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Cloning and characterization of a Marek's disease virus (MDV) gene homologous to herpes simplex virus type 1 (HSV-1) UL9 gene

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CLONING AND CHARACTERIZATION OF A MAREK'S DISEASE VIRUS (MDV) GENE HOMOLOGOUS TO HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) UL9 GENE

By

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ABSTRACT

CLONING AND CHARACTERIZATION OF A MAREK'S DISEASE VIRUS (MDV) GENE HOMOLOGOUS TO HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) UL9 GENE

By

Ting-Feng Wu

Marek's disease virus (MDV), a highly cell-associated avian herpesvirus, induces Marek's disease (MD) which is characterized by malignant lymphoma of T cells. Viral replication represents a point at which pharmacological control of herpesvirus infection may be most successful. Studies in HSV-1 DNA replication implicate the UL9 protein as a key initiator of replication. In this study, a protein with apparent molecular size similar to HSV-1 UL9, was identified in infected cell extracts by western blot analysis with anti-HSV-1 UL9 antibody. A putative MDV UL9 gene was subsequently identified through sequencing of MDV genome fragments (BamHI G and C). Extended DNA sequence analysis revealed an open reading frame (ORF) which could encode a protein homologous to HSV-1 UL9. The MDV UL9 ORF encodes an 841 amino acid polypeptide which is highly similar to HSV-1 UL9 and VZV gene 51 (VZV UL9). MDV UL9 shares numerous structural motifs with HSV-1 and VZV gene 51, including six conserved N-terminal helicase motifs, an N-terminal leucine zipper motif, a C-terminal pseudo-leucine zipper sequence, and a putative helix-turn-helix structure. The above results suggest that MDV UL9 gene may have the similar biochemical activities to HSV-1 UL9, specifically the origin-binding activity.

A MDV UL9 protein of 95 kd was detected in nuclear extracts of MDV infected cells by western blot analysis with anti-MDV UL9 antibody. PCR was used to clone the MDV UL9 gene. *In vitro* transcription-translation of this gene generated a protein with apparent molecular size of 95kd. Electrophoretic mobility shift assays (EMSAs) with *in-vitro* expressed MDV UL9 protein or the infected nuclear extracts showed that MDV UL9 protein could bind to the HSV-1 UL9 binding site I and MDV UL9 binding site II. Competitive EMSAs with a mutant MDV UL9 site II DNA indicated that the last nucleotide (T) within MDV UL9 binding site II was essential for the binding of MDV UL9 protein to MDV UL9 binding site II. Competitive EMSAs with a series of mutant MDV UL9 site I DNAs demonstrated that the last two nucleotides (TT) within HSV-1 UL9 binding site I were essential for the binding of MDV UL9 protein *in vitro*.

To my wife Shu-Ju

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List of Abbreviations

bp	base pairs
CEF	chick embryo fibroblast
C/EBP	CCAAT/enhancer binding protein
CHEF	contour-clamped eletric field
CMV	cytommegalovirus
CsCl	cesium chloride
DR	direct repeat
EBV	Epstein-Barr virus
EBNA-1	EBV nuclear antigen-1
EHV	equine herpes virus
EMSA	electrophoretic mobility gel shift assay
gB	glycoprotein B
GST	glutathione-S-transferase
HCMV	human cytomegalovirus
HSV-1	herpes simplex virus type 1
HVT	herpesvirus for turkey
ICP8	infected cell protein No. 1
IR_L	internal repeat long
IR_S	internal repeat short
kbp	kilobase pair
kDa	kilodalton

MD	Marek's disease
MDV	Marek's disease virus
Mta	(EBV) M transactivator
NC	nitrocellulose membrane
OBP	origin binding protein
Oct-1	octamer binding factor-1
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PI	post infection
RE	restriction endonuclease
Rta	(EBV) R transactivator
SDS	sodium dodecyl sulfate
TBP	TATA-binding protein
TRL	terminal long
TRS	terminal short
TPA	12-ortho-tetradecanoyl-phorbol-13-acetate
UL	unique long
US	unique short
VZV	varicella-zoster virus
Zta	(EBV) Z transactivator

Chapter 1

Literature review

1. Pathogenesis of Marek's disease virus

Marek's disease (MD) is a naturally occurring lymphomatous neoplasm of fowl (Calnek, 1980; Calnek and Witter, 1984) and is one of the most serious infectious diseases of poultry. MD was first described as inflammatory nerve lesions and classified as a polyneutritis in 1907 by Josef Marek (Marek, 1907). Later it became apparent that in addition to paresis and paralysis, lymphomas were also associated with the disease (Biggs, 1968). Therefore, MD has both neoplastic as well as inflammatory features. The neoplastic aspect of MD is characterized primarily by mononuclear cell infiltrations with the development of lymphomas in nerves, visceral organs, muscle and skin (Payne *et al.*, 1976; Calnek and Witter, 1991). In the late 1960s and early 1970s, MDV was found to be caused by a herpesvirus, Marek's disease virus (MDV) (Churchill and Biggs, 1968; Churchill *et al.*, 1969; Witter *et al.*, 1969; Calnek *et al.*, 1970). MDV transmission is strictly horizontal and infection is primarily by inhalation. Infectious virus is shed from the feather follicle epithelium and is often associated with dead keratinized cells as "dander" or attached to molted feathers (Beasley *et al.*, 1970; Calnek *et al.*, 1970). MDV generally gains entry via the respiratory tract but almost no infection can be detected in the trachea, lung, or air sacs (Adldinger and Calnek, 1973).

The pattern of MDV infection which occurs sequentially in genetically susceptible chickens can be generally divided into four phases (Calnek, 1985; Calnek and Witter,

1991): (1) early cytolytic infection, (2) latent infection, (3) permanent immunosupression and late cytolytic infection, and (4) transformation.

A semi-productive infection is evidenced in spleen, thymus and bursa of Fabricius by the third day postinfection (DPI) (Adldinger and Calnek, 1973). In those three organs, few or no enveloped virions are produced; rather, naked nucleocapsids are present in the infected cells (Adldinger and Calnek, 1973; Payne and Rennie, 1973). The infection in these three primary lymphoid organs is semi-productive and cytolytic (Jakowski *et al.*, 1969). The early necrotizing infection in these three primary lymphoid organs involves reticular cells as well as lymphocytes (Payne and Rennie, 1976). However, the primary target in cytolytic infection is bursa-derived lymphocytes (B cells) and a small percentage of cytolytically infected T cells (Shek *et al.*, 1983; Calnek *et al.*, 1984). Hyperplasia of reticular cells may occur, resulting in splenic enlargement but ultimately, atrophy of the bursa and thymus occurs (Payne *et al.*, 1976). Since these organs are the central organs in humoral and cell-mediated immunity, immunosuppression follows early infection. The early cytolytic infection in these organs reaches a peak by 4 to 5 DPI and then declines by 6 to 7 DPI.

Humoral and cell-mediated immune responses to MDV can be detected by 5 to 7 DPI (Sharma and Coulson, 1977; Confer and Adldinger, 1980). Concurrently, there is a switch in the type of infection seen in lymphocyte populations. Coincident with the decrease in the numbers of cytolytically infected cells, latently infected cells appear in the spleen and peripheral blood. In contrast to the early cytolytic infection which involves

mostly B-cells with only a few T-cells, latent infection involves primarily T-cells and few B-cells (Shek *et al.*, 1983; Calnek *et al.*, 1984).

After the 2nd or 3rd week postinfection, foci of infection appear in various epithelial tissues (Calnek and Hitchner, 1969; Spencer and Calnek, 1970). Latently infected lymphocytes may be the means by which infection spreads from the central lymphoid organs to other tissues in the body, although the pont have been proven. These infected areas are also of the semi-productive infection type. Kidney, adrenal gland, and feather follicle epithelium are good examples of tissues in which epithelial cells become infected (Calnek and Hitchner, 1969; Spencer and Calnek, 1970). The feather follicle epithelium is unique because it is the only tissue in which infection is fully productive (Calnek *et al.*, 1970; Nazerian, 1971). Enveloped virions are produced in infected feather follicle epithelial cells that are undergoing keratinization (Calnek *et al.*, 1970; Nazerian and Witter, 1970) and MDV is shed to the environment when either feathers are molted or when dead cells are lost in the form of dander. MDV from feather follicle epithelium is the sole source of infectious virions which can be transmitted to other birds. Cytolytic infection in other epithelial tissues however results in necrosis which in turn, induces an inflammatory response with infiltration of mononuclear cells.

At the same time cytolytic infection occurs in epithelial tissues, there is a reappearance of cytolytic infection in the central lymphoid organs. By 2 to 3 weeks postinfection, permanent immunosupression involving both humoral and cell-mediated immune responses manifests. It has been postulated that there is a cause-effect relationship

between permanent immunosupression and reappearance of cytolytic infection because the two events occur together.

The final manifestation of MD is cellular alteration, and the most obvious type of cellular alteration is the neoplastic transformation of lymphocytes with the development of gross lymphomas. After 3 or more weeks postinfection, lymphoma may develop in a variety of visceral organs, skin, muscle, and nerves. The composition of MD lymphomas is complex. The transformed cells in tumors have been identified as transformed thymus-derived lymphocyte (T-cells) which are activated T-cells. The transformed cells are the basic offending cells, however other cells also contribute to tumor formation, including immunologically committed or uncommitted T- or B- lymphocytes, macrophages, granulocytes, and plasma cells (Hudson and Payne, 1973; Payne and Rennie; 1976).

2. Marek's disease virus (MDV)

I. Biology of MDV

MDV is a cell-associated herpesvirus (Nazerian *et al.*, 1968; Solomon *et al.*, 1968) and the virion is composed of four components : (1) a core containing viral DNA; (2) a nucleocapsid; (3) an envelop ; and (4) a tegument between nucleocapsid and envelop. The nucleocapsid is approximately 85-100 nm and nucleocapsids with or without electron-dense nucleoids are usually found in the nucleus and occasionally in the cytoplasm of infected tissue culture cells (Kato and Hirai, 1985; Schat, 1985). In negatively stained preparations, nucleocapsids have cubic icosahedral symmetry and possess 162 hollow-center capsomeres. The nucleocapsid particles are cylindrical and measure 6 x 9 nm

(Nazerian, 1973). Enveloped particles 150-160 nm in diameter are principally associated with the nuclear membrane or nuclear vesicles (Kato and Hirai, 1985; Schat, 1985).

Three MDV serotypes have been identified based on agar precipitation and immunofluorescence (Bulow and Biggs, 1975a; Bulow and Biggs, 1975b): (1) Serotype 1 MDV includes all oncogenic strains and their attenuated derivatives; (2) Serotype 2 MDV consists of naturally occurring non-oncogenic chicken herpesviruses; and (3) Serotype 3 MDV is an antigenically related non-oncogenic herpesvirus of turkey (HVT).

II. The genome of MDV

The MDV genome is composed of a linear double-stranded DNA of 168-180 kilo-base pairs (kb) containing nicks and gaps which is common in other herpesvirus (Lee *et al.*, 1971; Hirai *et al.*, 1979; Cebrian *et al.*, 1982). The buoyant density of MDV DNA is 1.706 g/ml, with a base composition ratio of 46% guanine plus cytosine (Lee *et al.*, 1971; Hirai *et al.*, 1979; Cebrian *et al.*, 1982). MDV DNA is very difficult to separate from host cell DNA because the bouyant density is the same for both (Kaaden *et al.*, 1977; Wilson and Coussens, 1991). Originally, MDV was classified as a gammaherpesvirus based primarily on its lymphotrophic nature, similar to the lymphotropism of Epstein-Barr virus (EBV). However, recently MDV has been reclassified as an alphaherpesvirus based on genome structure, gene colinearity and gene homology that is closer to other alphaherpesvirus than to gammaherpesviruses (Brunovskis and Velicer, 1992; Buckmaster *et al.*, 1988; Cebrian *et al.*, 1982; Roizman, 1992).

The genome structure of herpesviruses can be divided into six groups based on complexity, designated by the letters A to F (Roizman, 1991). The genome structure of MDV belongs to the same *Herpesvidae* group E genome group as do HSV and VZV (Cebrian *et al.*, 1982). The group E genome structure consists of covalently linked long (L) and short (S) regions, each composed of unique sequences (U_S and U_L) flanked by inverted repeats (TR_S+IR_S and TR_L+IR_L, respectively)(Roizman, 1991) (Figure 1). The

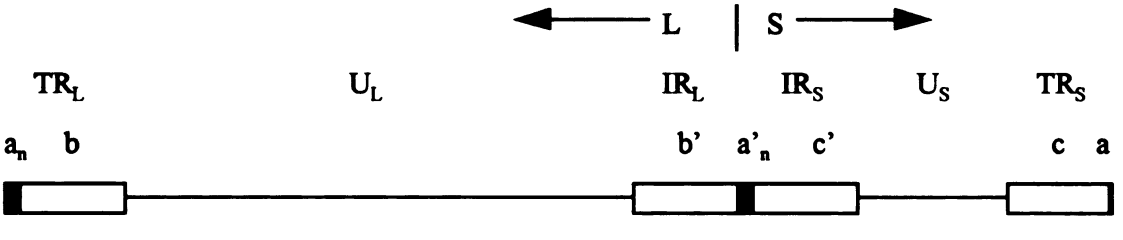

Figure 1. The group E herpesvirus genome structure

standard group E genome based on HSV genome is written as a_nb-U_L-b'a_nc'-U_S-ca. TR_L of group E genomes contain n copies of *a* sequence, whereas TR_S contains one copy of *a* sequence. Numerous studies suggest that the *a* sequences present in the L-S junction and both termini of the HSV genome contain two cis-acting recognition signals which are involved in the inversion of L-S components relative to each other (Morse *et al.*, 1977; Mocarski *et al.*, 1980; Chou and Roizman, 1985) and are also essential for proper cleavage and packaging of unit length viral DNA molecules (Deiss *et al.*, 1986; Deiss and Frenkel, 1986). A potential *a*-like sequence has been identified in the L-S junctions and both termini of serotype 1 MDV and HVT DNA molecules (Kishi *et al.*, 1991; Reilly and Silva, 1993).

Besides the standard group E genomic structure, serotype 1 MDV contains several sets of direct repeats consisting of more than 100-bp (designated DR1 to DR5) scattered through the genome (Hirai, 1988).

Physical maps of restriction endonuclease fragments have been constructed for the genomes of all three serotypes (Fukuchi *et al.*, 1985; Igarashi *et al.*,1987; Ono *et al.*, 1992) (Figure 2). The availability of RE maps and genomic clones of MDV DNA has greatly facilitated the cloning of MDV genes. Although all three MDV serotypes are antigenically related, their genomic RE patterns are very different (Ross *et al.*, 1983). This

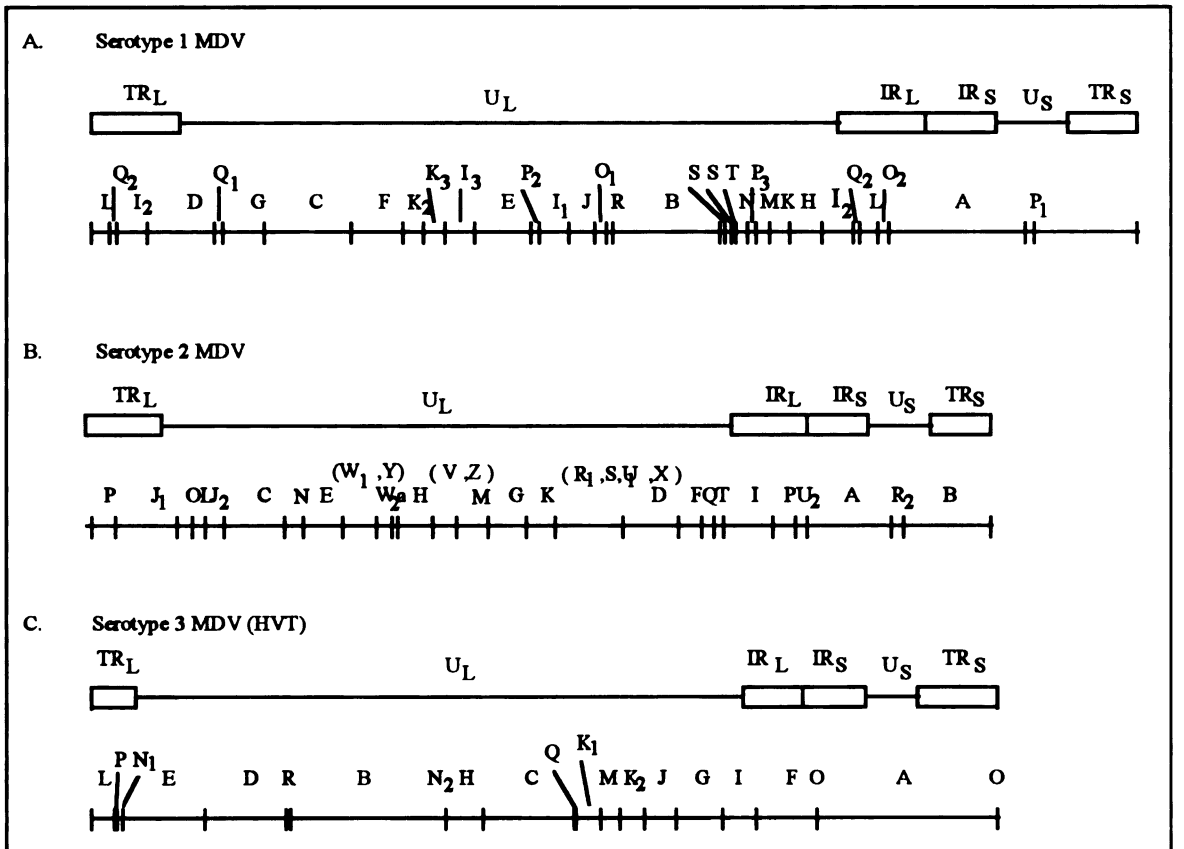

Figure 2. BamHI restriction endonuclease maps of (A) Serotype 1 MDV (B) Serotype 2 MDV (C) Serotype 3 MDV

difference can be used to identify new isolates (Silva and Barnett, 1991). Based on the

cross hybridization of cloned DNA fragments, the genomes of MDV and HVT are related and similarly organized. Recently, Ono *et al.* (1992) has shown that serotype 2 MDV is similar to serotype 1 MDV and HVT DNA molecules, with regard to genome structure and gene colinearity.

