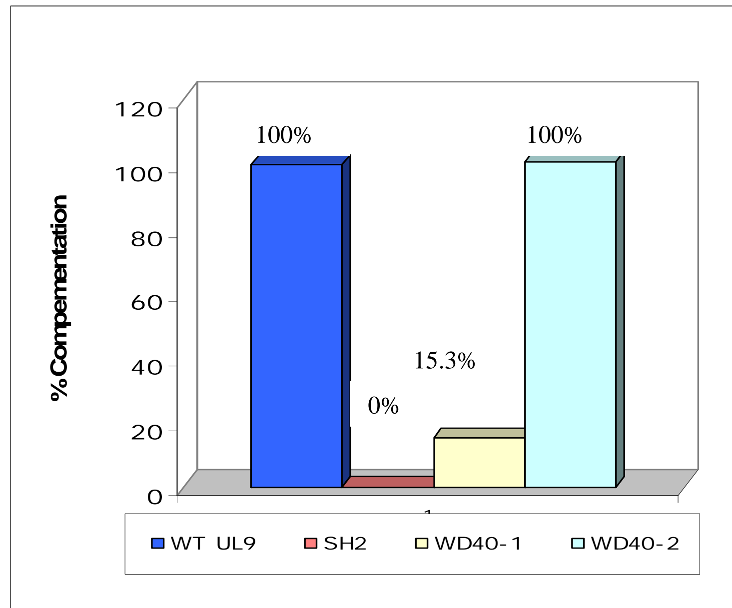


Fig. 13 SH2 mutant (SS304/305 AA) did not complement the UL9 deficient virus (Hr94), while WD40-2 (SS 380/384 AA) complemented as efficiently as wild type. On the other hand, WD40-1 (Y 368 A) complemented partially. In our complementation results we observed that the SH2 mutant (SS304/305 AA)), did not complement the null virus, while the WD40 -1 mutant complemented partially the null virus. In the other hand, the second motif for WD40 completely complemented the null virus compared to wild type UL9.

Complementation percentage was calculated as:

$$\% \text{ comp} : \frac{\text{number of plaques of mutant plasmid} - \text{number of plaques of empty plasmid}}{\text{number of plaques of UL9 wt plasmid} - \text{number of plaques of empty plasmid}}$$

*see Appendix 4 for details in titer, number of plaques and % in complementation .

Complementation Assay was performed three times from different experiments, and the results consistently showed that Mutation SH2 did not complement the null virus, Mutation WD40-1 complemented partially (~20%) and that Mutation WD40-2 complemented completely the null virus (100%) compared to wild type.

SH2 and WD40-1 motif mutants did not interact efficiently with the C-terminus of UL9:

a) Co-Immunoprecipitation with SH2-mutant and WD40-1 mutant (full length):

It has been observed previously that the N-terminal portion of UL9 interacts either with the full length or with the C terminus of the protein to form dimmers, so the full length UL9 protein with SH2 /WD40-1 mutations was cotransfected with a N-terminal fragment of UL9 (1-354) and co-Immunoprecipitation assay was carried out using the RH7 antibody as described in Materials and Methods section. The immunoprecipitated complex was resolved in a 10% SDS-PAGE and the membrane was probed with 17B antibody (described in Materials and Methods). It was observed that the N-terminal fragment was immunoprecipitated with the mutant proteins as efficiently as wild type UL9 (data not shown). But then, it was realized that as the SH2 and WD40-1 mutant were full length UL9, and that they have the C terminal region as well, so this area of the protein may also still be interacting with the N terminal fragment with which it was co-Immunoprecipitated, even if the mutant disrupts an interaction of UL9-UL9.

In order to overcome this problem C-terminal truncations of SH2 and WD40-1 mutations were generated. Thus, the N-terminal fragments with mutations can be used in Co-Immunoprecipitation Assay to measure their ability to interact with the C-terminal part of UL9.

