INTRODUCTION

HSV-1 is a common viral pathogen that infects millions of people worldwide causing widespreading disease. It causes cold sores to more serious complications that can lead to mental retardation, blindness and life threatening conditions as temporal encephalitis in immunocompromised patients and newborns (Whitley and Roizmann, 2003). All herpes virus cause a life long latent infection in the host, which usually results in recurrent lytic replication/productive infections. The inability to adequately control reactivation and lytic viral growth is problematic and ultimately leads to more severe illness.

Until now, there is no cure for latent herpes viruses, so this fact makes it important to find effective antiviral therapies that can block lytic infection, latent reactivation, and transmission. Several drugs are currently available, such as acyclovir and valcyclovir, that treat HSV-1 infections by targeting the viral polymerase (Kleynmann G. 2002). Although this treatment is good at reducing viral infection, there is an increase in resistance to these agents by the virus. Several viral proteins that are essential for the lifecycle of HSV-1 including those involved in DNA replication process may provide new potential targets for antiviral therapy with better outcomes for those afflicted by recurrent disease. There are some other drugs like penciclovir, famciclovir, idoxuridine,brivudine, ganciclovir, valgancclovir, cidofovir and foscarnet for the treatment of herpes viruses, and these can reduce or suppress the herpes disease symptoms, but not cure the disease or reduce frequency of recurrent outbreaks after discontinuouing treatment (Kleynmann, 2003). There are also some helicase-primase complex inhibitors like BAY 57-1293 that have shown to reduce frequency and severity of recurrent disease (Kleinmann, 2003), but still not completely.

In this work we will study UL9, the origin binding protein, a replication protein of the HSV-1. Many of its aspects during initiation of viral DNA replication and its regulation during infection remain unresolved. In this thesis we want to identify important motifs in this replication protein in order to better understand about its role during HSV-1 DNA replication initiation and its regulation. We will use the Minimotif Miner software (http://sms.engr.uconn.edu/servlet/SMSSearchServlet) in order to determine them and we will use mutagenesis approach as well as complementation assays to determine which of them are important for UL9.

Overview of Mechanism of Replication of HSV-1

The mechanism of replication in herpesviruses is not well understood mainly to the inability to reconstruct the process in the test tube. It is believed that replication in HSV-1 require circularization of linear viral genomes immediately following infection (there is still no much evidence to support this model). It was previously thought that linear virion DNA circularized in infected cells and that concatemers were generated by rolling circle replication; however, this model has never been directly confirmed experimentally. Several aspects of this model including the initial circularization and the rolling circle mechanism have recently been challenged experimentally. The HSV-1 genome is a 152,000 bp double stranded DNA and circular duplex DNAs are generally thought to replicate in a theta structure or a rolling circle mechanism. For example, bacteriophage lambda replicates initially as a theta form and switches to rolling circle replication later in infection. Although many details of the mechanism of HSV DNA replication remain unclear, several lines of evidence support the model that HSV-1 replication proceeds in two stages (Severini, 1994; Blumel, 1995). DNA replication very likely initiates in an origin-dependentmanner; however, at later times, replication apparently proceeds by an origin-independent manner, perhaps by a rolling circle and/or recombination-driven mechanism (reviewed in Marintchneva and Weller, 2001)

Replication Proteins:

There are seven replication proteins that are essential for HSV-1 replication in cell culture (Challberg, 1996). All of them code for proteins with biochemical activities expected for a replication fork: a DNA polymerase (UL30, UL42), an origin binding protein (UL9), a heterotrimeric helicase-primase complex (UL5, UL52 and UL8) and a ssDNA binding protein (UL29 or ICP8) (Olivo,et al 1989).

UL9, The Origin Binding Protein.

a) Biochemical properties and domain structure.

Biochemical analysis of purified UL9 protein indicates that UL9 possesses the following activities: it is a DNA dependent nucleoside triphosphatase, DNA helicase, it has the ability to form dimmers in solution and cooperative origin-specific DNA binding (reviewed in Chattopadhyay, et.al, 2006).

The origin binding protein is an 851 amino acid residue protein that contains ATP-binding and DNA helicase motifs, both of which are required for viral DNA replication. UL9 binds specifically as a homodimer to sites in the origin of replication containing the DNA sequence CGTTCGCACTT (Elias and Lehman, 1988). The binding of UL9 to the origin is believed to cause a bend in the DNA allowing for the distortion of the DNA structure to form singlestranded stem loop structure. The ssDNA and UL9 may recruit the single-stranded DNA binding protein, ICP8, which in turn stimulates the helicase activity of UL9 (reviewed in Taylor, 1988).

UL9 is organized in two different domains: the N terminal domain(residues 1-534) and the C terminal domain (535 to 851). Protein sequence analysis shows that the N-terminal domain contains seven conserved helicase motifs characteristic of Super Family II of helicases which have been shown to be essential for its function in vivo by site directed mutagenesis and complementation analysis (Martinez et al, 1992). The N terminus contains a cooperative binding domain, and it interacts with the helicase primase complex, UL8 and UL42 (McLean et al 1994, Monahan et al, 1998)) . On the other hand the C terminal also functions as a separate domain, it interacts with ICP8 (Boehmer 1994), it has the nuclear localization signal and it contains the DNA binding domain (Arbuckle, et al , 1993).