III. DNA replication

Mammalian DNA viruses have been useful model systems for the study of eucaryotic DNA replication (Challberg and Kelly, 1989; Stillman, 1989). Mammalian DNA viruses offer many advantages as models for DNA replication because their relatively simple genome structures allow easy manipulation at the molecular level. Studies of Simian virus 40 (SV40) and adenovirus DNA replication, which rely mainly on the host-cell replication machinery, have revealed many essential eucaryotic cellular proteins which are involved in the viral DNA replication process (Challberg and Kelly, 1989). In contrast to SV40 and adenovirus, herpesvirus genomes are more complex and encode most of the proteins required for their DNA synthesis. Thus herpesviruses are an attractive model for studying the interactions between virus-encoded and cellular proteins involved in DNA synthesis .

Study of MDV DNA replication is important for several reasons. First, Marek's disease is highly contagious and results in tremendous losses to the poultry industry. In the interest of disease prevention, it is essential to understand the mechanism of MDV DNA replication. A better understanding of viral DNA replication may lead to better management of the disease in chickens. Second, an understanding of the mechanism of MDV DNA replication may help to elucidate replication mechanisms in closely related

herpesviruses. In addition, knowledge of MDV DNA replication would contribute to the generation of a general model of DNA replication for α herpesviruses. Third, Marek's disease is a good model for herpesvirus oncology because MD is a naturally occurring disease which can be reproduced experimentally using natural methods of exposure in the natural host. The mechanisms for establishment of latency and transformation by MDV infection are not clear at present. However, restriction of DNA replication has been implicated in DNA virus transformation. Also, integration of the virus genome into host cell chromosomes can lead to an imbalance of host gene expression and thereby contribute to the transformation process. Understanding MDV DNA replication will help to shed light on the mechanisms MDV uses for integration, and therefore provide insight to the transformation process by MDV.

The literature review will be focused on comparisons of DNA replication from all three herpesvirus classes (alpha, beta, and gamma-herpesviruses).

(i) Alphaherpesvirus DNA replication

Herpes simplex virus (HSV) is an alphaherpesvirus and is the most extensively characterized of all alphaherpesviruses. As mentioned previously, MDV genome structure is similar to that of HSV-1. Location of the origin of replication within the complex HSV-1 genome involved studies of both standard and defective viruses. Electron microscopic studies of replicating standard wild type HSV-1 DNA isolated from infected cells have suggested that the genome contains two origins of replication, one near the middle of U_L and the other within the repeats flanking the U_S (Friedmann *et al.*, 1977; Hirsch *et al.*, 1977). Studies of defective molecules of HSV-1, which are generated during serial

passage of the virus at high multiplicities of infection, provided indirect evidence of two origins (Kaerner *et al.*, 1979; Locker and Frenkel, 1979; Frenkel, 1981).

. Defective DNA molecules fall into two classes. Each of two defective DNA molecules consists of tandem duplications of small subsets of viral DNA sequence. Class I defective genomes contain sequences from the repeats which bracket U_S (Kaerner *et al.*, 1979; Locker and Frenkel, 1979; Frenkel, 1981) whereas class II defective genomes contain sequences from U_L (Kaerner, 1979; Frenkel, 1981).

Direct evidence that the repeat units of class I and II defective genomes contain origins of replication was first provided by Frenkel and his colleagues (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982). These investigators demonstrated that monomeric units of class I and II defective DNAs are amplified to generate tandemly repeated DNA structures when cotransfected with wild type HSV-1 DNA which provides essential helper functions *in trans*. By using the cloned HSV-1 fragments as seed units in the presence or absence of superinfecting wild type helper virus, the replication origins of HSV-1 were identified. From class I defective genomes, two copies of lytic origins have been identified within the inverted repeats flanking the S component (ori_S) (Figure 3) (Stow, 1982). A third origin, ori_L, was localized to the center of L component (ori_L) (Figure 3) (Spaete and Frenkel, 1982). Ori_S is located in a 90-bp fragment containing an imperfect palindrome with a central AT-rich region (Stow and McMonagle, 1983). The disruption of the palindrome abolishes the function of origin, suggesting that the palindrome is essential for origin function (Lockshon and Galloway, 1988). More accurate deletion mapping has refined the minimal sequence for origin function to a 75-bp fragment

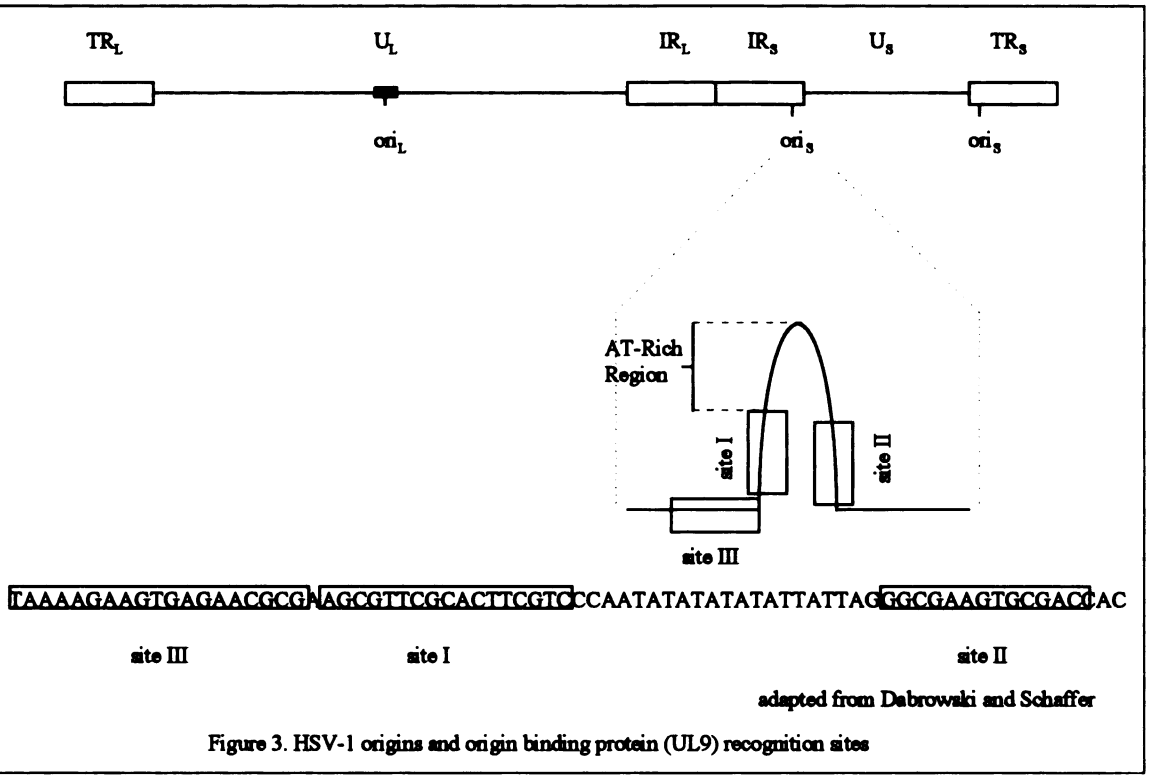

Figure 3. HSV-1 origins and origin binding protein (UL9) recognition sites

(Deb and Doelberg, 1988). This core region includes the 46-bp palindrome centered on AT-rich region and a region left of the left arm of palindrome. Replacement of AT base pairs in the AT-rich region with GC base pairs eliminated origin activity (Lockshon and Galloway, 1988), suggesting that the AT-rich sequence at the center of palindrome is required for origin activity. Ori$_L$ is located in a 425-bp fragment containing a perfect 144-bp palindrome (Weller, 1985). Deletion analysis revealed that the sequence within the palindrome is essential for origin function (Weller, 1985). Ori$_S$ and ori$_L$ share extensive nucleotide sequence similarity, except for the sequences extending to rightward end from the AT-rich palindrome (Weller, 1985). To determine functional significance of the three separate origins, several mutant viruses were created. Mutant viruses lacking ori$_L$ or with one copy of ori$_S$ replicated normally *in vitro* (Longnecker and Roizman, 1986; Polvino-Bodnar *et al.*,1987), while attempts to construct mutant viruses lacking both ori$_S$ have not

been successful, implying that HSV-1 DNA replication requires at least one copy of ori_S or, alternatively, at least two origin sequences (ori_S and ori_L or two copies of ori_S).

Seven genes necessary for HSV-1 DNA replication have been identified (Wu *et al.*, 1988): a helicase and primase complex (UL5, UL8, and UL52), the origin-binding protein (OBP) (UL9), the major single-stranded DNA-binding protein (ICP8), DNA polymerase (UL30), and a polymerase accessory protein (UL42). The HSV-1 UL9 is an origin-specific binding protein and plays a role in initiation of HSV-1 DNA replication. Studies of OBP's function indicate that it serves as an initiator for the HSV-1 DNA replication. A more extensive literature review on the description of HSV-1 OBP will be addressed in a later section entitled "*origin-binding proteins of alphaherpesviruses*".

Two high affinity origin binding protein (OBP) sites (designated as sites I and II) at the ends of each arm of the ori_S palindrome have been identified (Figure 3) (Weller *et al.*, 1985; Elias *et al.*, 1986; Elias and Lehman, 1988; Olivo *et al.*, 1988; Hazel *et al.*, 1989; Martin *et al.*, 1991). Site I has a 5 to 10-fold higher affinity for OBP than site II (Elias and Lehman, 1988). Using DNAase I footprinting, methylation interference, and electrophoretic mobility gel shift assay (EMSA) with mutant oligonucleotides for site I, the HSV-1 OBP binding site was mapped to a domain of 11 nucleotides in site I (CGTTCGCACTT) (Koff *et al.*, 1988; Deb *et al.*, 1989; Elias *et al.*, 1990; Hazuda *et al.*, 1991). The 11-bp element within site II is different in two positions from that within site I (Elias *et al.*, 1990) (Figure 3). The presumed HSV-1 OBP binding site (11-bp element) is conserved in both ori_L and ori_S of HSV-1 and HSV-2 as well as in VZV (Stow *et al.*, 1986; Polvino-Bodnar *et al.*, 1987; Baumann *et al.*, 1989). It was proposed that HSV-1

OBP binds as a dimer to two inverted, overlapping pentanucleotides within site I (5'-GTTCGCAC-3'/3'-CAAGCGTG-5') (Koff *et al.*, 1988; Fierer and Challberg, 1995). An oscillating activity was observed with plasmids containing different copy numbers of the AT dinucleotide within the AT-rich region. This phenomenon suggested that OBP may be required to bind to the cognate binding sites located on the same side of the DNA helix (Lockshon and Galloway, 1988). Deletion and mutation analysis have shown that both sites I and II are required for the efficient activity of ori_S (Deb and Deb, 1989; Weir and Stow, 1990; Hernandez *et al.*, 1991, Martin *et al.*, 1991). Thus, interaction of OBP with ori_S is critical to optimal HSV-1 DNA replication.

The Ori_S region contains a sequence which has strong sequence similarity to OBP-binding sites I and II, and has been designated as site III (Figure 3). Site III is located to the left of site I. No sequence-specific OBP binding, however, has yet been demonstrated to this site (Elias *et al.*, 1990; Weir and Stow, 1990). Analysis of origin sequences in HSV-2 have shown that deletion of part of a sequence corresponding to site III in HSV-1 resulted in a dramatic loss in DNA replication (Lockshon and Galloway, 1988), while the deletion of site III in HSV-1 ori_S affected replication only moderately (Weir and Stow, 1990; Martin *et al.*, 1991). Thus, although OBP was not shown to bind to site III *in vitro*, *in vivo* studies suggested that all three OBP-binding sites were required for optimal replication of HSV-1.

Like other DNA-containing viruses, the three origins of HSV-1 DNA replication are flanked by sequences containing transcriptional regulatory elements. Ori_L is positioned between the divergent transcriptional start sites of the genes encoding the major DNA-

binding protein (ICP8) and DNA polymerase (Weller *et al.*, 1985; Polvino-Bodnar *et al.*, 1987). Ori_S, like ori_L, is also located between divergently transribed genes. These genes encode the immediate-early proteins ICP4 and ICP22/47 (Stow and McMongle, 1983). The transcriptional regulatory elements within which ori_S resides are well characterized and collectively exhibit the properties of an enhancer element (Preston *et al.*, 1984; Preston and Tannahill, 1984; Preston *et al.*, 1988). A variety of recognized transcription factors have been shown to bind specifically to ori_S-flanking sequences. These include the potent HSV-1 transactivator VP16 (apRhys *et al.*, 1989) and cellular transcription factors Sp1, and nuclear factor III (NF-III) (Bzik *et al.*, 1986). It has been shown that the promoter-regulatory elements surrounding the ori_S act to increase the overall replication efficiency of ori_S-containing plasmids (Wong and Schaffer, 1991). Wong and Schaffer (1991) postulated that the *trans*-acting factors that bind to regulatory elements of immediate early genes may serve specifically to make ori_S more accessible to proteins of the initiation complex. Alternatively, the proteins which bind to and regulate the immediate-early genes may interact directly with DNA replication proteins and assist in promoting localized strand separation.

Several studies have suggested that the linear genome of HSV-1 circularizes upon entry into susceptible cells and replicates predominantly by a rolling circle mechanism (Ben-Porat *et al.*, 1976; Mocarski and Roizman, 1982; Roizman, and Sears, 1991). Based on pulse-chase experiments and electron microscope observations of HSV DNA replication intermediates from bouyant density centrifugation, Jacob and Roizman (1979) observed that there were five types of HSV-1 DNA replication intermediates : (1) linear,

full size molecules with internal gaps and single-stranded regions at the termini; (2) molecules with a lariat structure; (3) circular, double-stranded molecules; (4) molecules with "D" loop; and (5) large, tangled masses of DNA. By using restriction enzyme analysis of extracted HSV-1 DNA replication intermediates, Jacob and Roizman (1979) reported that head-to-tail concatemers accumulated in the nuclei of infected cells. Based on these observations, it was proposed that HSV-1 DNA replicates by a rolling circle mechanism. It is possible that the linear HSV-1 DNA can fold back upon itself and the ends ligate together to form circular molecules through the *a* sequence. The resulting circular molecules may then serve as the templates to generate linear concatemers consisting of tandemly repeated genome size units of HSV-1 via a rolling circle mechanism.

Recent studies on the mechanism of HSV-1 DNA replication further support the rolling circle mechanism for HSV-1 DNA replication. Rabin and Hanlon (1990) performed *in-vitro* DNA syntheses with HSV-1-infected-cell extracts using a preformed replication fork that was made from a nicked, doubled-stranded, circular DNA molecule with a 5' single-stranded tail. The product of this *in-vitro* reaction was a linear concatemer, as demonstrated by electron microscopy. Skaliter (1996) performed an *in-vitro* DNA synthesis with HSV-1-infected-human cell extracts using pUC18 plasmid as the template to generate linear concatemers composed of tandemly repeated plasmid size units of pUC18. Therefore, the rolling circle mechanism is involved in HSV-1 DNA replication. However, other recent studies on the interactions between HSV-1 origin binding protein (UL9) and other replication proteins have suggested that HSV-1 DNA initially replicates by an early origin-dependent theta-circle replication step followed by subsequent rolling

circle replication to generate concatemers that are packaged into viral particles. By immunoprecipitation, Lee *et al.* (1995) showed that the UL9 protein co-immunoprecipitated with the 180 kDa catalytic subunit of cellular DNA polymerase α-primase but not with HSV-1 DNA polymerase, suggesting that the UL9 protein interacts with the cellular polymerase but not with the viral polymerase and initiation at viral origins may be accomplished by UL9 and a cellular polymerase. Skaliter and Lehman (1994) reported that extracts of insect cells infected with baculovirus recombinants containing seven HSV-1 genes required for replication but not the UL9 gene could promote rolling circle replication of pUC18 plasmid, suggesting that rolling circle replication is independent of the UL9 gene and the origins but does require other viral replication proteins. These results strengthens the model that HSV-1 DNA initiates replication at the viral origin and later replicates via the rolling circle mechanism.

Several lines of evidence suggest that HSV viral genome maturation involves site-specific cleavage of viral DNA concatemers to unit size monomers of viral genome (Deiss *et al.*, 1986a; Deiss *et al.*, 1986b; Varmuza and Smiley; 1985). The *cis*-acting sequence required for cleavage is located within the *a* sequence (Varmuza and Smiley; 1985; Deiss *et al.*, 1986; Deiss and Frenkel, 1986). The *a* sequence is present as a direct repeat at both termini and in inverted orientation at the L-S junction. The *a* sequence is present in a single copy at the S terminus of the viral genome and in one to several copies at the L terminus and at the L-S junction (Mocarski and Roizman). Two separate *cis*-acting signals within the *a* sequence (called pac-1 and pac-2) appear to be essential for the cleavage/package process (Varmuza and Smiley, 1985; Deiss *et al.*, 1986). The signals

essential for the packaging/processing reaction are structurally conserved among many herpesviruses (Davison, 1984; Matsuo *et al.*, 1984; Albrecht *et al.*, 1985; Bankier *et al.*, 1985; Tamashiro and Spector, 1986; Marks and Spector, 1988).

(ii) Beta-herpesvirus DNA replication

Cytomegaloviruses (CMV) is a betaherpesvirus. In comparison to alphaherpesviruses which have relatively short reproductive cycles and spread rapidly in culture, betaherpesviruses have a long reproductive cycle and grow slowly in culture. CMV has the largest genome among herpesviruses, equivalent to approximately 240 kliobase pairs (kb). The genome of human CMV also belongs to group E genome (Roizman, 1991). CMV infection is very complex. Infection of the host by CMV can become latent or lead to productive infection that can either persist asymptotically, or cause disease.