Co-Immunoprecipitation was performed using UL9-1-354 N-terminal fragment as a control of strong interaction with the C-terminus. As shown in Figure 14, it was observed that the SH2 and WD40-1 mutants were not detected in the Western blot when they were transfected alone (lanes 6 and 7). As expected, UL9-1-354 fragment was pulled by the C-terminus efficiently (lane 10), but interestingly a faint band of these

mutants (lanes 8 and 9) were detected as compared to the UL9-1-354 fragment (lane 10). This result suggests that these two mutants were not pulled by the RH-7 antibody or did not bind to the beads non-specifically, but they were able to maintain a weak interaction with the C-terminus of UL9.

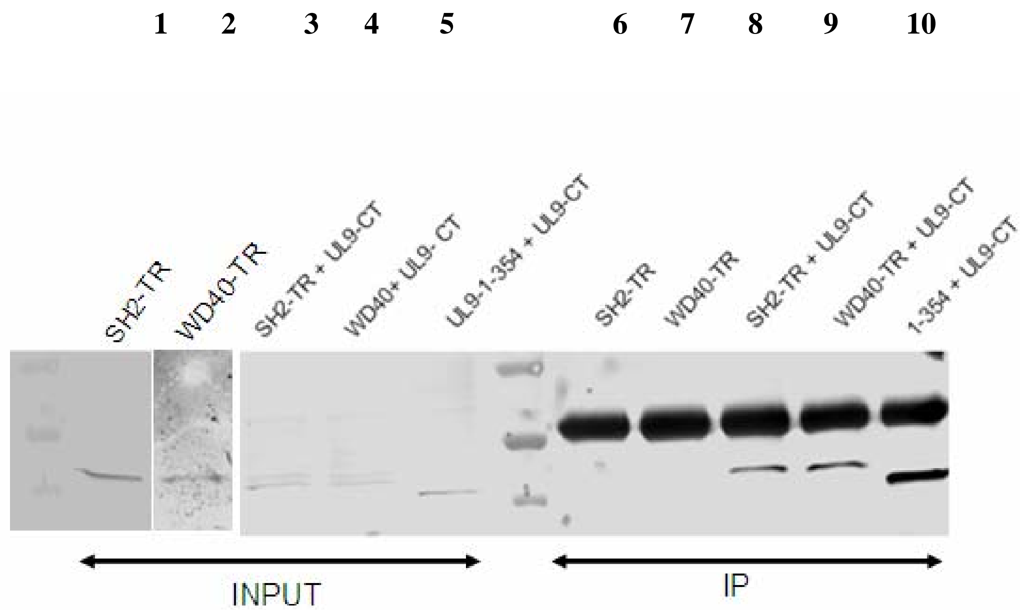


Figure 14. N-terminal truncations of SH2 and WD40-1 motif mutants exhibit decreased ability to interact with the C-terminus. Lines 1 to 5 include the input samples without the co-Immunoprecipitation analysis, lines 6 to 10 include the co-Immunoprecipitated samples. Lines 1 and 6 include the SH2 truncations, Lines 2 and 7 the WD40-1 truncations, Lines 3 and 8 the SH2 truncation and a UL9 C-Terminal fragment, Lines 4 and 9 the WD40 truncation and a UL9 C Terminal Fragment, Lines 5 and 10 a UL9 wild type N Terminal Fragment and a UL9 wild type C terminal fragment. The Western Blot analysis shows in the input that the sample was present before the Co-Immunoprecipitation and in the IP that the SH2 and WD40 mutations are still forming dimmers, but their ability is decreased.

It was also reported that the residues of the N-terminus can modulate the DNA binding ability of the C-terminus (Chattopadhyay, 2006), so the SH2 and WD-40 mutants were also tested in a Plaque Reduction Assay to asses the effect of these mutants in inhibition of replication.

Plaque Reduction Assay showed that SH2 motif mutant fragment showed relief on inhibition, compared to wild type fragments.

Plaque Reduction Assays were performed by co-transfecting KOS infectious DNA and plasmid of interest as described in Materials and Methods, to measure inhibition of replication (or relief of inhibition) by the number of plaque forming units (P.F.U). When UL9 is overexpressesd we can see less plaques than compared to the KOS infectious DNA alone (as shown in Table 2).