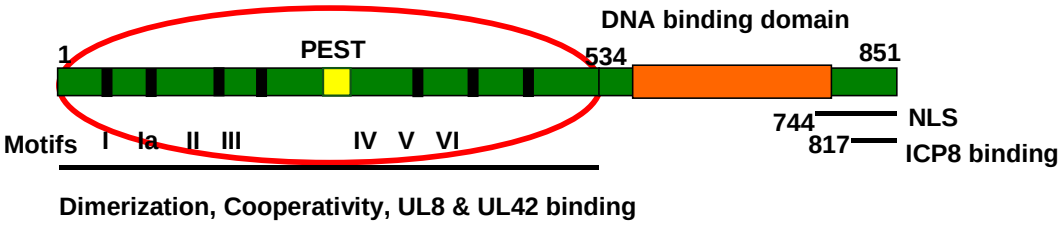

Fig. 1 The N terminal domain of UL9.

The N-Terminal domain of the UL9 protein has a PEST sequence involved on its degradation, it has also the dimerization and cooperativity domain and it interacts with UL8 and with UL42.

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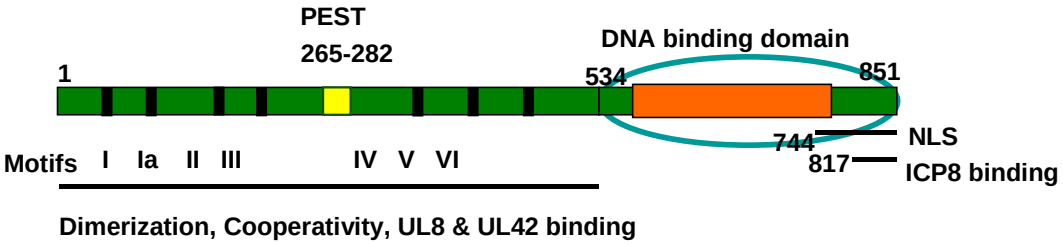


Fig. 2 The C terminal domain of UL9 The C- Terminal Domain of UL9 has the DNA Binding Domain, it includes a Nuclear Localization Signal and the ICP8 binding region.

The region required for interaction with ICP8 is in the last 27 aminoacids of the C terminal region , while the UL8 and UL42 interactions have been localized in the N terminal region (but they have not been specifically mapped yet).

b) Inhibition of Replication.

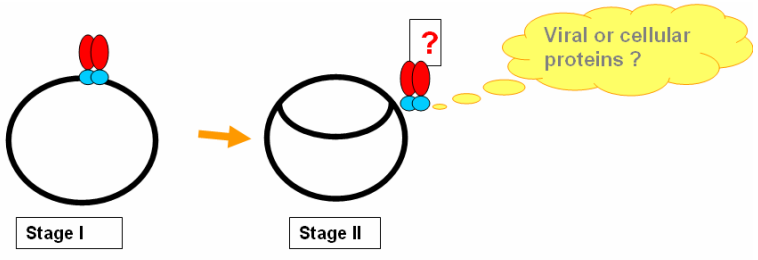
HSV DNA synthesis is believed to begin at one of the three viral origins of replication. UL9 exists as dymers that bind to box I and to box II, altering the conformation of the origin region, distorting the A/T rich region, facilitating the unwinding of DNA via the ATP-dependent helicase activity of UL9 (reviewed in Marintchneva and Weller, 2001).

UL9 apparently binds to box I in OriS as a dimer , and another dimer of UL9 binds site II in a cooperative manner.

With the help of ICP8 the A/T rich spacer becomes distorted. It is possible that unwinding at the origin requires other cellular or/and viral proteins (reviewed in Marintchneva and Weller, 2001). Once the origin of replication is unwound or distorted it is proposed that the helicase primase complex can be recruited. Different evidences suggest the existence of a subassembly consisting of these five viral proteins: ICP8, UL9, UL5, UL52 and UL8. Various interactions between these proteins have been observed. Afterwards, the two subunit polymerase can be recruited at the replication fork to begin DNA synthesis.

UL9 is present and important in early HSV-1 infection but not required late in infection (Blumel and Matz ,1995). Blumel and Matz (1995) showed by thermossensitive mutants that the function of UL9 was required at early times during DNA synthesis whereas upward temperature shifts at later time did not considerably inhibit DNA synthesis. So it is suggested that UL9 has a role in the initiation phase, but not at the elongation phase of viral DNA synthesis.

As it was mentioned before, the exact HSV-1 replication mechanism is not very clear. It is suggested that there is a two stage model that proposes that a replication via theta mechanism happens at the early stage of replication which is dependent of UL9 and that a rolling circle mechanism which is independent of UL9, occurs at late stages of replication (Severini at al., 1994).