Based on a novel approach utilizing gancyclovir-induced chain termination, Hamzeh et al (1990) identified an authentic lytic origin (oriLyt) of human CMV DNA replication within the center of unique long region (EcoRI-V fragment). Subcloning and deletion analyses of the region containing the authentic lytic origin defined a 2.4-kb core region containing elements required for the oriLyt function (Anders, *et al.*, 1992). In contrast to the alphaherpesvirus lytic origin of DNA replication, the human CMV lytic origin is large and complex. The overall base composition of this region is similar to that for the entire genome, but is asymmetric. At the left boundary is an AT-rich region (up to 72%) while at the right boundary is a 62% GC-rich region (Anders *et al.*, 1992). The region within and around the boundaries contain numerous repeated motifs, including

known transcription factor recognition sequences. OriLyt contains two kinds of 10-bp repeats, a 12-bp repeat, a 15-bp repeat and a 14-bp repeat (Anders *et al.*, 1992). Numerous known transcription factor recognition sequences are present in the human CMV oriLyt, including ATF/CREB sequences, MLTF/USF sequences, and Sp1 motifs (Anders *et al.*, 1992). TATA, CAAT and polyadenylation sequences are also present in the human CMV oriLyt (Anders *et al.*, 1992).

The lytic origin of simian CMV has also been identified and analyzed. Subcloning and deletion analyses defined a 1.3-kbp core region sufficient for origin function in the apparently noncoding region upstream of the single-stranded DNA-binding protein gene (dbp) (Anders and Punturieri, 1991). As with the oriLyt of human CMV, the oriLyt of simian CMV is also complex. Nucleotide sequence analysis has revealed four distinct domains : (1) a 9-bp repeated sequence; (2) an AT-rich segment; (3) an 11-bp direct repeat; and (4) a 47-bp direct repeat (Anders and Punturieri, 1991).

Like alphaherpesvirus, CMV requires virus-encoded proteins for DNA replication. Eleven loci encoding *trans*-acting factors required for transient complementation of human CMV oriLyt-mediated DNA replication have been identified (Pari and Anders, 1993; Pari *et al.*, 1993). In human CMV-infected cells, blocking expression of individual proteins expressed by members of this set of loci inhibited viral DNA replication (Ripalti *et al.*, 1995; Smith and Pari, 1995), suggesting that these eleven loci are required for viral DNA replication *in vivo*. Six of the defined loci encode homologs of HSV-1 replication genes (Pari and Anders, 1993). UL54 encodes a DNA polymerase that shows sequence similarity to a variety of alphaherpesvirus DNA polymerases. UL44 encodes a putative polymerase

accessory protein homologous to the HSV-1 UL42. UL57 encodes a single-stranded-DNA binding protein homologous to HSV-1 major DNA-binding protein (ICP8). UL70 encodes a homolog of HSV-1 UL52. HSV-1 UL52 encodes a primase activity which is a component of a three-subunit primase-helicase complex. UL70, UL101-102, UL105 are homologous to HSV-1 helicase-primase subunits. Five additional loci are required to complement human CMV DNA replication in transient assays. In contrast to HSV-1 DNA replication, the homologous proteins are not required to complement viral DNA replication in transient assays. Three of the five additional loci (UL36-38, UL122-123 and IRS1/TRS1) encode viral transactivators. It is not surprising that the viral transactivators are involved in DNA replication. It was established that origin efficiency was augmented or controlled by elements that also regulate transcription (Depamphilis, 1988), as found for HSV-1 (Wong and Schaffer, 1991). It has been shown that four of the eleven loci (UL36-38, UL112-113, IRS1/TRS1, and the major immediate early region UL122-123) required for transient complementation of human CMV DNA replication cooperate to activate expression of the replication genes (UL54, UL44, UL57, UL70, UL102 and UL1105) (Iskenderian, *et al.*, 1996).

The overall mechanism of CMV DNA replication is not clear. However, it is thought that after entering permissive cells, linear CMV genome circularizes (LaFemina and Hayward, 1983) and then replicates in the nucleus by a mechanism producing concatemers that are subsequently cleaved and packaged during virion assembly (Stinski, 1991). Transient transfection assays revealed that oriLyt containing plasmids can induce

the amplification of tandem oligomers (Anders *et al.*, 1992) and suggests that CMV DNA replicates via a rolling circle mechanism similar to HSV-1.

(iii) Gammaherpesvirus DNA replication

Epstein-Barr virus (EBV) is a human herpesvirus belonging to the gammaherpesvirus family. Human B lymphocytes can be infected and immortalized by EBV (Henle, 1967; Epstein and Achong; 1979). EBV is associated with mononucleosis, nasopharyngeal carcinoma and Burkitt's lymphoma (zur Hausen, 1981). The EBV genome is a linear, double-stranded 172 kb DNA molecules. The structure of EBV genome is composed of five unique sequence domains which are divided by four classes of internal repeats (Irs) and a variable number of directly repeated 0.5-kbp sequences (TR) located at both ends of EBV genome (Dambaugh, 1980). The overall EBV genome can be designated as TR-U1-IR1-U2-IR2-U3-IR3-U4-IR4-U5-TR.

In cells immortalized by EBV, multiple copies of the EBV genome are maintained as 172 kb supercoiled plasmids (Lindahl *et al.*, 1976; Gussander and Nonoyama, 1984). A *cis*-acting sequence that is required for plasmid replication and maintenance in immortalized cells has been identified. This sequence, designated oriP for origin of plasmid replication, is located on a 1.8 kb segment of the EBV genome which is located in the U1 region. (Yates *et al.*, 1984; Lupton and Levine, 1985). Deletion mapping has identified two separate regions in oriP, each required *in cis* for plasmid maintenance (Lupton and Levine, 1985; Reisman *et al.*, 1985). Region I is composed of 20 imperfect copies of a tandemly repeated 30 bp sequence. Region II, located 960 bp away, consists of a 65 bp sequence forming a dyad symmetry. Region I essential for long-term maintenance of the

plasmid form of EBV (Lupton and Levine, 1985; Reisman *et al.*, 1985) contains a termination site for replication (Gahn and Schildkraut, 1989) and functions as a transcriptional enhancer for RNA polymerase II-transcribed genes (Reisman and Sugden, 1986). Region I also plays a role in plasmid segregation during cell division. Region II is at the initiation site of latent cycle DNA replication and contains the actual origin of replication (Gahn and Schildkraut, 1989). In latently infected cells, oriP only replicates once during the S phase of the cell cycle in a coordinate fashion with cellular DNA synthesis (Hamper *et al.*, 1974; Adams, 1987). The close proximity of initiation and termination sites in oriP results in replication of the plasmid proceeding in a predominantly unidirectional manner (Gahn and Schildkraut, 1989).

In latently infected B lymphocytes, infectious virus is not produced and only a small fraction of the EBV genome is expressed. At least nine genes are expressed in latently infected cells. Six of these genes encode nuclear proteins (EBNA-1, -2,-3A, -3B, -3C and -LP) . Of these six nuclear proteins, EBNA-1 is the best characterized protein. EBNA-1 is the only EBV protein required for EBV genome replication during the latent cycle. (Yates *et al.*, 1984; Lupton, S., and Levine, 1985; Yates *et al.*, 1985). All other proteins required for plasmid replication are provided by the host cell.

There are multiple EBNA-1 binding sites within oriP. Each of the twenty 30 bp repeats within the region I of oriP contains one EBNA-1 binding site (Ambinder *et al.*, 1991; Rawins *et al.*, 1985) and region II contains four EBNA-1 binding sites (Ambinder *et al.*, Rawins *et al.*, 1985). Multiple EBNA-1 binding sites are required for the function of oriP (Wysokenski and Yates, 1989). Deletion analysis and site-directed mutagenesis of

oriP-containing recombinant plasmid revealed that at least six to eight copies of EBNA-1 binding sites within region I are required for plasmid maintenance and only two EBNA-1 binding sites are required for the function of region II (Chittenden *et al.*, 1989; Harrison *et al.*, 1994).

EBNA-1 is a multifunctional protein. It cooperatively assembles on oriP as a dimer via a direct interaction (Summers, 1996). It is the only viral protein which is required for plasmid replication. The family of repeats within region I , when bound by EBNA-1 , can activate replication from region II, enhance latent transcription and control the stable segregation of EBV episomes during cell division (Lupton and Levine, 1985; Reisman *et al.*, 1985; Reisman and Sugden, 1986; Gahn and Schildkraut, 1989). DNase I and $KMnO_4$ footprinting *in vitro* and *in vivo* on oriP bound by EBNA-1 has revealed that EBNA-1 can induce distortion of region II but is unable to distort the duplex in region I (Hsieh *et al.*, 1993). Investigation of the interaction of pure EBNA-1 with oriP DNA has shown that EBNA-1 dimers bound to region I and II interact efficiently with each other, bringing the two elements together (Frappier and O'Donnell, 1991; Su *et al.*, 1991; Middleton and Sugden, 1992). This interaction results in the generation of looped DNA molecules and cross-linking of multiple DNA molecules via EBNA-1. Interactions between EBNA-1 molecules bound to region I and II stabilize EBNA-1 on region II and likely are an important part of the mechanism by which region I activates replication from region II. Purified EBNA-1 lacks helicase activities and does not appear to act by performing any enzymatic function (Yates and Camiolo, 1988a). Thus, initiation of replication at oriP is dependent upon a cellular DNA helicase for the initial unwinding of DNA.

Functional domains of EBNA-1 have been investigated extensively. Deletion analysis of the domain which consists of a repetitive array of glycine and alanine residues has revealed that this domain is not essential for function of EBNA-1 (Yates *et al.*, 1985; Polvino-Bodnar *et al.*, 1988; Yates and Camiolo, 1988b). The domains essential for function of EBNA-1 are all localized within the C-terminal domain, including the domains for nuclear localization, for dimerization, for DNA binding, for transactivation and for DNA-looping (Ambinder *et al.*, 1991; Inoue *et al.*, 1991; Laine and Frappier; 1995).

Lytic EBV replication occurs in the mucosal epithelial cells of the oropharynx and gential tract (Sixbey, 1989) and can be induced by treating latently infected B cells with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (zur Hausen *et al.*, 1978) or by introduction of the EBV Zta transactivator (Countryman and Miller, 1985). Lytic phase replication proceeds via a separate origin, oriLyt, which is different from oriP and results in 100- to 1,000-fold amplification of the genome via concatemeric intermediates (Hammerschmidt and Sugden, 1988; Sato *et al.*, 1990). There are two copies of oriLyt in the intact EBV genome. One copy is centrally located within the EBV genome, approximately 40 kb away from oriP and the other is located at the right end of EBV genome. EBV isolates containing one copy of oriLyt replicate as efficiently as EBV isolates containing two copies of oriLyt (Hammerschmidt and Sugden, 1988), suggesting that one copy is sufficient for lytic phase replication. OriLyt is complex and contains arrays of direct and inverted repeats. OriLyt can be divided into three essential domains: (i) The first domain is the promoter and leader of the BHLF1 gene. The BHLF1 promoter contains four binding sites for the Zta (BZLF1) transactivator and is strongly Zta

responsive in transient expression assays (Lieberman *et al.*, 1989; Lieberman *et al.*, 1990). (ii) The second domain is a central 225-bp region whose prominent features include two related AT-rich palindromes of 18 and 20 bp and an adjacent polyurine-polypyrimidine tract. Elements of this type may serve as sites for initiation of DNA replication or transmission of localized unwinding in origins of replication (Kowalski, 1989; Wells, 1988). (iii) The third domain is an enhancer that responds to the Rta transactivator and contains two binding sites for Rta and one for Zta (Cox, 1990; Gruffat, 1990; Liberman, 1990).

By using transient replication assays, Fixman *et al.* (1992) identified the EBV genes essential for transient complementation of oriLyt-mediated DNA replication, which included six EBV genes (BALF5, BMRF1, BALF2, BBLF4, BSLF1, and BBLF2/3), the viral lytic-cycle transactivators, Zta, Rta and Mta, and an unidentified gene in the SalI F fragment. These six EBV replication genes are homologous to six of seven essential genes for HSV-1 DNA replication. BALF5 shares 33% identity to the HSV-1 DNA polymerase, BMRF1 is a positional and functional homolog of the HSV-1 DNA polymerase processivity factor, BALF2 shares 25% identity with the HSV-1 single stranded DNA-binding protein (ICP8), and BBLF4 as well as BSLF1 have significant sequence identity with the HSV-1 UL5 and UL52 genes. BBLF2/3 is likely to be a homolog of HSV-1 UL8. The activity in *Sal*I-F was shown to be encoded by BKRF3 which encodes an enzyme, uracyl DNA glycosylase. This enzyme is dispensable for replication (Fixman *et al.*, 1995). One of the replication genes that has not been identified is an oriLyt origin-binding protein equivalent to the HSV-1 origin-binding protein, UL9. Fixman *et al.* (1995) reported that

EBV does not encode an equivalent of HSV-1 UL9 and that Zta is the sole virally encoded protein that serves as an essential origin-binding protein.

(iv) Marek's disease virus DNA replication

Replication origins for all three MDV serotypes have been identified. A functional origin of replication for serotype 2 MDV was identified (Camp *et al.*, 1991) using a defective MDV genome and transient replication assay. The serotype 2 MDV replication origin is located in the inverted repeats flanking the unique long region (Figure 4), suggesting that there are at least two copies of the origin. The replication origin of serotype 2 MDV was located to a 90-bp region (Figure 4). Like HSV-1 ori_S and ori_L, it contains an imperfect palindrome with 30 bp of alternating AT sequence located at the enter. The structure and sequence of the serotype 2 MDV replication origin is very similar to HSV-1 ori_L and ori_S, VZV origin, and equine herpes virus type 1 (EHV-1) origin (Camp *et al.*, 1991). In addition, the serotype 2 MDV replication origin contains a 9-bp motif which is located both at the left and right arms of the palindrome (Figure 4). This 9-

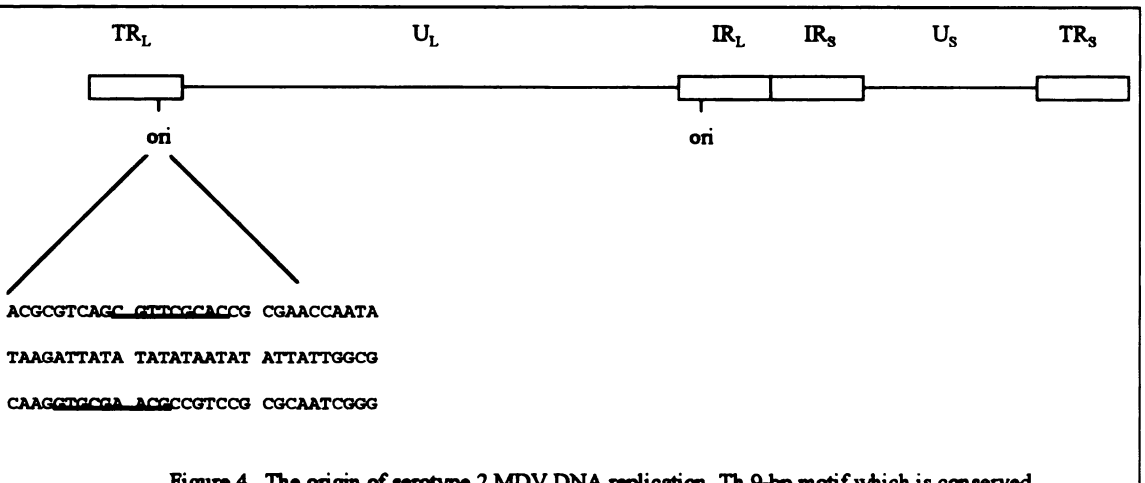

Figure 4 . The origin of serotype 2 MDV DNA replication. Th 9-bp motif which is conserved among alphaherpesviruses is indicated by the bars under sequence

bp motif is highly conserved among alphaherpesviruses. The 9-bp motif sequence is a

subset of a 11-bp motif that is recognized by HSV-1 origin binding protein (UL9). The presence of two copies of the 9-bp motif suggests that there are two potential binding sites for MDV origin-binding protein.

Based on nucleotide sequence analysis, a putative replication origin of serotype 1 MDV was reported (Bradley *et al.*, 1991). The putative serotype 1 MDV replication origin is located in the inverted repeats flanking the unique long region of serotype 1 genome and contains several transcription factor binding sites (sp1 and Oct-1 binding sites) within the flanking sequence. As with HSV-1, CMV and EBV, these auxiliary elements may play a role in MDV DNA replication. Morgan et al (1991) reported that there was a decrease in MDV plaque formation when cells were cotransfected with viral DNA and plasmids containing the auxiliary components flanking the putative serotype 1 MDV replication origin.

Based on nucleotide analysis and DpnI resistance assays, an HVT replication origin was identified (Smith *et al.*, 1995). The HVT replication origin is located in the junction between U_L and IR_L and is very similar to the origins of replication of MDV-1, -2, HSV-1, VZV and EHV-1. It also contains a 9-bp motif which is conserved among alphaherpesviruses. As found for the MDV-1 replication origin, there are CAAT sequences, sp1 binding motifs and an Oct-1 binding motif present in the HVT replication origin.

The mechanism of MDV DNA replication has not been determined. However, based on the structural similarity and gene colinearity between HSV-1 and MDV, it is likely that MDV replicates via rolling circle mechanism. Based on nucleotide analysis,

Camp et al (1993) reported that an HSV *a*-like sequence was identified within the 5'-end of 4 kb MDV replicon (a single monomeric repeat unit of serotype 2 defective MDV genome).

3. Origin binding protein (OBP) of alphaherpesviruses

I. The OBP of herpes simplex virus type 1 (HSV-1)

HSV-1 UL9 is a multifunctional origin-binding protein. HSV-1 UL9 binds directly and cooperatively to two UL9 binding sites (sites I and II) within ori_S (Olivo *et al.*, 1988; Elias *et al.*, 1990). It also exists as a dimer in solution (Bruckner *et al.*, 1991), unwinds the partial DNA duplex *in vitro* (Fierer and Challberg, 1992), exhibits DNA-helicase activity (Bruckner *et al.*, 1991; Boehmer *et al.*, 1993) and has DNA-dependent nucleoside 5'-triphosphatase activity (Dodson and Lehman, 1993). In addition, purified HSV-1 UL9 forms a high order nucleocomplex with plasmids containing HSV-1 ori_S via inter- and intra-molecular interactions (Rabkin and Hanlon, 1991). Based on DNase I and $KMnO_4$ footprinting with purified HSV-1 UL9, Koff et al (1991) reported that HSV-1 UL9 loops and distorts HSV-1 ori_S.