Plaque Reduction Assay was performed with the N terminal fragments with point mutations in SH2 and WD40 motifs. The percentages are summarized in table 2, and they were estimated comparing the number of plaques of empty plasmid (considering it 100%).

Table 2. Percentages in Number of Plaques compared to Number of Plaques counted for empty plasmid

Empty Plasmid	100%
UL9 Wild Type	2%
1-394 Wild Type Fragment	60%
SH-2 Truncation	20%
WD40 Truncation	80%
SH2 Full Length Mutant	1%
WD40 Full Length Mutant	1%

The Plaque Reduction Assay for full length mutations in SH2 and WD40 motifs did not showed much difference compared to wild type. The truncations do not include the DNA binding domain, so they did show a strong effect on relief of inhibition. Although, there was some effect in inhibition of replication compared to wild type same size fragment in the N terminal domain. This may be suggesting a transdominant effect in the protein, observing how mutations in one domain may be affecting the functional abilities of the other. Further experiments, like DNA Binding Assay will be useful to determine if there is an effect of these mutants in binding to DNA even if they are not in the DNA binding domain.

4.- Mutations LC8 and MDM2 .

Detection of Mutant Protein Fragments:

Vero cells were transfected with plasmids with the mutations (with the LC8 and MDM2 fragments) as described in Materials and Methods. Cell Lysates were prepared using SDS buffer, boiled for 10 minutes, and loaded into 10% SDS-PAGE. As shown in Figure 16, mutant proteins were expressing protein (marked by asterisk). Proteins were detected with KST-1 antibody, which generated many background (unspecific bands in the blot).

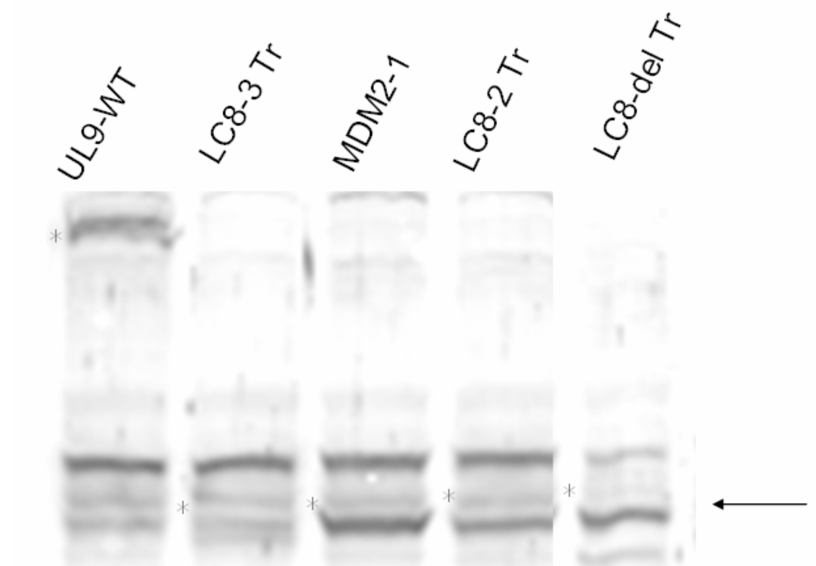
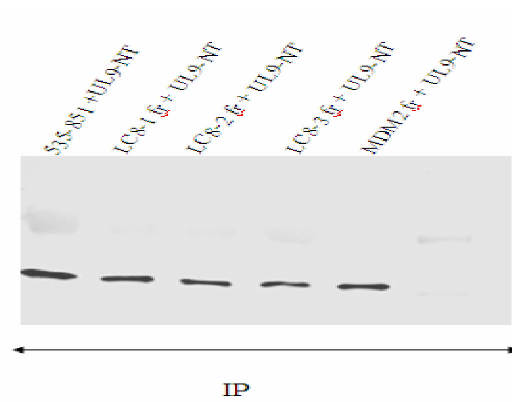


Figure 15. C-terminus fragments with LC8 and MDM2 mutations were expressing protein. An analysis by Western Blot showed that the mutants expressed protein in equal levels. Lines 2, 3, 4 and 5 include the samples for the mutant proteins of LC8 and MDM2 while lines 1 include the samples for the wild type protein.