Severini et al., 1994; Weller, 1995; Marintcheva and Weller, 2001

Fig. 3 Two Stage Model .This model suggests that UL9 is required in the first stage of replication but is dispensable, or not important for the later stage, where it´s not bound anymore to the origin of replication.

Little is known about the regulation of the UL9 gene during HSV-1 infection. UL9 is necessary in early infection and dispensable late in infection (Blumel, 1995). Furthermore, overexpression of wild type UL9 or the C-terminal origin binding domain is inhibitory for HSV-1 infection as judged by transfection/superinfection and plaque reduction assays (Perry et al., 1993). Thus, in the second stage, UL9 is not needed, and the overexpression of the protein causes inhibition of replication. The inhibition of infection by expression of the UL9 proteins raises the possibility that the activity of UL9 may be regulated during infection. It is possible that UL9 activity is altered allowing activity in the early stage and inactivation and/or degradation at later stages.

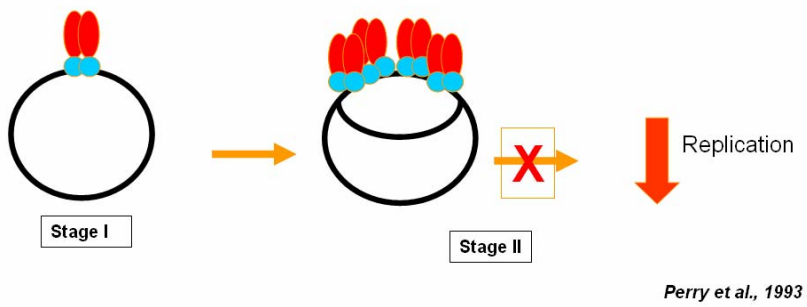


Fig.4 The over expression of UL9 wild type inhibits viral replication It has been shown that when UL9 is overexpressed and bound to the origin of replication the replication of the virus is inhibited dramatically.

c) Regulation of UL9 activity.

Phosphorylation is a very common mechanism of regulation of protein activity in the cell. It was actually shown that UL9 is phosphorylated in the context of HSV-1 infection (Isler and Schaffer, 2001). Replication initiator proteins in other systems are known to be regulated by phosphorylation. Using a metabolic labelling and immunoprecipitation approach, Isler and Schaffer obtained evidence that UL9 is

phosphorylated during infection. Assessment of the phosphorylation state of UL9 has been challenging due to the very low level of UL9 expressed in infected cells. Blumel and Schaffer demonstrated that UL9 is phosphorylated during HSV infection and that cellular or viral factors enhance the level of phosphorylation. They also showed that HSV induced phosphorylation is dependent on early synthesis and independent on DNA replication. Also, Yong Eom and Lehman (2003), demonstrated that UL9 binds to NFB42 (part of ubiquitin-ligase complexes, which mediate ubiquitination and preoteasome degradation of phosphorylated proteins) and is degraded via the ubiquitin-proteasome pathway. They coexpressed NFB42 and UL9 and treated the cells with the 26S-proteosome inhibitor MG132 and restored the normal level of UL9 protein. They also observed that UL9 was polyubiquitinated *in vivo* and that the interaction between NFB42 and UL9 is dependent upon phosphorylation. These results suggest that the interaction of the UL9 protein with NFB42 finishes in its polyubiquitiantion and degradation by the proteosome.

Although the two step model we mentioned before, is consistent with most of the experimental data, there are many unanswered questions, Which viral or cellular proteins contribute to the switch between stages? What happens to the origin binding protein after initiation of replication? It is interacting with other partners and changing its conformation so it is not bound anymore to DNA? Is it regulated via phosphorylation?

In this study we would like to answer which are the important functional motifs of UL9 that might be involved in either UL9-UL9 interaction, interaction of UL9 with other viral and cellular proteins or that may be involved in regulation of its activity during infection? (via phosphorylation for example).

In order to identify these motifs we will use Minimotif Miner which is a tool for analysing protein function (Balla et al, 2006, http://sms.engr.uconn.edu/servlet/SMSSearchServlet). Minimotif Miner (MnM) analyzes protein queries for the presence of short functional motifs that have been demonstrated to be involved in posttranslational modifications, binding to other proteins, nucleic acids, or small molecules, or proteins trafficking (Balla et al, 2006).

Protein domains are highly conserved throughout evolution. It is logical to expect that their binding partners or substrates would also be conserved. These short motifs (less than 15 residues) provide complementary information about protein function. There are many databases that catalog small collections of motifs by specific functions such as phosphorylation. These databases are usefull if we know that the protein in study has such functionality (Balla, et al, 2006). These databases are not, however designed to identify unknown functions in proteins, but this program can be used to search known or new proteins for the presence of motifs in the Minimotif database. The purpose of Minimotif analysis is to predict new putative functions in proteins, and that they can be then confirmed experimentally (Balla et al., 2006).

In order to confirm if these motifs are required or important for HSV-1 replication, mutants in these motifs will be created and tested by Complementation Assay and Plaque Reduction Assay. These assays are used to test functionality of the protein and will be described in the Methodology section of the thesis.