Since HSV-1 UL9 is an origin binding protein, it is not surprising that HSV-1 UL9 interacts with other replication proteins. Several studies have revealed that HSV-1 UL9 interacts with single-stranded DNA binding protein (ICP8) *in vitro* and *in vivo* (Boemer *et al.*, 1993; Boemer *et al.*, 1994; Gustafsson *et al.*, 1995) and that this interaction is influenced by DNA ligands (single-stranded or double-stranded DNA). HSV-1 UL9 interacts with ICP8 and double-stranded DNA to form a triple complex but the complex between HSV-1 UL9 and ICP8 can be destroyed by single-stranded DNA, suggesting that

the interaction between HSV-1 UL9 and ICP8 serves to position the single stranded DNA-binding protein with high precision onto the single-stranded DNA at the replication fork or at the origin of replication. By using immunoprecipitation, Lee et al (1995) showed that the HSV-1 UL9 interacts with the catalytic subunit of DNA polymerase α. HSV-1 UL9 also has been shown to interact specifically with HSV-1 UL8 (Mclean *et al.*, 1994). By using the indirect immunofluoresence, Liptak et al (1996) showed that the HSV-1 replication proteins assemble in an orderly fashion to form a prereplicative complex, and suggested that the HSV-1 UL9 is a component of the replication complex.

The structure and function of HSV-1 UL9 have been investigated extensively. The N-terminal domain of HSV-1 UL9 encodes helicase activity (Martinez *et al.*, 1992) and is responsible for dimerization and cooperativity (Hazuda *et al.*, 1992; Elias *et al.*, 1992). The helicase activity of HSV-1 UL9 is consistent with the presence of six helicase conserved motifs within the N-terminal domain. Single amino-acid substitutions at conserved residues in each of six helicase motifs inactivated HSV-1 UL9 in transient origin-dependent replication assays (Martinez *et al.*, 1992). Insertion mutagenesis of the HSV-1 amino-terminal leucine zipper domain dramatically affected cooperativity and dimerization of HSV-1 UL9 in solution (Hazuda *et al.*, 1992), suggesting that the leucine zipper within the N-terminal domain was essential for cooperativity and dimerization. Stabell and Olivo (1993) reported that the truncated form of HSV-1 UL9 which contains only the C-terminal origin binding domain, binds to ori_S but does not induce conformational changes in ori_S, suggesting that besides the helicase activity and dimerization, the N-terminal of HSV-1 UL9 is necessary for a UL9-induced

conformational change on ori_S. The C-terminal domain of HSV-1 UL9 encodes DNA-binding activity (Deb and Deb, 1991; Arbuckle and Stow, 1993; Martin *et al.*, 1994). It contains two known structural motifs (a pseudo-leucine zipper and a helix-turn-helix motif). By insertion and deletion mutagenesis, the pseudo-leucine zipper was shown to be important for DNA-binding activity (Deb and Deb, 1991; Arbuckle and Stow, 1993; Martin *et al.*, 1994). However, importance of the helix-turn-helix motif for DNA-binding activity remains to be elucidated (Deb and Deb, 1991; Arbuckle and Stow, 1993; Martin *et al.*, 1994).

A highly conserved region is found within the very C-terminal end of HSV-1 UL9. This sequence, termed the VZV homology region (Martin *et al.*, 1994), is very similar to a corresponding sequence in VZV gene 51 (a homolog of HSV-1 UL9). Despite a lack of similarity to any known structural motif, the VZV homology region is critical in maintaining DNA-binding activity of HSV-1 UL9 (Deb and Deb, 1991; Arbuckle and Stow, 1993; Martin *et al.*, 1994). In addition to DNA-binding activity, the C-terminal domain is also needed for association with ICP8. Boehmer et al (1994) showed that a UL9 mutant protein that lacks the C-terminal 27 amino acids exhibits the normal origin-specific DNA binding and retains its DNA-dependent ATPase and helicase activities, but has greatly reduced affinity for ICP8 (Boehmer et al, 1993), indicating that the C-terminal 27 amino acids are essential for ICP8 association.

The biochemical activities of HSV-1 UL9 are similar to those of large T antigen of simian virus 40 (SV40). In SV40, large T antigen not only binds to the replication origin as two hexamers and unwinds it locally for recruitment of other replication proteins but

also serves as the DNA helicase for subsequent steps in replication (Boroweic *et al.*, 1990). It play an essential role in initiation of SV40 DNA replication. The precise role of HSV-1 OBP in HSV-1 DNA replication has not been established but it is suggested that it plays a role in initiation of HSV-1 DNA replication similar to the role of SV40 large T antigen serving as an initiator protein for viral DNA replication.

II. The OBP of varicella-zoster virus (VZV)

VZV gene 51 is a homolog of HSV-1 UL9. The predicted amino acid sequence of gene 51 protein was shown to be 44% identical to HSV-1 UL9 (Chen and Olivo, 1994). VZV gene 51 protein binds to three sites (A, B and C) within the VZV origin but with different affinity for each of these three sites (Stow *et al.*, 1990). All three binding sites for VZV gene 51 protein are very similar to the HSV-1 UL9 binding site I. They all contain an 11-bp motif which is recognized by HSV-1 UL9. Based on deletion analyses, Chen and Oilvo (1994) showed that the C-terminal domain of VZV gene 51 protein encoded DNA binding activity. By alignment of the predicted amino acid sequences of HSV-1 UL9 and VZV gene 51, Chen and Olivo (1994) showed that VZV gene 51 contained all the motifs essential for function of HSV-1 UL9, including six helicase conserved motifs as well as a leucine zipper within the N-terminus and a pseudoleucine zipper as well as a helix-turn-helix motif within the C-terminus. The spatial arrangement of all three conserved motifs within VZV gene 51 protein are the same as that within HSV-1 UL9. The similarity between HSV-1 UL9 and VZV gene 51 suggests that the VZV gene 51 has biochemical activities similar to HSV-1 UL9. Consistent with this prediction, Webster *et al.* (1995) reported that VZV OBP can substitute for the HSV-1 OBP in a transient origin-dependent DNA replication assay in insect cells using the recombinant baculoviruses which can

express the seven genes essential for HSV-1 DNA replication. In addition, VZV gene 51 can support the replication of a HSV-1 UL9 null mutant (Chen *et al.*, 1995). The ability of VZV gene 51 to complement UL9 suggests that alphaherpesviruses have a highly conserved mechanism of initiation of viral DNA synthesis.

III. The OBP of equine herpes virus type 1 (EHV-1)

Based on the predicted amino acid sequence, the EHV-1 gene 53 was shown to have a strong similarity to HSV-1 UL9 (Martin and Deb, 1994). Using PCR to clone the EHV-1 gene 53 and *in-vitro* coupled transcription-translation to express the EHV-1 gene 53, Martin and Deb (1994) showed that EHV-1 gene 53 protein binds to HSV-1 UL9 binding site I as well as to the EHV-1 origin and that a 9-bp motif which is a subset of the 11-bp motif recognized by HSV-1 UL9 is essential for binding the EHV-1 gene 53 protein. By deletion analysis, Martin and Deb (1994) reported that the C-terminal domain encodes the DNA-binding activity. By alignment of the predicted amino acid sequences of HSV-1 UL9, VZV gene 51 and EHV-1 gene 53, Martin and Deb (1994) showed that all the structural motifs which are essential for function of HSV-1 UL9 are conserved among all three OBPs and that the spatial arrangement of all conserved motifs is the same for all three OBPs. The similarity between HSV-1, VZV and EHV-1 OBPs suggests that OBPs are highly conserved among alphaherpesviruses and the OBP of MDV should be structurally similar.

Chapter 2

Cloning and sequence analysis of a Marek's disease virus origin binding protein (OBP) reveals strict conservation of structural motifs among OBPs of divergent alphaherpesviruses

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Virus Genes in press

Abstract

Marek's disease virus (MDV) is a highly cell-associated avian herpesvirus. In it's natural host, MDV induces Marek's disease (MD), a lethal condition characterized by malignant lymphoma of T cells. Although symptoms of MD may be prevented by vaccination, no practical pharmacological method of control has been widely accepted. Viral replication represents a point at which pharmacological control of herpesvirus infection may be most successful. However, this requires detailed knowledge of viral replication proteins. Studies in HSV-1 DNA replication implicate the UL9 protein as a key initiator of replication. For example, binding of UL9 to HSV-1 origins is a prerequisite for assembly of additional replication proteins. In this study, a protein, whose apparent molecular size is similar to that of HSV-1 UL9, was identified in extracts of MDV infected cells by western blot analysis with anti-HSV-1 UL9 antibody. A putative MDV UL9 gene was subsequently identified through sequencing of MDV genome fragments (*BamH*I G and C). Extended DNA sequence analysis revealed an open reading frame (ORF) which could encode a protein homologous to HSV-1 UL9. The MDV UL9 ORF encodes 841 amino acids, producing a sequence 49 % identical to HSV-1 UL9 and 46% identical to VZV gene 51 product (VZV UL9). MDV UL9 shares numerous structural motifs with HSV-1 and VZV UL9 proteins, including six conserved N-terminal helicase motifs, an N-terminal leucine zipper motif, a C-terminal pseudo-leucine zipper sequence, and a putative helix-turn-helix structure.

Introduction

Marek's disease virus (MDV) is a highly cell-associated avian herpesvirus. In chickens, MDV is the etiologic agent of Marek's disease (MD), characterized by malignant lymphoma of T cells (Calnek *et al.*, 1991). MDV cell tropism and pathology are similar to those of gammaherpesviruses (Roizman and Knipe, 1990). However, based on overall genomic structure and colinearity of many MDV genes to those of alphaherpesviruses, such as herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV), MDV has been classified as an alphaherpesvirus (Cebrian *et al.*, 1982; Bucmaster *et al.*, 1988; Brunovski *et al.*, 1992; Roizman, 1992). Consistent with this classification, MDV genomes are linear double-stranded DNA molecules 160 to 180 Kbp in length, consisting of two unique regions (U_S and U_L) flanked by inverted repeats (IR_S, IR_L, TR_S, and TR_L). Furthermore, two functional replication origins, with high similarity to HSV-1 replication origins, have been identified in different MDV serotypes (Camp *et al.*, 1991; Smith *et al.*, 1995)

Viral replication represents a point at which pharmacological control of herpesvirus infection may be most successful. However, this requires detailed knowledge of viral replication mechanisms and proteins involved in the replication process. Thus, better management of MD in poultry requires an understanding of MDV DNA replication mechanisms. In addition, information on MDV DNA replication may help elucidate similar mechanisms in closely related herpesviruses and contribute to generation of a general model of DNA replication for herpesviruses.

Much of what is currently known regarding herpesvirus replication stems from studies of HSV-1. HSV-1 encodes seven proteins that are both necessary and sufficient for

origin-dependent DNA synthesis (Wu *et al.*, 1988). The seven genes encode a two-subunit DNA polymerase (UL30 and UL42), a single-stranded DNA binding protein (UL29 or ICP8), a three protein complex with helicase-primase activities (UL5, UL8 and UL52) and an HSV-1 origin-specific DNA binding protein (UL9). The UL9 protein binds to specific sequence elements within the viral DNA replication origin, a critical event which represents the first step toward initiation of DNA replication.

There are two types of HSV-1 DNA replication origins: (1) ori_S, located in inverted repeats flanking the U_S region (Stow and Mcmonagle, 1983); and (2) ori_L, centrally located within the unique long region (Weller *et al.*, 1985). Both origins feature palindromic DNA sequences with central A+T-rich regions and share considerable homology (Weller *et al.*, 1985). Disruption of palindromic sequences abolishes origin function (Stow, 1984; Lockshon and Galloway, 1988), suggesting that these sequences are essential for replication. Two high-affinity UL9 binding sites occur within regions of dyad symmetry in both ori_S and ori_L (Weller *et al.*, 1985; Elias *et al.*, 1988; Olivo *et al.*, 1988; Martin *et al.*, 1991). Mutagenesis experiments indicate that UL9 binding sites are also critical for origin function (Deb and Deb, 1989; Weir and Stow, 1990). HSV-1 UL9, purified from recombinant expression systems, binds cooperatively to the two UL9 binding sites (sites I and II), within ori_S (Olivo *et al.*, 1988; Elias *et al.*, 1990), exists as a dimer in solution (Bruckner *et al.*, 1991), exhibits DNA-helicase activity (Bruckner *et al.*, 1991; Boehmer *et al.*, 1993) and DNA-dependent nucleoside 5'-triphosphatase activity (Dodson and Lehman, 1993).

There are six conserved helicase motifs within the N-terminus of HSV-1 UL9 (Martinez *et al.*, 1992) and a C-terminal domain encodes DNA binding activity (Deb and Deb, 1991). UL9 homologs have been identified in other alphaherpesviruses, such as VZV and equine herpesvirus type 1 (EHV-1) (Chen and Olivo, 1994; Martin and Deb, 1994). Replication origins and binding sites for UL9 homologs of VZV and EHV-1 are highly similar to HSV-1 ori_S (Stwo and Davison, 1986; Bauman *et al.*, 1989; Chen and Olivo, 1994; Martin and Deb, 1994). Similarly, domain structures of VZV and EHV-1 origin binding proteins are conserved relative to those of HSV-1 UL9 (Chen and Olivo, 1994; Martin and Deb, 1994).

Functional MDV replication origins have been identified in serotype 2 MDV defective virus (Camp *et al.*, 1991) and in serotype 3 MDV (Smith *et al.*, 1995). The serotype 2 MDV replication origin is located within inverted repeats flanking the unique long region (IR_L) in intact genomes (Camp *et al.*, 1991). This origin is highly similar to HSV-1 ori_S and ori_L and contains a 9-bp sequence (CGTTCGCAC) which is highly conserved in alphaherpesvirus replication origins. This 9-bp sequence is a subset of an 11-bp motif (CGTTCGCACTT) shown to be required for HSV-1 UL9 binding (Deb and Deb, 1989; Elias *et al.*, 1990). Similarity of MDV replication origins to those of HSV-1 and VZV, combined with preliminary data indicating MDV encodes a protein capable of sequence-specific interaction with these origin sequences (Camp, 1993), suggested that MDV encodes a functional UL9 homologue.

In this study, a protein whose apparent molecular size is similar to that of HSV-1 UL9, was identified in extracts of MDV infected cells by western blot analysis with anti-HSV-1

UL9 antibody. A putative MDV UL9 gene was subsequently identified through partial sequencing of genome fragments. An open reading frame (ORF) within a transcriptionally active region of the MDV genome, which encodes a protein homologous to HSV-1 UL9 was identified. The MDV UL9 ORF encodes an 841 amino acid polypeptide, which bears 49 % identity to HSV-1 UL9 and 46% identity to the VZV gene 51 product (VZV UL9). MDV UL9 shares several structural motifs with HSV-1 and VZV UL9 proteins. For example, the amino terminal domain of MDV UL9 contains six conserved helicase motifs and a leucine zipper motif, which is important for cooperativity and dimerization of HSV-1 UL9 (Elias *et al.*, 1992; Hazudz *et al.*, 1992). In addition, MDV UL9 contains a pseudo-leucine zipper motif and a helix-turn-helix motif in the carboxyl-terminal domain. A similar pseudo-leucine zipper motif, found in the C-terminal portion of HSV-1 UL9, is important for sequence-specific DNA binding activity (Deb and Deb, 1991; Arbuckle and Stow, 1993; Martin and Deb, 1994).

Material and Methods

Cells and Viruses

Chicken embryo fibroblast (CEF) cells were prepared, maintained and infected with MDV according to previously described procedures (Glaubiger *et al.*, 1983). Two MDV serotype 1 strains, GA (passage 14), Md11 (passage 16) and Md11 (passage 89) were used for this study. CEF cultures were grown in Leibovitz-McCoy medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 4% calf serum at 37^{o}C in a humidified atmosphere containing 5% CO_2. Calf serum concentration was reduced to 1% following MDV infection.

Western blot analysis

Mock-infected and MDV-infected CEF cell lysates were prepared by sonication (Dabrowski and Schaffer, 1991). Lysates were separated on 12% SDS-PAGE minigels (Bio-Rad, Hercules, California) and transferred to nitrocellulose (NC) membrane (Schleicher & Schuell, Keene, New Hampshire). NC membranes were blocked using 5% dry milk. Rabbit antiserum against HSV-1 UL9 (generous gift of Dr. M. Challberg, National Institute of Allergy and Infectious Disease, National Institues of Health) was used at 1:200 dilution and donkey anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary antibody. Proteins bound by antibody were visualized using an Amsherm ECL system according to the manufacturer's specification.

DNA sequencing

Five internal *EcoR*I subclones from the MDV strain GA *BamH*I C fragment were generated in pUC19. DNA sequencing was performed on double-stranded plasmids by dideoxy chain termination using [^{35} S]ATP (NEN Research Products, Williamston, DE) and sequenase DNA sequencing kits (United States Biochemical Corp., Cleveland, OH). DNA sequences were analyzed using Genepro Software (Riverside Scientific enterprises, Bainbridge Island , Washington) and Wisconsin Sequence Analysis Package (Genetics Computer Group, University Research Park, Madison, WI). Directed oligonucleotide primers and nested unidirectional deletion clones were used to complete sequencing of the MDV UL9 gene in both directions. *EcoR*I clone junctions were confirmed by direct sequencing from the *BamH*I C clone. *BamH*I G-C junction sequences were confirmed by sequencing a PCR clone generated using opposing primers which amplified this region from intact MDV DNA. The upstream primer (GGATGGTTACTATGGTGAGAA) was located approximately 50 bp within the *BamH*I G fragment. The downstream primer (TTTCTAGACCAACGTTCAGAGCCGCTGATGC) was located within the C-terminal domain of MDV UL9. PCR reactions were performed in 100-uL using a GeneAmp® PCR System 9600 DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT). Each reaction contained 50mM KCl, 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 0.001% gelatin (W/V), 5.0 pmole of each primer, 200uM of each deoxyribonuceloside triphosphate (dNTP), 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer) and 1ug DNA template. Template for PCR reactions was total cellular DNA isolated from CEF infected with MDV GA strain. Cycling conditions were 30 cycles of: 95^{o}C for 1min; 55^{o}C for 1 min; and 72^{o}C for 1

min. The resulting PCR product was cloned into the pGEM-T vector (Promega, Madison, WI) prior to DNA sequence analysis using commercially available primers.