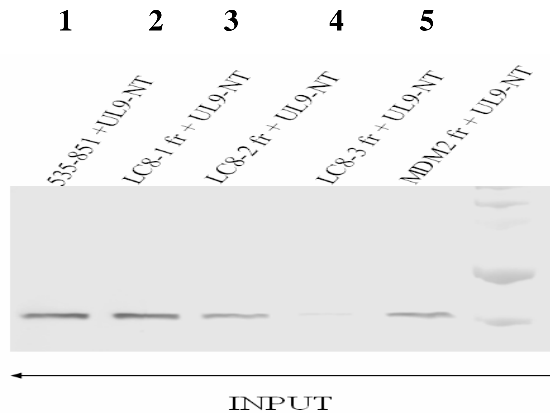


Figure 18. C-terminal fragments of LC8 and MDM2 motif mutants exhibit normal ability to interact with the N-terminus. The second Western Blot includes the input samples without the co-Immunoprecipitation analysis, while the first one includes the co-Immunoprecipitated samples. Line 1 in both Blots includes the wild type N-Terminal Fragment and the wild type C-Terminal Fragment, Lines 2, 3 and 4 the LC8 mutant fragments and a wild type UL9 N-Terminal fragment, Line 5 includes the MDM2 mutant fragment with a wild type UL9 N-Terminal fragment The Western Blot analysis shows in the input that the sample was present before the Co-Immunoprecipitation and in the IP that the LC8 and MDM2 mutations are still forming dimmers with normal interaction within its domains.

The UL9 fragments containing the LC8 and MDM2 motif mutations were cotransfected with a N-terminal fragment of UL9 (1-354) and co-Immunoprecipitation assay was carried out using the RH7 antibody as described in Materials and Methods section. The immunoprecipitated complex was resolved in a 10% SDS-PAGE and the membrane was probed with 17B antibody (described in Materials and Methods). It was observed that the N-terminal fragment was immunoprecipitated with the mutant proteins as efficiently as wild type UL9. Co-Immunoprecipitation was performed using UL9-535-851 C-terminal fragment as a control of strong interaction with the N-terminus. As shown in Figure 19, N-terminus was pulled by the UL9 535-851 fragment efficiently as expected, and so did the 535-851 fragments containing the LC8 and MDM2 motif mutants, mostly with the same intensity. These results indicate that this motifs are not involved in the N and C-terminal interaction of UL9. Input blot is to show that the proteins were in the cell lysate before performing Co-IP.

Plaque Reduction Assay showed that LC8 motif mutant fragments caused release on inhibition, compared to wild type fragments.

The DNA binding domain of UL9 is mapped within residues 564 to 832. This domain is responsible for binding to the origin DNA to start initiation of replication in the first stage of replication, however, it was proposed that the DNA binding ability of this protein causes inhibition of replication probably in the second stage of replication as over-expression of UL9 was inhibitory. Since the MDM2 and LC8 motifs are mapped within the DNA binding domain of this protein, therefore these mutants were analyzed to check their inhibitory ability in plaque reduction assay.

Plaque Reduction Assays were performed by co-transfecting KOS infectious DNA and plasmid of interest as described in Materials and Methods, to measure inhibition of replication (or relief of inhibition) by the number of plaque forming units (P.F.U). When UL9 is overexpressesd we can see less plaques than compared to the KOS infectious DNA alone (as shown in Table 3).

Plaque Reduction Assay was performed with the C terminal fragments with point mutations in MDM2 and LC8 motifs. It was shown by the number of plaques that LC8 deletion was causing a considerable relief on inhibition compared to the C-terminal wild type fragment of UL9. On the other hand, the MDM2 motif fragment did not have a considerable effect on inhibition compared to wild type.

Table 3. Percentages in Number of Plaques compared to Number of Plaques counted for empty plasmid.

Empty Plasmid	100%
UL9 Wild Type	2%
535-851 Wild Type Fragment	1%
535-851 LC8-d Fragment	75%
535-851 MDM2 Fragment	20%

In order to confirm the DNA binding ability of the mutant protein, DNA binding assay was performed by expressing mutant protein in *In vitro* transcription and translation using Rabbit reticulocyte system (done by Soma Chattopadhyay). As shown in Figure 16 the mutant proteins were expressed well in this assay.

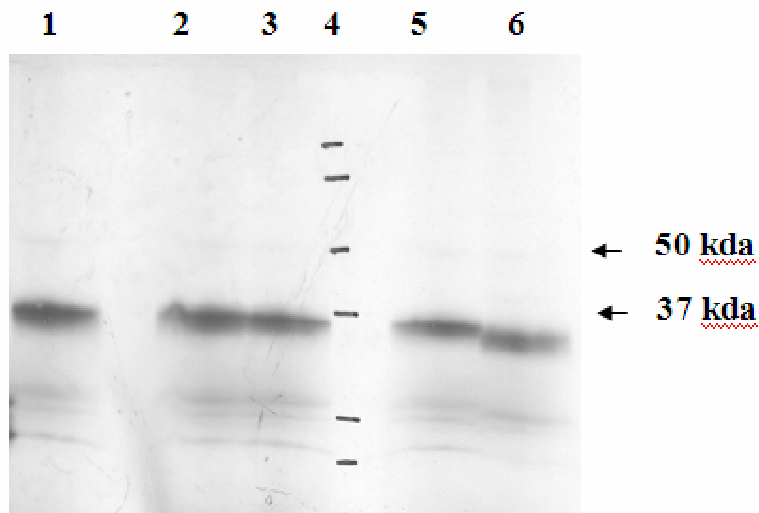


Fig. 16

a) Mutant proteins were expressed in In Vitro Transcription-Translation using Rabbit reticulocyte (Chattopadhyay, unpublished data). This assay shows that the plasmid was still expressing protein even in an *in vitro* assay and that the levels of expression

were similar. Line: 1 UL9-C (535-851), Line: 2 LC8-1 (535-851), Line: 3 LC8-2 (535-851), Line: 4 , Line: 5 MDM2-1(535-851), Line: 6 dLC8 (535-851)

DNA Binding Assay showed LC8 motif mutants were defective for their ability to bind to the origin. On the other hand MDM2 motif mutant binds to origin DNA as efficiently as the C-terminal wild type fragment (experiment done by Soma Chattopadhyay, unpublished data).

Gel Shift, Band Shift Assay, or Electrophoretic Mobility Shift Assay (EMSA) is a technique for determining protein-DNA interactions. The assay is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel shift assay is carried out by first incubating a protein such as nuclear or cell extract with a 32P end-labeled DNA fragment containing the putative protein origin binding site. The reaction products are then analyzed on a nondenaturing polyacrylamide gel.

The DNA Binding assay was performed by Soma Chattopadhayay to determine if the mutants were affecting DNA binding capacity of the protein to the origin of replication.

The DNA Binding region was mapped in the C-terminal domain (Arbuckle et al, 1993) of UL9, so fragments containing the C terminal domain can bind to DNA, while wild type fragments of the N terminal domain cannot. This DNA Binding Assay showed LC8 motif mutants were defective for their ability to bind to the origin. In the other hand MDM2 motif mutant binds to the origin DNA almost as efficiently as the C-terminal wild type fragment (experiment done by Soma Chattopadhyay, unpublished data). These data correlates with the Plaque Reduction Assay results in which the LC8 mutant forms more plaques compared to wild type, it releases inhibition because it is causing a defect in DNA binding. The deletion (LC8-3) did not bind DNA, and the two and three residue mutants (LC8-1 and LC8-2) affected also DNA binding. MDM2 is probably not as efficient as wild type, but still binds to DNA.