Total cellular RNA isolation and Northern blot analysis

Total cellular RNA was isolated from mock-infected and MDV strain GA or Md11-infected CEF cells using a guanidium-phenol:chloroform method as described by Chomczynski et al (1987) (Chomczyski and Sacchi, 1987). The isolated RNA was treated with RQ1 DNase (Promega, Madison, WI) at 37°C for 30 min in DNase I buffer containing 40 mM Tris-HCL (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$, followed by phenol/chloroform extraction to remove the enzyme. Total RNA (15 μg) was loaded onto 1.2% agarose gels containing 5% formaldehyde and electrophoresed for 12 hr at 30 V. RNA was transferred onto Hybond-N membrane (Amersham Corp., Heights, IL) as described by Sambrook et al (1989) (Sambrook *et al.*, 1989). A 0.3Kbp *BamH*I-*Bgl*II subfragment from the MDV strain GA *BamH*I-G fragment, a 0.59Kbp *Eco*RI-*Ava* I, a 3.1Kbp *Eco*RI and a 1.3 Kbp *Eco*RI-*BamH*I subfragment from the MDV strain GA *BamH* I-C fragment were labeled using [^{32}P]dCTP (NEN research Products) and used as probes. Northern blot hybridization was performed using standard procedures (Sambrook *et al.*, 1989). Transcript size was determined by comparison to an ethidium bromide stained RNA ladder marker (Life Techonologies, Inc, Gaithersburg, MD).

Genebank accession number

Nucleotide sequence data presented in this paper has been asigned Genebank Accession Number U28785

Results

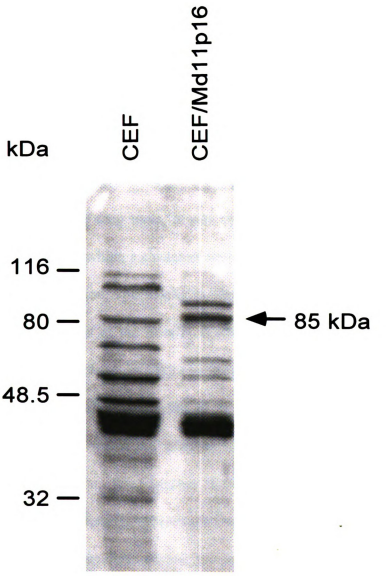
Identification of a protein similar in size and immunologically related to HSV-1 UL9 in MDV infected cells

Based on gene colinearity between MDV and other alphaherpesviruses, as well as origin similarities, it seemed likely that MDV encodes a UL9 homolog which is critical for initiation of DNA replication. Conservation of a potential UL9 binding sites within the MDV core origin of replication and specific recognition of a 22-mer oligonucleotide probe representing these sequences by both MDV and HSV-1 proteins further suggested that MDV UL9 may be highly similar to HSV-1 UL9 proteins (Camp, 1993). To analyze this possibility, we performed Western blot analyses of MDV-infected and uninfected cell extracts using a polyclonal anti-HSV-1 UL9 protein antibody (generous gift of Dr. M. Challberg, National Institute of Allergy and Infectious Disease, National Institues of Health). An 85 kd protein was specifically detected in extracts from CEF infected with MDV, strain Md11 (Figure 1). A similar protein was not detected in mocked-infected CEF. The apparent molecular size of this protein is consistent with that reported for HSV-1 UL9 (83 kd) (Bruckner *et al.*, 1991).

Identification and nucleotide sequence analysis of an MDV UL9 gene

Sequence-specific interaction of an MDV-encoded protein with the serotype 2 origin sequence (Camp, 1993), and antigenic recognition of a protein similar in size to HSV-1 UL9 by heterologous polyclonal antisera strongly suggested that MDV encodes a

Figure 1: Detection of MDV UL9 in MDV-infected cells. Western blot hybridization was performed as described in Material and Methods. Mock-infected CEF cell lysate (CEF) was used as negative control. Position of the putative MDV UL9 protein identified in lysates of CEF cells infected with MDV strain Md11p16 (CEF/Md11p16) is indicated by an arrow to the right. Protein sizes were determined by comparison to standards of known size.

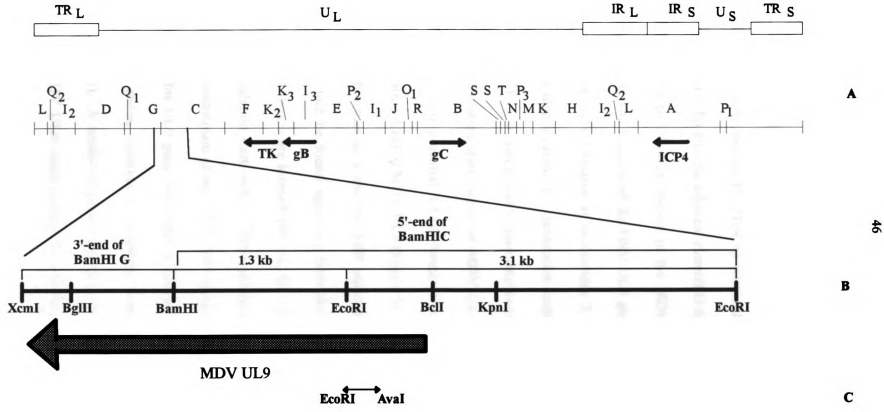

kDa
CEF
CEF/Md11p16
116
80
48.5
32
85 kDa

UL9 homolog. We therefore proceeded with localization and identification of an MDV gene capable of encoding this polypeptide.

Identification of a functional MDV replication origin (Camp *et al.*, 1991) and initial mobility shift assay results (Camp, 1993) were obtained with extracts from cells infected with serotype 2 MDV. However, subsequent Western blot analysis, cloning, and DNA sequence analysis were performed with serotype 1 MDV strains. Serotype 1 MDV has been more extensively characterized with regard to gene content and colinearity with HSV-1 than serotype 2, thus providing substantially more reference points for genome comparisons. Using relative map units, it was possible to predict that an MDV UL9 homolog would be arranged within a cluster of replication protein genes near the leftward end of the unique long region, specifically in *BamH*I fragments G and C (Figure 2). Preliminary sequence analysis of the *BamH*I G fragment from serotype 1 MDV (strain GA), indicated that the leftward terminus of *BamH*I G could encode the N-terminal portion of a MDV UL5 homolog. Using this as an anchor, a major portion of MDV UL9 was predicted to be located within the leftward end of MDV *BamH*I C. To precisely locate the MDV UL9 gene, five internal *EcoR*I subclones from *BamH*I-C were constructed in pUC19 and terminal sequences determined using standard forward and reverse pUC19 primers. Comparison of deduced terminal amino acid sequences with that of HSV-1 UL9 revealed one subclone, with an insert size of 3.1 kb, whose deduced amino acid sequence displayed 57% identity with the N-terminal portion of HSV-1 UL9. Directed oligonucleotide sequencing and ExoIII nested deletion clone sequencing were used to expand our analysis (Figure 2) from the 3.1 Kb *EcoR*I subclone into an adjacent

Figure 2: Location of the MDV UL9 gene within the MDV genome of the GA strain.

(A) *BamH*I restriction map of the MDV genome showing approximate location of several major genes, including those encoding thymidine kinase (TK), glycoprotein B (gB), glycoprotein C (gC), and ICP4. Restriction map is adapted from Fukuchi *et al.* (1984). (B) Predicted localization of the MDV UL9 gene within the *BamH*I G and C fragments. Locations of several restriction enzyme cleavage sites used in cloning and sequencing are also shown, as are the boundaries of MDV *BamH*I G and C fragments. (C) An *Eco*RI-*Ava*I subfragment located at the N-terminal domain of the MDV UL9 gene which was used as a probe for Northern blot hybridization. Drawing is not to scale.


TR L
U L
IR L
IR S
U S
TR S
A
L
I2
D
Q2
Q1
G
C
F
K2
K3
I3
E
P2
I1
J
O1
R
B
S S T
P3
N
M K
H
I2
Q2
L
A
P1
TK
gB
gC
ICP4
5'-end of
BamHIC
3'-end of
BamHI G
1.3 kb
3.1 kb
B
XcmI
BglII
BamHI
EcoRI
BclI
KpnI
EcoRI
MDV UL9
EcoRI
AvaI
C

subclone and the *BamH*I G fragment. The *EcoR*I junction region was sequenced from an intact *BamH*I C clone and the *BamH*I C-G junction sequence was confirmed by analysis of a 600bp PCR fragment (see Materials and Methods).

The MDV UL9 gene extends for 2104 bp within the serotype 1 MDV *BamH*I C fragment and extends 419 bp into the adjacent *BamH*I G fragment (Figure 2). MDV UL9 is transcribed from right to left with respect to the MDV genome and, as expected, is consistent with direction and location of the HSV UL9 gene. There are several potential translation start codons (ATG) located at nucleotides 3, 17, 21, 33, 52, 354 and 502 (Figure 3). Based on identification of termination codons between nt 3 and nt 502, comparison with HSV-1 UL9, and Kozak's consensus sequence for eukaryotic translation (Kozak, 1989), the translation start codon of MDV UL9 has been assigned at nt 502 (Figure 3). There are four potential TATA boxes located 62, 239, 286, and 297 bp upstream from the putative ATG of MDV UL9 (Figure 3). At present, it is unclear which, if any, of these may function as a site for TBP interaction. Several binding sites for transcription factors could be found upstream from the translation start codon. One CAAT box and five GC boxes are located 406, 39, 85, 120, 342 and 376 nt, respectively, upstream from the translation start codon. There are no potential poly(A) signals within the known sequence downstream of the MDV UL9 open reading frame (ORF). In HSV-1, the poly(A) signal for UL9 gene transcripts is located 2490 nt downstream from the UL9 translation stop codon, creating a bicistronic transcript encoding UL9 and UL8 (Mcgeoch *et al.*, 1988). A similar situation may exist in MDV UL9 transcription. The ORF predicted for MDV UL9 could potentially encode an 841 amino acid polypeptide

Figure 3: Nucleotide sequence of a 3108-bp segment of MDV strain GA DNA from *BamHI* fragments C and G. Predicted amino acid sequence is indicated under the DNA sequence. Four potential TATA boxes are denoted by asterisks beneath the DNA sequence. The CAAT box and five GC boxes are indicated by dotted lines under the DNA sequence. To provide continuity between Figures 3 and 4, conserved structural motifs are indicated by solid bars under the amino acid sequence and conserved regions without homology to known structural or functional domains are indicated by open boxes.

```
AAATGCACCACTCCCCATGTATGCGCACCCACATGTTTCTTGCTCGATATAATGCACCAA 60
CAGTAATATAACATACGAGAAACACGAAAACAATACTCGTTGCATAGAAGAAAAAAACTA 120
                          "CAAT box"
CCGCCTTTGTCTGGAAGAATAATACTGGAACCACGCCCCCCAGCCTGGGATAGGTGAGAC 180
"GC box"                              "GC box"
CCGTGTGCACTAGTGTCGTATTAACTGTTCTATAATCTACAACGGCTGCGAAGAAGCACG 240
                    *******     ******
GGAAACCCTCAGTTAAATTTATAGACGCGGCAACAAGTGTTCCAAAGAATACCAGTACGG 300
                     ****
CGATCCCGAAGCACACCGCTTGGACTACCCACATCTTTTTGTGCACGTAGTCTATGTGAC 360
TCAGTCCTCTGTAATTCCGCTCCATTCGTGCTCGACTGGCCATAGCGACGACCGCCGGTA 420
                "GC box"                                "GC box"
GTTTTCTGATCAGTGTTATACGATAGACACTCAGCCAGGGCGACTTTCTTCCGTAACGTT 480
                    ****                "GC box"
AGTAATCAGAGACAACTCAAAATGATAGACTATGCATCCAGCGCCTCCTTGTCTAGAATG 540
                        M  I  D  Y  A  S  S  A  S  L  S  R  M  13
TTATATGGAGAGGATCTTATAGACTGGATTATCAAGAACAGACCGGGAATAACAACAGAG 600
L  Y  G  E  D  L  I  D  W  I  I  K  N  R  P  G  I  T  T  E  33
CGTCAATCCGACGGTCCCGTTACTTTTCCGTCACCTTTGTACCCGAGAACGCGCAATGTC 660
R  Q  S  D  G  P  V  T  F  P  S  P  L  Y  P  R  T  R  N  V  53
CTTATAGTACGTGCACCTATGGGATCTGGCAAGACTTCTGCTCTCATGAACTGGTTGCAG 720
L  I  V  R  A  P  M  G  S  G  K  T  S  A  L  M  N  W  L  Q  73
                  motif       I
TGTATTTTATGCAATTCAAATATGAGCGTTTTGATTGTGTCTTGTAGACGCAGTTTTACC 780
C  I  L  C  N  S  N  M  S  V  L  I  V  S  C  R  R  S  F  T  93
AATACATTATCCGAAAAAATTAATAGGGCTGGCATGTCAGGATTTTGTACCTATCTGTCA 840
N  T  L  S  E  K  I  N  R  A  G  M  S  G  F  C  T  Y  L  S  113
TCCAGCGATTATATTATGCGAGGTAGAGAGTTTTCTAGACTCTTAGTACAAATTGAATCT 900
S  S  D  Y  I  M  R  G  R  E  F  S  R  L  L  V  Q  I  E  S  133
                                          Leucine
CTACATCGCGTAGATTCGAAACTTCTTGATAATTATGACATCGTTATATTGGATGAAATC 960
L  H  R  V  D  S  K  L  L  D  N  Y  D  I  V  I  L  D  E  I  153
   Zipper
                                  motif
ATGTCGACCATCGGCCAGCTTTTCTCTCCAACTATGAAACATTTATGTCAGGTCGATAAT 1020
M  S  T  I  G  Q  L  F  S  P  T  M  K  H  L  C  Q  V  D  N  173
   II
ATATTGACATCTCTTCTCAGATATCGTCCGAAGATTGTAGCAATGGACGCTACTATAAAT 1080
I  L  T  S  L  L  R  Y  R  P  K  I  V  A  M  D  A  T  I  N  193
                                      motif
ACTCAATTGATAGATATGTTAGCCATCATGAGAGGTGAAGAAAATATACATGTTATCGTA 1140
T  Q  L  I  D  M  L  A  I  M  R  G  E  E  N  I  H  V  I  V  213
   III
GGTGAATATGCAGCATCCGGGTTTTCTAGAAGGTCATGTACGATCCTCCGTAGTTTGGGA 1200
G  E  Y  A  A  S  G  F  S  R  R  S  C  T  I  L  R  S  L  G  233
ACTAATATCCTTCTTTCGGTGATGAATGAATTCAAACAACTTCCATCTCATACTCAGCCT 1260
T  N  I  L  L  S  V  M  N  E  F  K  Q  L  P  S  H  T  Q  P  253
ATATTTAAACAGAGTACAGGCGTCAACGGTTCTTTGGATATAAGTCTCCATGATCGGACG 1320
I  F  K  Q  S  T  G  V  N  G  S  L  D  I  S  L  H  D  R  T  273
TTTTTTTCAGAACTCACTAGACGGCTTGAGGGGGGGTTGAATATTTGTTTGTTTTCCTCA 1380
F  F  S  E  L  T  R  R  L  E  G  G  L  N  I  C  L  F  S  S  293
                                          motif
```

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ACTATATCATTTTCAGAGATTGTGGCACGTTTCTGCCTCGCATACACGGATTCGGTTTTA 1440
T  I  S  F  S  E  I  V  A  R  F  C  L  A  Y  T  D  S  V  L   313
         IV
GTGTTAAATTCCACAAGAAATACGCCAATAGATATAAATTCATGGTCTAACTACCGCGTC 1500
V  L  N  S  T  R  N  T  P  I  D  I  N  S  W  S  N  Y  R  V   333

GTAATTTATACTACCGTAGTAACCGTTGGTCTTAGTTTTAACGATTCCCATTTTCATAGT 1560
V  I  Y  T  T  V  V  T  V  G  L  S  F  N  D  S  H  F  H  S   353
      motif                  V
ATGTTTGCATACATCAAGCCTACGATAAATGGTCCCGAAATGGTATCGGTTTACCAATCT 1620
M  F  A  Y  I  K  P  T  I  N  G  P  E  M  V  S  V  Y  Q  S   373
                                          motif
TTGGGTCGAATTAGATCACTGCGTCTTAATGAAGTGCTAATTTATATCGATGCATCGGGA 1680
L  G  R  I  R  S  L  R  L  N  E  V  L  I  Y  I  D  A  S  G   393
         VI
GCTGGGTCGGAGCCGGTTTTTACGCCCATGCTACTAAATCACGTAATCGCCAATGGAGGA 1740
A  G  S  E  P  V  F  T  P  M  L  L  N  H  V  I  A  N  G  G   413
      Conserved              Box                 II
GGATGGCCAACGCGCTTTTCTCAAGTTACCAATATGTTGTGTCACAATTTCAGAAGAGAT 1800
G  W  P  T  R  F  S  Q  V  T  N  M  L  C  H  N  F  R  R  D   433

TGTATACCAACGTTCAGAGCCGCTGATGCATTATACATATTCCCACGATTTAAGTACAAA 1860
C  I  P  T  F  R  A  A  D  A  L  Y  I  F  P  R  F  K  Y  K   453

CATTTATTTGAGCGATGTACACTAAACAATGTAAGTGATAGTATTAATATTCTACATGCT 1920
H  L  F  E  R  C  T  L  N  N  V  S  D  S  I  N  I  L  H  A   473
     Conserved              box                 III
CTCCTTGAATCAAATTTAATACACGTGCGCTTCGATGGCTGTGATCTCCAATTAAATGCT 1980
L  L  E  S  N  L  I  H  V  R  F  D  G  C  D  L  Q  L  N  A   493

GAAGCCTTCTGTGATTTTTTAGTAATTCTTAGAGCAGATTCTATAACTGCCCAACGCGAT 2040
E  A  F  C  D  F  L  V  I  L  R  A  D  S  I  T  A  Q  R  D   513

ATGAAAACTCTGCGCAAAAATGCTACCTGCCCTTTACCGGTAGAGGTCGACGTAATTGAT 2100
M  K  T  L  R  K  N  A  T  C  P  L  P  V  E  V  D  V  I  D   533

AGTGATGCGGTAGCGTGTTTTGTTCAGAAATATCTAAGACCTACTGTGCTCGCCAATGAT 2160
S  D  A  V  A  C  F  V  Q  K  Y  L  R  P  T  V  L  A  N  D   553
CTCACAGAACTCCTAACAAAATTAGCGGAACCCATTACTAGAGAACAGTTCATAAACATC 2220
L  T  E  L  L  T  K  L  A  E  P  I  T  R  E  Q  F  I  N  I   573
ACTATGCTGGAAGCATGTCGTGCAACCCCGGCGGCTCTTTACAGTGAAGCGGTATTTTGT 2280
T  M  L  E  A  C  R  A  T  P  A  A  L  Y  S  E  A  V  F  C   593

CGTATATATGATTATTATGCATCTGGAAATATACCTATAATTGGACCAAGTGGAACCTTA 2340
R  I  Y  D  Y  Y  A  S  G  N  I  P  I  I  G  P  S  G  T  L   613
            conserved          box              I
GATACAACGATACTGACATGCGATTTTAATACATCTGGAAGATGGGACTTATACAGGGTA 2400
D  T  T  I  L  T  C  D  F  N  T  S  G  R  W  D  L  Y  R  V   633

TGTTGTAAATGGGCCGAATTATTGGGCATCAACCCTTTAGAAGGACCCAATGCTGATATA 2460
C  C  K  W  A  E  L  L  G  I  N  P  L  E  G  P  N  A  D  I   653
         helix                                turn
```

```
GATCCAACAAAACTGTTGCACGTCATGAAAGACGACTATGATATTTATGCTCGTTCTGTG 2520
D  P  T  K  L  L  H  V  M  K  D  D  Y  D  I  Y  A  R  S  V   673
                  helix
CTGGAAATTGCGCGATGTTACTTGATTGACGCCCAAACTGCCTTAAAACGCCCTGTGCGA 2580
L  E  I  A  R  C  Y  L  I  D  A  Q  T  A  L  K  R  P  V  R   693
         Leucine                                 Zipper
GCAACAAAATGTGCCTTGAGTGGGATCCAAAATTCTCACCATAGTCAACCATCCACTCAG 2640
A  T  K  C  A  L  S  G  I  Q  N  S  H  H  S  Q  P  S  T  Q   713

AGCCATGCAGTGTCTTTATTTAAAGTCACATGGGAGATTCTCTTCGGACTCCGCCTCACA 2700
S  H  A  V  S  L  F  K  V  T  W  E  I  L  F  G  L  R  L  T   733

AAGAGTACAACAACATTTCCGGGTAGAACAAAAGTAAAGAATTTACGGAAGGCGGAGATA 2760
K  S  T  T  T  F  P  G  R  T  K  V  K  N  L  R  K  A  E  I   753

GAAGCTCTGTTAGACGGAGCGGGTATTGATAGAACGTCATGCAAAACTCACAAGGATCTC 2820
E  A  L  L  D  G  A  G  I  D  R  T  S  C  K  T  H  K  D  L   773
      Varicella   Zoster   Virus   Homolgy
TACACCCTCTTGATGAAAAGCAAGTCATTATTTCGCAATATGCGCTATGATATTCGACGC 2880
Y  T  L  L  M  K  S  K  S  L  F  R  N  M  R  Y  D  I  R  R   793

CCGAAGTGGTACGACCTATTAAGATCTCGTTTAGACAAAGAGTTGGGTATATATCATGAT 2940
P  K  W  Y  D  L  L  R  S  R  L  D  K  E  L  G  I  Y  H  D   813
CTGGTAGATTTGGAATCTGTGTTGGCGGAAATTCCGTCAGCACTCTGGCCACGCGTAGAA 3000
L  V  D  L  E  S  V  L  A  E  I  P  S  A  L  W  P  R  V  E   833
GGTGCTGTAGATTTTCATCGTTTATAATTATTGGAACCGAATGCGTCAAACCATATCAAC 3060
G  A  V  D  F  H  R  L                                       841
GATGGCAGCATCGTCAAAAACTAATATCATGCAGATAATGCGAGGATG             3108
```

with a calculated molecular weight of 95 kd, similar to that predicted for the HSV-1 UL9 ORF, which encodes 851 amino acids.

Predicted amino acid sequence of MDV UL9 demonstrates strict conservation of structural motifs.

Overall, the predicted amino acid sequences of HSV-1 and MDV UL9 share 49% identity and 66% similarity while those of VZV and MDV UL9 share 46% identity and 63% similarity (Figure 4). MDV UL9 thus represents the second most highly conserved MDV protein identified to date, following glycoprotein B (gB), which had a mean identity of 50% between MDV, HSV-1 and VZV (Ross *et al.*, 1989). Furthermore, all three UL9 proteins: HSV-1 UL9 (851 amino acids); MDV UL9 (841 amino acids); and VZV UL9 (835 amino acids) are of similar size. Alignment of MDV, HSV-1, and VZV UL9 protein predicted amino acid sequences clearly demonstrates this sequence conservation (Figure 4). Consistent with location of important functional motifs, the N-terminal domains of MDV, HSV-1, and VZV UL9 proteins are more highly conserved than the C-terminal domains (Figure 4).

Similarity among alphaherpesvirus UL9 proteins from divergent sources supports the notion that UL9 plays a central role in alphaherpesvirus DNA replication. Much of the similarity between MDV, HSV-1, and VZV UL9 protein N-terminal sequences occurs within several highly conserved motifs. For example, all putative helicase motifs found within the N-terminal portion of HSV-1 and VZV UL9 proteins are present in MDV UL9. Furthermore, the spatial arrangement in these motifs is conserved between the three origin

Figure 4: Aligment of the predicted amino acid sequences of MDV, HSV-1 and VZV UL9 proteins. The top sequence shows the predicted amino acid sequence of MDV UL9 protein (mdvul9), HSV-1 UL9 amino acid sequence is listed in the second row (hsvul9) and the VZV UL9 sequence is listed in the third row (vzvul9). A consensus sequence derived from examination of the three UL9 proteins is presented in the fourth row (within the shaded box). Upper case letters in the consensus sequence indicate an amino acid is the same in all three proteins while lower case letters represent amino acids which are identical in two of the three proteins. Structural motifs and conserved regions are labeled and indicated by boxes surrounding the appropriate amino acids.

```
          1                                                  50
mdvul9    .......... .......... ...MIDYASS ASLSRMLYGE DLIDWIIKNR
hsvul9    MPFVGGAESG DPLGAGRPIG DDECEQYTSS VSLARMLYGG DLAEWVPRVH
vzvul9    .......... ....MSPNTG ESNAAVYASS TQLARALYGG DLVSWIKHTH

con       ----------------------------YaSS -sLaRmlYGg DL--Wi---h

          51                                                 100
mdvul9    PGITTERQSD GPVTFPSPLY PRTRNVLIVR APMGSGKTSA LMNWLQCILC
hsvul9    PKTTIERQQH GPVTFPNASA PTARCVTVVR APMGSGKTTA LIRWLREAIH
vzvul9    PGISLELQLD VPVKLIKPGM SQTRPVTVVR APMGSGKTTA LLEWLQHAL.

con       PgiT-ErQ-d gPV-fp-p-- p-tR-VtvVR APMGSGKTtA L--WLq-al-
                                          ATP binding motif
                                            (motif   I)
          101                                                150
mdvul9    NSNMSVLIVS CRRSFTNTLS EKINRAGMSG FCTYLSSSDY IMRGREFSRL
hsvul9    SPDTSVLVVS CRRSFTQTLA TRFAESGLVD FVTYFSSTNY IMNDRPFHRL
vzvul9    KADISVLVVS CRRSFTQTLI QRFNDAGLSG FVTYLTSETY IM...GFKRL

con       --d-SVlvVS CRRSFTqTL- -rfn-aGlsg FvTYlsS--Y IM--r-F-RL
                                                               -
          151                                                200
mdvul9    LVQIESLHRV DSKLLDNYDI VILDEIMSTI GQLFSPTMKH LCQVDNILTS
hsvul9    IVQVESLHRV GPNLLNNYDV LVLDEVMSTL GQLYSPTMQQ LGRVDALMLR
vzvul9    IVQLESLHRV SSEAIDSYDV LILDEVMSVI GQLYSPTMRR LSAVDSLLYR

con       IVQ-ESLHRV -s-lldnYDv liLDEvMSti GQLySPTM-- L--VD-Il-r
             Leucine   Zipper
                          motif  II
          201                                                250
mdvul9    LLRYRPKIVA MDATINTQLI DMLAIMRGEE NIHVIVGEYA ASGFSRRSCT
hsvul9    LLRICPRIIA MDATANAQLV DFLCGLRGEK NVHVVVGEYA MPGFSARRCL
vzvul9    LLNRCSQIIA MDATVNSQFI DLISGLRGDE NIHTIVCTYA GVGFSGRTCT

con       LLr-cp-IiA MDAT-N-Qli D-l-glRGee NiHviVgeYA --GFS-R-Ct
                     motif   III
          251                                                300
mdvul9    ILRSLGTNIL LSVMNEFKQL PSHTQPIFKQ STGVNGSLDI SLHDR..TFF
hsvul9    FLPRLGTELL QAALR..... .....PP GPPSGPSPDA SPEARGATFF
vzvul9    ILRDMGIDTL VRVIK..... .....  .RSPEHEDVR TIHQLRGTFF

con       iLr-lGt-lL --v---------------- ------s-d- s-h-r--TFF
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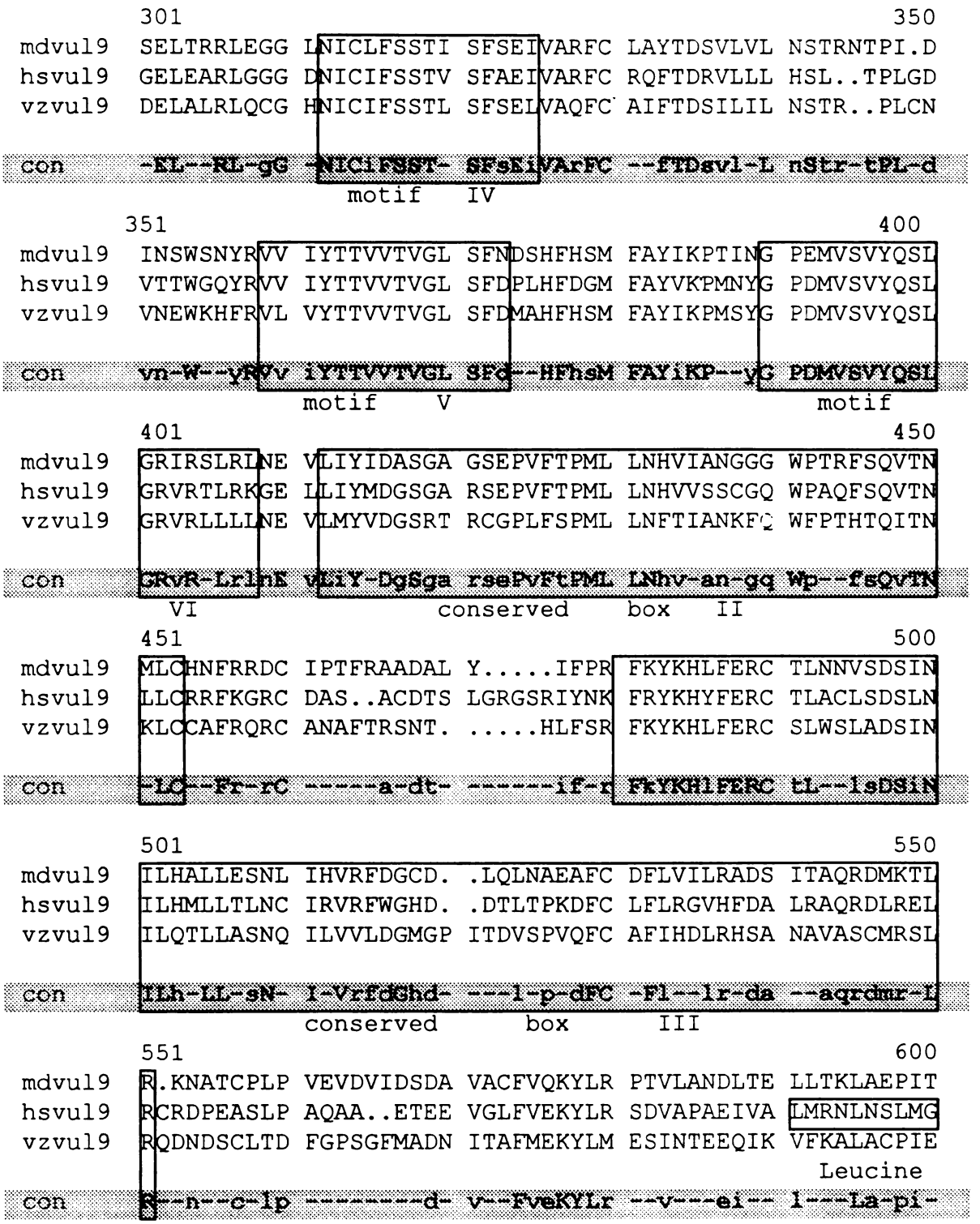
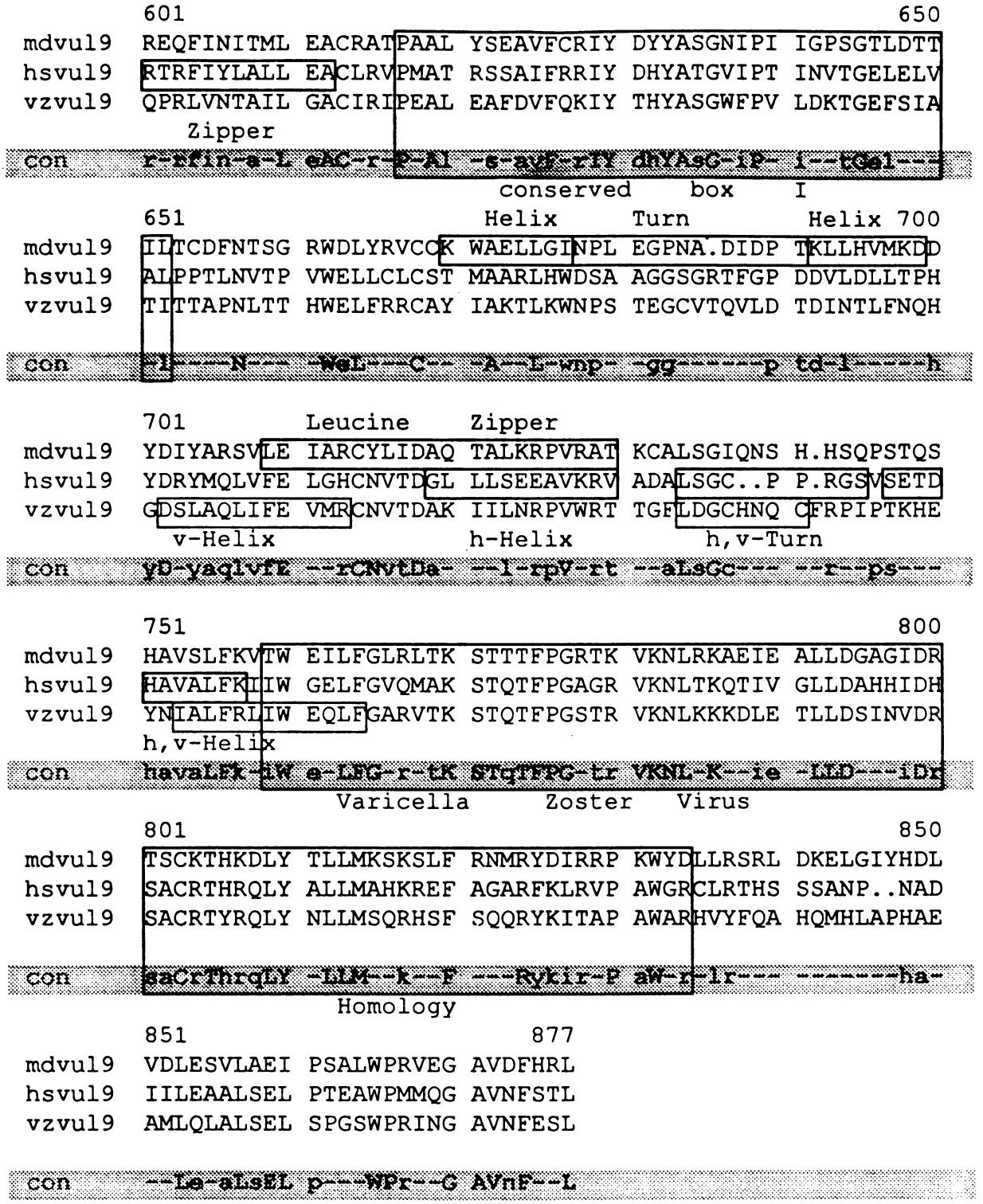