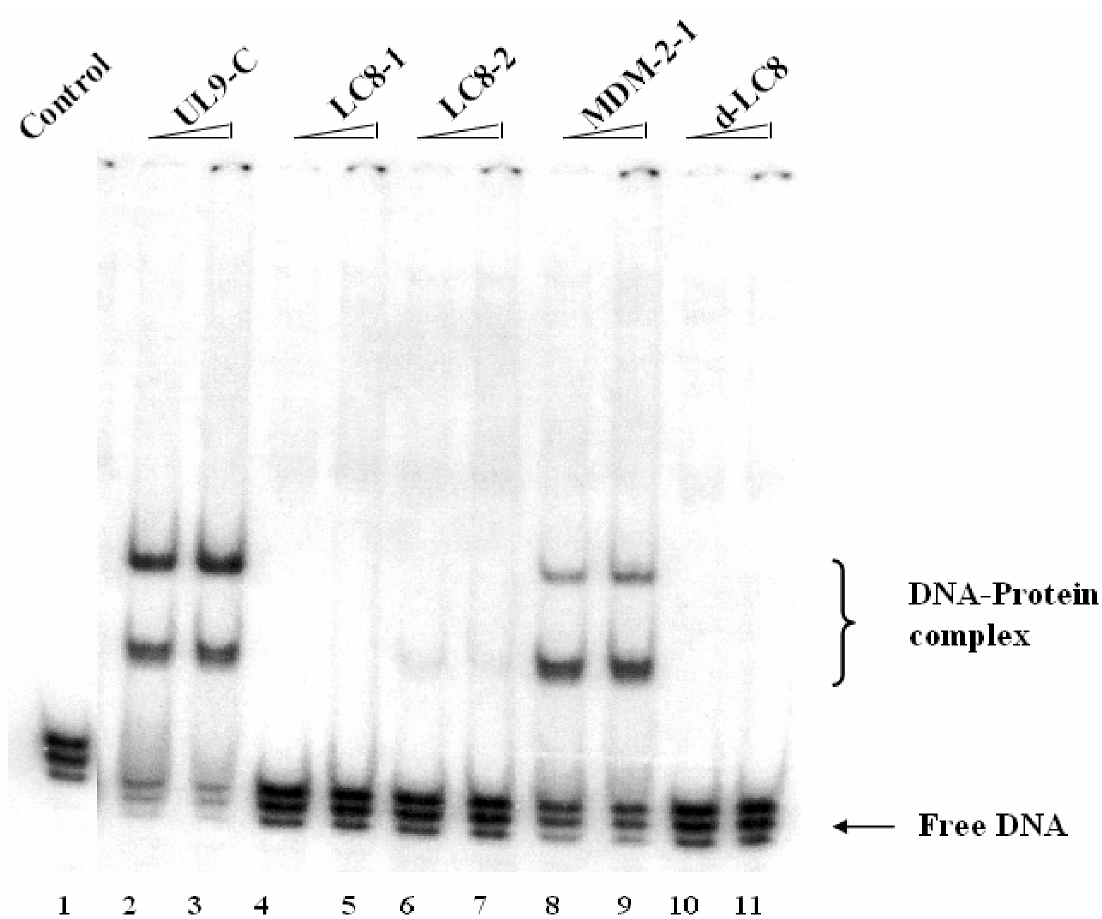


Fig. 17

b) DNA Binding Assay showed LC8 motif mutants were defective for their ability to bind to the origin (Chattopadhyay, unpublished data). DNA Binding Assay showed LC8 motif mutants were defective for their ability to bind to the origin. On the other hand MDM2 motif mutant binds to origin DNA as efficiently as the C-terminal wild type fragment (experiment done by Soma Chattopadhyay, unpublished data).

In this Assay, it was observed that the LC8 motif mutants were defective for their ability to bind to the origin. In the other hand MDM2 motif mutant binds to origin DNA as efficiently as the C-terminal wild type fragment. This results also correlate with the Plaque Reduction Assay in which LC8 cause release of inhibition.