301 350
mdvul9 SELTRRLEGG LNICLFSSTI SFSEIVARFC LAYTDSVLVL NSTRNTPI.D
hsvul9 GELEARLGGG DNICIFSSTV SFAEIVARFC RQFTDRVLLL HSL..TPLGD
vzvul9 DELALRLQCG HNICIFSSTL SFSELVAQFC AIFTDSILIL NSTR..PLCN
con -EL--RL-gG -NICiFSST- SFsEiVArFC --fTDsvl-L nStr-tPL-d
motif IV
351 400
mdvul9 INSWSNYRVV IYTTVVTVGL SFNDSHFHSM FAYIKPTING PEMVSVYQSL
hsvul9 VTTWGQYRVV IYTTVVTVGL SFDPLHFDGM FAYVKPMNYG PDMVSVYQSL
vzvul9 VNEWKHFRVL VYTTVVTVGL SFDMAHFHSM FAYIKPMSYG PDMVSVYQSL
con vn-W--yRVv iYTTVVTVGL SFd--HFhsM FAYiKP--yG PDMVSVYQSL
motif V motif
401 450
mdvul9 GRIRSLRLNE VLIYIDASGA GSEPVFTPML LNHVIANGGG WPTRFSQVTN
hsvul9 GRVRTLRKGE LLIYMDGSGA RSEPVFTPML LNHVVSSCGQ WPAQFSQVTN
vzvul9 GRVRLLLLNE VLMYVDGSRT RCGPLFSPML LNFTIANKFQ WFPTHTQITN
con GRvR-LrlnE vLiY-DgSga rsePvFtPML LNhv-an-gq Wp--fsQvTN
VI conserved box II
451 500
mdvul9 MLCHNFRRDC IPTFRAADAL Y.....IFPR FKYKHLFERC TLNNVSDSIN
hsvul9 LLCRRFKGRC DAS..ACDTS LGRGSRIYNK FRYKHYFERC TLACLSDSLN
vzvul9 KLCCAFRQRC ANAFTRSNT. .....HLFSR FKYKHLFERC SLWSLADSIN
con -LC--Fr-rC -----a-dt- ------if-r FkYKHlFERC tL--lsDSiN
501 550
mdvul9 ILHALLESNL IHVRFDGCD. .LQLNAEAFC DFLVILRADS ITAQRDMKTL
hsvul9 ILHMLLTLNC IRVRFWGHD. .DTLTPKDFC LFLRGVHFDA LRAQRDLREL
vzvul9 ILQTLLASNQ ILVVLDGMGP ITDVSPVQFC AFIHDLRHSA NAVASCMRSL
con ILh-LL-sN- I-VrfdGhd- ---l-p-dFC -Fl--lr-da --aqrdmr-L
conserved box III
551 600
mdvul9 R.KNATCPLP VEVDVIDSDA VACFVQKYLR PTVLANDLTE LLTKLAEPIT
hsvul9 RCRDPEASLP AQAA..ETEE VGLFVEKYLR SDVAPAEIVA LMRNLNSLMG
vzvul9 RQDNDSCLTD FGPSGFMADN ITAFMEKYLM ESINTEEQIK VFKALACPIE
Leucine
con R--n--c-lp --------d- v--FveKYLr --v---ei-- l---La-pi-

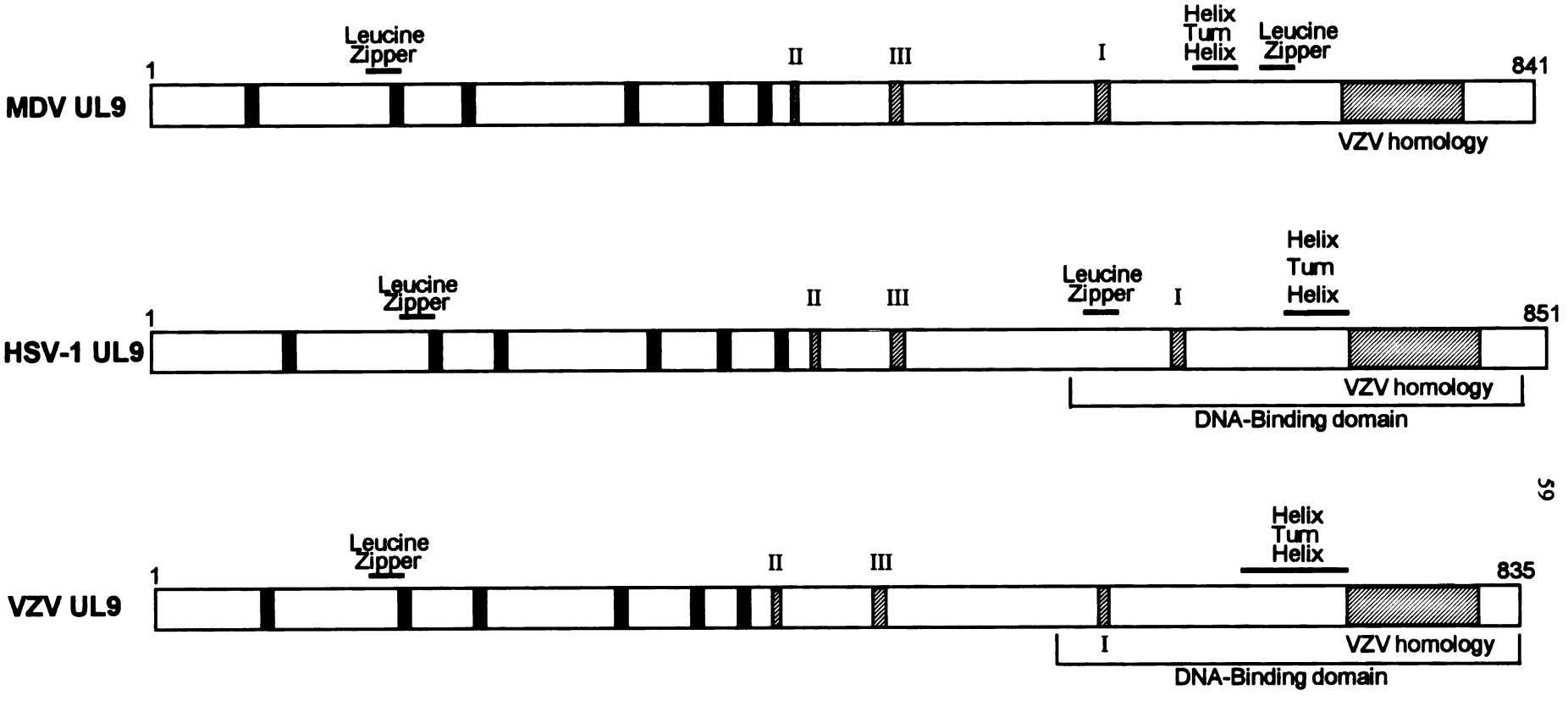

601
650
mdvul9 REQFINITML EACRATPAAL YSEAVFCRIY DYYASGNIPI IGPSGTLDTT
hsvul9 RTRFIYLALL EACLRVPMAT RSSAIFRRIY DHYATGVIPT INVTGELELV
vzvul9 QPRLVNTAIL GACIRIPEAL EAFDVFQKIY THYASGWFPV LDKTGEFSIA
Zipper
con r-rfin-a-L eAC-r-P-Al -e-avf-rIY dhYAsG-iP- i--tGel---
conserved box I
651
Helix
Turn
Helix 700
mdvul9 ILTCDFNTSG RWDLYRVCCK WAELLGINPL EGPNA.DIDP TKLLHVMKDD
hsvul9 ALPPTLNVTP VWELLCLCST MAARLHWDSA AGGSGRTFGP DDVLDLLTPH
vzvul9 TITTAPNLTT HWELFRRCAY IAKTLKWNPS TEGCVTQVLD TDINTLFNQH
con -I----N--- -WeL---C-- -A--L-wnp- -gg-----p td-l-----h
701
Leucine Zipper
mdvul9 YDIYARSVLE IARCYLIDAQ TALKRPVRAT KCALSGIQNS H.HSQPSTQS
hsvul9 YDRYMQLVFE LGHCNVTDGL LLSEEAVKRV ADALSGC..P P.RGSVSETD
vzvul9 GDSLAQLIFE VMRCNVTDAK IILNRPVWRT TGFLDGCHNQ CFRPIPTKHE
v-Helix
h-Helix
h,v-Turn
con yD-yaqlvfE --rCNvtDa- --l-rpV-rt --aLsGc--- --r--ps---
751
800
mdvul9 HAVSLFKVTW EILFGLRLTK STTTFPGRTK VKNLRKAEIE ALLDGAGIDR
hsvul9 HAVALFKIIW GELFGVQMAK STQTFPGAGR VKNLTKQTIV GLLDAHHIDH
vzvul9 YNIALFRLIW EQLFGARVTK STQTFPGSTR VKNLKKKDLE TLLDSINVDR
h,v-Helix
con havaLFk-iW e-LFG-r-tK STqTFPG-tr VKNL-K--ie -LLD---iDr
Varicella Zoster Virus
801
850
mdvul9 TSCKTHKDLY TLLMKSKSLF RNMRYDIRRP KWYDLLRSRL DKELGIYHDL
hsvul9 SACRTHRQLY ALLMAHKREF AGARFKLRVP AWGRCLRTHS SSANP..NAD
vzvul9 SACRTYRQLY NLLMSQRHSF SQQRYKITAP AWARHVYFQA HQMHLAPHAE
con saCrThrqLY -LLM--k--F ---Rykir-P aW-r-lr--- -------ha-
Homology
851
877
mdvul9 VDLESVLAEI PSALWPRVEG AVDFHRL
hsvul9 IILEAALSEL PTEAWPMMQG AVNFSTL
vzvul9 AMLQLALSEL SPGSWPRING AVNFESL
con --Le-aLsEL p---WPr--G AVnF--L

binding proteins (Figures 4 and 5). Average amino acid identity within helicase motifs in MDV, HSV-1, and VZV UL9 proteins is 73%. Most striking among these conserved domains is an ATP-binding motif (motif I), which is virtually identical in these three origin binding proteins (Figure 4). HSV-1 UL9 possesses an intrinsic helicase activity (Boehmer, 1993). Single amino-acid substitutions at conserved residues in five helicase motifs inactivated the function of HSV-1 UL9 in transient origin-dependent replication assays (Martinez *et al.*, 1992). Conservation of these motifs in MDV UL9 suggests that, like HSV-1 UL9, MDV UL9 possesses an intrinsic helicase activity within the N-terminal portion.

A leucine zipper region, also within the N-terminal domain of MDV UL9 (overlapping conserved helicase motif II), has 67% and 59% homology with corresponding sequences in HSV-1 and VZV UL9, respectively (Figure 4). Insertion mutagenesis of the HSV-1 amino-terminal leucine zipper domain dramatically affects cooperativity and dimerization of HSV-1 UL9 in solution (Hazuda *et al.*, 1992). As with the helicase motifs, spatial arrangement of the leucine zipper motifs is conserved between MDV, HSV-1, and VZV UL9 proteins (Figures 4 and 5). In addition to conservation of known structural/functional motifs, there are two highly conserved regions in MDV, HSV-1, and VZV UL9 (conserved boxes II and III) which do not show clear homology to previously identified functional domains (Figure 4).

The C-terminal domain of MDV UL9 contains two structural motifs in common with the C-terminal domain of HSV-1 UL9, a pseudo-leucine zipper (not clearly defined in VZV UL9) and a helix-turn-helix motif (Figure 4). By insertion and deletion mutagenesis,

Figure 5: Schematic summary comparing conservation and spacial arrangement of structural motifs in HSV-1, MDV and VZV UL9 proteins. Solid black boxes indicate conserved helicase motifs. A large hatched box highlights the VZV homology domain. Positions of conserved leucine zippers and helix-turn-helix domains are indicated by text and highlighted by solid bars. Small hatched boxes represent conserved regions (boxes I, II, and III). DNA-binding domains for HSV-1 and VZV UL9 are also indicated.

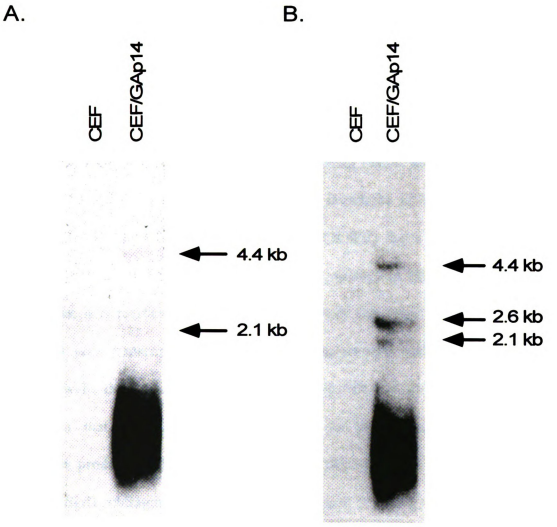

MDV UL9
1
Leucine Zipper
II
III
I
Helix Turn Helix
Leucine Zipper
841
VZV homology
HSV-1 UL9
1
Leucine Zipper
II
III
Leucine Zipper
I
Helix Turn Helix
851
VZV homology
DNA-Binding domain
VZV UL9
1
Leucine Zipper
II
III
Helix Turn Helix
835
I
VZV homology
DNA-Binding domain

the pseudo-leucine zipper was shown to be important for DNA-binding activity of HSV-1 UL9 (Deb and Deb, 1991; Arbuckel and Stow, 1993). However, importance of the helix-turn-helix motif for the DNA-binding activity remains to be elucidated (Deb and Deb, 1991; Arbuckel and Stow, 1993). In addition to these two recognizable structural motifs, there are two, less obvious regions, which may be important for UL9 DNA-binding activity. From comparison of MDV, HSV-1, and VZV UL9 proteins, a region from amino acids 583 to 618 (conserved box I) was found to be highly conserved between all three proteins (Figures 4 and 5). Another highly conserved region is found within the very C-terminal end of MDV UL9. This sequence, termed the VZV homology region (Martin *et al.*, 1994), is highly similar to corresponding sequences in HSV-1 and VZV UL9 proteins (Figure 4). Despite a lack of similarity to any known DNA binding motif, the VZV homology region is critical in maintaining the DNA-binding activity of HSV-1 UL9 (Deb and Deb, 1991; Arbuckel and Stow, 1993).

Detection of MDV UL9 transcripts

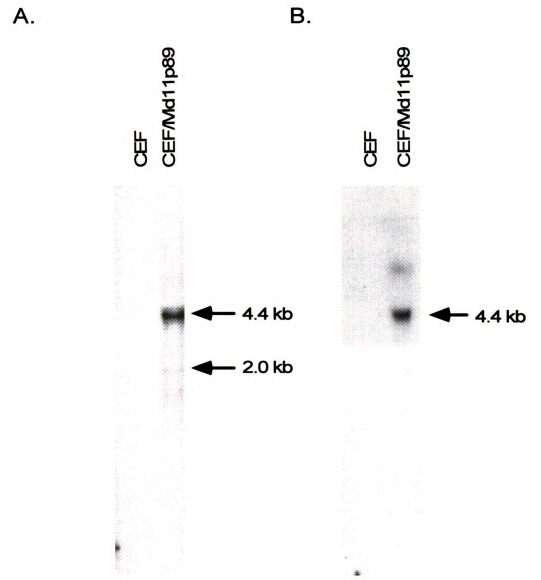
Northern blot hybridization was performed to detect transcripts arising from the MDV UL9 gene region. An *EcoR*I-*Ava*I DNA fragment (0.59 kb) (Figure 2), which maps within the 5'-end of the MDV UL9 gene, was used as probe for detection of transcripts. The *EcoR*I-*Ava*I probe hybridized to two transcripts of 4.4 and 2.1 kb in RNA derived from CEF cells infected with MDV strain GA (Figure 6A, lane 2). No transcripts were detected by the *Ava*I-*EcoR*I probe in mock-infected CEF cells (Figure 6A, lane 1). An *EcoR*I subfragment (3.1 kb) clone, originally used to identify the MDV UL9 gene within

Figure 6: Northern blot analysis of MDV UL9 gene transcription. Total RNA was isolated from uninfected CEF cells and cells infected with MDV strain GAp14 as described in Materials and Methods. Panel A: RNA was hybridized with a 0.59 Kbp *EcoR*I-*Ava*I probe from MDV *BamH*I fragment C. Panel B: RNA was hybridized with a 3.1 Kbp *EcoR*I clone from MDV *BamH*I fragment C. In both panels, Lane 1 contains RNA isolated from mocked-infected CEF while Lane 2 contains RNA from CEF infected with MDV strain GA. The large diffuse band of hybridization below 1.0 kb in MDV-infected cell lanes of both panels likely represents viral DNA not completely removed by RQ1 DNase treatment (see Materials and Methods).


A.
CEF
CEF/GAp14
4.4 kb
2.1 kb
B.
CEF
CEF/GAp14
4.4 kb
2.6 kb
2.1 kb

the *BamH*I C fragment, was also used as probe for detection of transcripts. The 3.1 Kb *EcoR*I probe hybridized to three transcripts of 4.4, 2.6 and 2.1 kb (Figure 6B, lane 2). In HSV-1, the UL9 coding region is 2.5 kb while the predominant transcript of the HSV-1 UL9 gene is about 5.2 kb, being 3'-coterminal with UL8 gene transcripts (Baradaran *et al.*, 1994). HSV-1 UL10 is transcribed from a gene immediately downstream of HSV-1 UL9 oriented in the opposite direction, relative to the HSV-1 genome. The transcription start site for HSV-1 UL10 is within the HSV-1 UL9 coding region and the coding region of HSV-1 UL10 is 1.4 kb (Mcgeoch *et al.*, 1988; Baradaran *et al.*, 1994). Given the similarity of MDV and HSV-1 gene organization, it is likely that a similar organization of replication protein genes exists in MDV. Based on analogy to HSV-1 and our Northern blot analysis, it is reasonably possible that the 4.4-kb transcript represents the primary MDV UL9 gene transcription product, possibly co-terminal with an MDV UL8 gene transcript, while the 2.1-kb transcript represents the MDV UL10 gene. To confirm the presumption that the 4.4-kb transcript represents the primary MDV UL9 gene transcription product, while the 2.1-kb transcript likely represents the MDV UL10 gene, an *BamH*I-*Bgl*II subfragment from *BamH*I G fragment (Figure 2) was used as probe for northern blot hybridization. This probe contains sequence from the 3'-end of the MDV UL9 gene and would not be expected to hybridize with MDV UL10 gene sequence. The *BamH*I-*Bgl*II subfragment hybridized to two transcripts (4.4 kb and 2.0 kb) (Figure 7A, lane 2). An *EcoR*I-*BamH*I subfragment from *BamH*I C fragment (Figure 2), representing central portions of the MDV UL9 gene, was also used as probe for detection of transcripts. The *EcoR*I-*BamH*I fragment hybridized to only one transcript (4.4kb) (Figure

Figure 7: Northern blot analysis of MDV UL9 transcription. Total RNA was isolated from uninfected CEF cells and cells infected with MDV strain Md11p89 as described in Materials and Methods. Panel A: RNA was hybridized with a 0.3 Kbp *BamH*I-*Bagl*II probe from MDV *BamH*I G fragment. Panel B: RNA was hybridized with a 1.3 Kbp *EcoR*I-*BamH*I probe from MDV *BamH*I fragment C near the junction between *BamH*I C and G fragments. In both panels, Lane 1 contains RNA isolated from mocked-infected CEF while Lane 2 contains RNA from CEF infected with MDV strain Md11.


A.
B.
CEF
CEF/Md11p89
4.4 kb
2.0 kb
CEF
CEF/Md11p89
4.4 kb

7B, lane 2). These two results suggest that 4.4-kb transcript represents the primary MDV UL9 gene transcription product and it is reasonably possible that the 2.1 kb band represents MDV UL10.

Discussion

We have identified and sequenced the MDV serotype 1 UL9 gene, which is located within *BamH*I-G and *BamH*I-C fragments of MDV strain GA DNA. Predicted amino acid sequences of MDV UL9 is highly similar to UL9 sequences from other alpha-herpesviruses, particularly HSV-1 and VZV. A summary of structural motifs and conserved regions within MDV, HSV-1 and VZV UL9 proteins is presented in Figure 5. The N-terminal domain of MDV UL9 contains six conserved helicase motifs and a putative leucine zipper sequence which, by analogy to HSV-1 UL9, may be important for activity and dimerization, respectively, of MDV UL9. Spatial arrangement of both the helicase motifs and leucine zipper within MDV UL9 are similar to that within origin-binding proteins of HSV-1 and VZV (Martinez *et al.*, 1992; Chen and Olivo, 1994; Martin *et al.*, 1994) (Figure 5). Using a model DNA substrate, Boehmer *et al.* (1993) has shown that HSV-1 UL9 possesses intrinsic helicase activity, consistent with a hypothesis that UL9 protein serves as an initiator of viral DNA replication. Presumably, UL9 unwinds origin regions, allowing entry of DNA replication machinery. Conservation of helicase motifs and a putative leucine zipper in MDV UL9 suggests that the N-terminal domain of MDV UL9 may encode a helicase activity and be necessary for dimerization and cooperativity of MDV UL9 monomers. Thus, MDV UL9 may serve as an initiator for MDV viral DNA replication. In addition to conservation of helicase and leucine zipper elements within the UL9 amino terminal one-half, two regions of high homology (conserved boxes II and III), occur in UL9 proteins from MDV, HSV-1, and VZV.

Sequences within conserved boxes II and III do not correspond to any recognizable structural motifs and, to date, have no assigned or obvious function.

Sequence-specific DNA binding activity of HSV-1 UL9 is localized to amino acids 564 to 832 (Deb and Deb, 1991) while that of VZV UL9 is localized to amino acids 551 to 835 (Chen and Olivo, 1994). Although the C-terminal domain of MDV UL9 exhibits lower similarity than other regions, when compared with HSV-1 and VZV UL9, it contains several elements which are important for DNA-binding activity of HSV-1 UL9 (Arbuckle and Stow, 1993; Martin *et al.*, 1994), a pseudo-leucine zipper sequence, a helix-turn-helix motif, and a VZV homology region (Martin *et al.*, 1994). In contrast to the amino-terminal helicase and leucine zipper motifs, spatial arrangement of C-terminal pseudo-leucine zipper and helix-turn-helix domains within MDV UL9 is different from that within HSV-1 and VZV UL9 (Figure 5). At present, consequences of altered spatial arrangement for C-terminal elements are unknown. However, presence of essential motifs for DNA-binding activity suggests that the C-terminal portion of MDV UL9 may function in this capacity. In addition to elements mentioned above, a region from amino acids 583 to 618 within the C-terminal portion of MDV UL9 is highly conserved among UL9 proteins from diverse alphaherpesviruses. This conserved region does not resemble any known structural motifs, however, It may be important for DNA-binding activity (Martin *et al.*, 1994). Overall, MDV UL9 appears to retain the same domain structure as UL9 proteins from HSV-1 and VZV, that is the N-terminal portion encodes dimerization signals, helicase activity and an ATP binding site, while the C-terminal portion encodes

DNA-binding activity. Experiments designed to test this hypothesis using in vitro translated MDV UL9 protein are currently in progress.

In HSV-1, UL9 plays a central role in initiation of viral replication. UL9 protein binding to an origin sequence is the first committed step for initiation of replication. Following UL9 binding, other replication proteins bind to the origin and form a functional replication complex. Reflecting this central role, MDV UL9 is the second most highly conserved MDV protein, relative to HSV-1 and VZV, behind the essential glycoprotein B (Ross *et al.*, 1989). One MDV replication origin, which is highly similar to origins of other alphaherpesviruses has been identified (Camp *et al.*, 1991). This origin contains two sequences which are highly similar to HSV-1 UL9 recognition sites. Each of these putative UL9 recognition sites contains one 9 bp sequence (CGTTCGCAC) which is a subset of an 11-bp motif (CGTTCGCACTT) shown to be recognized by the HSV-1 origin binding protein. A viral specific band was detected in electrophoretic mobility shift (EMS) assays with MDV-infected cell extracts using an oligonucleotide containing a potential MDV UL9 binding site (Camp, 1993). Identity of MDV encoded proteins which bind to this site have yet to be determined. However, given the conservation of UL9 binding sites in HSV-1 and VZV and the high degree of similarity between HSV-1, MDV and VZV UL9, it is likely that each of the 9-bp repeats serves as a site of MDV UL9 recognition. EMSA assays with in vitro translated MDV UL9 will address this issue.

In other alphaherpesviruses, replication protein genes are clustered within the unique long region. Transcription of these genes often leads to bicistronic or polycistronic transcripts (Mcgeoch *et al.*, 1988). Based on Northern blot hybridization, the MDV UL9

gene appears to be transcribed in a similar manner. Two transcripts (4.4 kb and 2.1 kb) were detected in RNA of cells infected with MDV, using a probe from within the UL9 coding region. A third transcript (2.6 kb) was observed when a large (3.1 Kb) probe containing sequences immediately downstream of MDV UL9 was used. The 4.4 kb transcript was also observed in RNA of cells infected with MDV when two probes, one from the 3'-end of MDV UL9 gene and another centrally located along the MDV UL9 sequence, were used. Based on gene colinearity between MDV and HSV-, it is a reasonable possibility that the 2.1-kb transcript, whose 5'-end overlaps the N-terminal domain of MDV UL9 coding region, is transcribed from the opposite direction and represents the MDV UL10 gene transcript. The 4.4-kb transcript likely represents the predominant MDV UL9 encoding message. In support of these predictions, open reading frame analysis of known DNA sequence revealed that predicted amino acid sequences of one ORF, located upstream from the MDV UL9 ORF and oriented in a direction opposite the UL9 gene, is homologous to N-terminal sequences of HSV-1 UL10 (47% similarity). Thus, gene organization of at least UL9 and UL10 within the MDV genome appears similar to that within the HSV-1 genome. Lack of a consensus polyadenylation signal downstream of MDV UL9 supports the suggestion that the 4.4 kb UL9 transcript either contains a long 3' cotranslated region or is bicistronic.

Acknowledgements

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Chapter 3

Marek's disease virus (MDV) DNA replication : a MDV gene homologous to herpes simplex virus type 1 (HSV-1) UL9 gene encodes an origin binding protein (OBP)

Ting-Feng Wu, and Paul M. Coussens*

Abstract

In our previous studies, we identified an MDV UL9 homolog gene which lies between the *BamH*I C and *BamH*I G fragments of the serotype 1 MDV strain GA genome. Computer analysis of the predicted amino acid sequences of MDV and HSV-1 UL9 proteins revealed that MDV UL9 is highly similar to HSV-1 UL9 and shares numerous motifs with HSV-1 UL9. It is suggested that MDV UL9 may have the similar biochemical activities to HSV-1 UL9 protein, specifically the origin-binding activity. In this study, a MDV UL9 protein of 95 kd was detected in nuclear extracts prepared from chick embryo fibroblast (CEF) cells infected with serotype 1 MDV by western blot analysis with antiserum against the MDV UL9 protein. PCR was used to clone the MDV UL9 gene. *In vitro* transcription-translation of this gene generated a protein of 95 kd as measured by sodium dodecyl sulfate-polyacrylamide analysis. Further characterization of this protein was accomplished via the use of Electrophoretic mobility shift assays (EMSA). EMSAs with *in-vitro* expressed MDV UL9 protein or MDV-infected CEF nuclear extracts showed that the MDV UL9 protein could bind to the HSV-1 UL9 binding site I and one of two potential OBP binding sites within the serotype 2 MDV origin. A mutant MDV UL9 site II DNA containing a point mutation which render the MDV UL9 binding site II to become MDV UL9 binding site I was used in competitive EMSAs to showed that the last nucleotide (T) within MDV UL9 binding site II was essential for the binding of MDV UL9 protein to MDV UL9 binding site II. A series of oligonucleotides containing substitutions within the flanking sequence around the MDV UL9 binding site I was used in competitive

EMSAs to showed that the last two nucleotides (TT) within HSV-1 UL9 binding site I were essential for the binding of MDV UL9 protein *in vitro*.

Introduction

Marek's disease virus (MDV) is a highly cell-associated avian herpesvirus. In chickens, MDV is the etiologic agent of Marek's disease (MD), characterized by malignant T-cell lymphomas(Calnek *et al.*, 1991). Originally MDV was classified as a gammaherpesvirus based on cell tropism and pathology (Roizman, 1990). However, based on overall genomic structure and colinearity of many MDV genes to those of alphaherpesviruses, such as herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV), MDV has been reclassified as an alphaherpesvirus (Brunovskis and Velicer, 1992; Buckmaster *et al.*, 1988; Cebrian *et al.*, 1982; Roizman, 1992). Consistent with other alphaherpesviruses, MDV genomes are linear double-stranded DNA molecules 160 to 180 Kbp in length, and consist of two unique regions (U_S and U_L) flanked by inverted repeats (IR_S, IR_L, TR_S, and TR_L).

Replication of viral genomes depend on *cis*-acting elements which function as origins of viral DNA replication, and *trans*-acting proteins. The replication origin of HSV-1 have been functionally identified by using assays based on amplification of plasmid DNA molecules containing suspected origins in transfected tissue culture cells expressing viral replication proteins (Weller et al, 1985; Stow et al, 1983). Two HSV-1 origins have been identified, one (ori_L) lies close to the center of unique long (U_L) region (Weller et al, 1985), while two identical copies of the second (ori_S) are located within the inverted repeats flanking the unique short (U_S) region (Stow et al, 1983). Both origins feature palindromic DNA sequences with central A+T-rich regions and share considerable sequence homology (Weller *et al.*, 1985). Functional MDV origins of replication have

been identified in serotype 2 MDV defective virus (Camp *et al.*, 1991) and in serotype 3 MDV (Smith *et al.*, 1995). The serotype 2 MDV origin of replication is located within the inverted repeats flanking the unique long region (U_L) (Camp *et al.*, 1991). The serotype 2 MDV origin of replication has a structure similar to the replication origins of HSV-1, VZV and EHV-1. The HVT replication origin is located in the junction between U_L and IR_L and is highly similar to the origins of replication of MDV-2, HSV-1, VZV and EHV-1

HSV-1 encodes seven proteins that are both necessary and sufficient for origin-dependent DNA synthesis (Wu *et al.*, 1988). HSV-1 UL9, one of seven essential HSV-1 genes, encodes the origin binding protein (OBP) (Olivo et al , 1988). OBP has been shown in several studies to be essential for viral DNA replication and to bind specifically to sequences within HSV-1 ori_s (Elias et al, 1990; Weir et al, 1990; Deb et al, 1989; Weir et al, 1989; Elias et al, 1988; Koff et al, 1988; Olivo et al , 1988; Elias et al, 1986). HSV-1 ori_s contains three OBP-binding sites, site I and II are located within the right and left arms of palindromic sequences flanking the central A+T core and site III is located to the left of site I (Elias et al 1990; Weir et al, 1990; Weir et al, 1989; Elias et al, 1988; Deb et al, 1988; Lockshon et al, 1988). All three OBP-binding sites are highly homologous however, site I has 5 to 10-fold higher affinity for OBP than site II (Elias et al, 1990; Weir et al, 1990; Elias et al, 1988). All three OBP-binding sites have been shown to be essential for HSV-1 viral replication *in vivo* (Hernandez et al, 1991; Weir et al, 1990). Using DNAase I footprinting, methylation interference, and filter binding assay with mutant oligonucleotides for site I, the HSV-1 OBP binding site was mapped to within a domain of 11 nucleotides of site I (CGTTCGCACTT) (Hazuda et al, 1991; Elias et al, 1990; Deb

and Deb, 1989; Koff et al, 1988). The 11-base-pair (bp) element within site II is different in two positions from that within site I, while the motif within site III differs only in one position (Elias et al, 1990). The presumed HSV-1 OBP binding site (11-bp element) is conserved in both ori_L and ori_S of HSV-1 and HSV-2 as well as in a VZV origin of replication. It was proposed that the HSV-1 OBP binds as a dimer to two inverted, overlapping pentanucleotides within site I (5'-GTTCGCAC-3'/3'-CAAGCGTG-5') (Koff et al, 1988).

Two distinct regions which are highly homologous to the conserved 11-bp element are identified within the serotype 2 MDV origin. These two regions are located within the left and right arms of a serotype 2 MDV origin palindrome. Each region contains a 9-bp sequence (CGTTCGCAC) which is highly conserved in the ori_L and ori_S of HSV-1 and HSV-2 as well as the origins of VZV and EHV-1 (Camp et al, 1991). This 9-bp element is a subset of the conserved 11-bp element. Thus, there are two potential OBP binding sites within MDV serotype 2 origin. The HVT replication origin also contains a 9-bp motif which is conserved among the alphaherpesviruses.

In our previous studies, we identified an MDV UL9 homolog gene which lies between the *BamH*I C and *BamH*I G fragments of the serotype 1 MDV strain GA genome. The predicted amino acid sequences of the HSV-1 and MDV UL9 proteins share 49% identity and 66% similarity while those of VZV and MDV UL9 share 46% identity and 63% similarity. Computer analysis of the predicted amino acid sequences of MDV and HSV-1 UL9 proteins revealed that MDV UL9 shares numerous motifs with HSV-1 UL9 and VZV gene 51 (a HSV-1 UL9 homolog). Conserved motifs include several helicase

moieties and a leucine zipper in the N-terminus, a C-terminal pseudo-leucine zipper, and a putative helix-turn-helix structure.

In this study, we detected MDV UL9 protein in nuclear extracts prepared from chick embryo fibroblast (CEF) cells infected with serotype 1 MDV strains GA and Md11 by western blot analysis with antiserum against the MDV UL9 protein. Electrophoretic mobility shift assays (EMSA) with *in-vitro* expressed MDV UL9 protein showed that the MDV UL9 protein could bind to the oligonucleotide for the HSV-1 UL9 binding site I and also bind to one of two potential OBP binding sites within the serotype 2 MDV origin. EMSAs with MDV-infected CEF nuclear extracts showed that the MDV UL9 protein present in the infected CEF nuclear extract could bind to the HSV-1 UL9 binding site I and one of two potential OBP binding sites within the serotype 2 MDV origin.

Materials and Methods

Cells and Viruses

Chicken embryo fibroblast (CEF) cells were prepared, maintained and infected with MDV as previously described (Glaubiger *et al.*, 1983). Two serotype 1 MDV strains, GA (passage 14) and Md11 (passage 89) were used for this study. CEF cultures were grown in Leibovitz-McCoy medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 4% calf serum at 37°C in a humidified atmosphere containing 5% CO_2. Calf serum concentration was reduced to 1% following MDV infection.

Preparation of nuclear extracts

CEF cells were infected with serotype 1 MDV strains GA and Md11. Cells infected with MDV strain GA or Md11 were harvested 48 hr or 24 hr later, respectively. The harvested cells were lysed in buffer A (15mM KCl, 10mM HEPES pH 7.6, 2mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40) for 10 minutes on ice. Nuclei were pelleted by centrifugation at 500 xg at 4°C for 10 min. Pelleted nuclei were lysed in buffer B (1 M KCl, 25mM HEPES pH 7.6, 0.1 mM EDTA, 1 mM DTT), at 4°C for 15 minutes with frequent vortexing, and then centrifuged at 14000 rpm for 20 minutes. The supernatants were collected and diluted with buffer C (20% glycerol, 25 mM HEPES pH 7.6, 0.1 mM EDTA, 1 mM DTT) at 1:3.75 ratio.

Western blot analysis

Mock-infected and MDV-infected CEF cell nuclear extracts were separated on 10% SDS-PAGE minigels (Bio-Rad, Hercules, California) and transferred to nitrocellulose

(NC) membrane (Schleicher & Schuell, Keene, New Hampshire). NC membranes were blocked using 5% dry milk. Rabbit antiserum against MDV UL9 protein was used at 1:100 dilution and donkey anti-rabbit IgG conjugated with horseradish peroxidase was used as the secondary antibody. Proteins bound by antibody were visualized using an Amsherm ECL system according to the manufacturer's specification.

Expression of GST fusion protein

The pGEX-5x-3 vector system (Pharmica Biotech, Uppsala, Sweden), which encodes a 27 kDa bacteria GST ORF under the control of an inducible Lac promoter, was used to express the GST-MDV UL9 fusion protein. A *BamH*I-*Kpn*I fragment from the 3' end of MDV UL9, which encodes amino acids 702 to 841 of MDV UL9 and has a significantly hydrophilic characteristic, was treated with T4 polymerase in the presence of dGTP to remove the 3'-protruding end of the *Kpn*I site. The digested fragments were cloned between the *BamH*I and *EcoR*I sites of pGEX-5x-3, had the *EcoR*I site blunt-ended with klenow enzyme. DH5∝ cells containing the GST-MDV UL9 ORF fusion protein plasmid were cultured at 37°C until the $O.D._{600}$ reached between 1.0 and 2.0. Then Isopropylβ-D-thiogalactoptranoside (IPTG) was added to a final concentration of 0.3 mM at 37°C for 3 hrs to induce fusion protein expression and then centrifuged. Pelleted cells were suspended in 25ml ice-cold PBS and GST fusion protein was purified by glutathione beads following the manufacturer's recommendation (Parmacia). Purified GST fusion protein was analyzed on a 12% SDS-polyacrylamide gel (PAGE). Purified GST-MDV UL9 fusion protein was

mixed with adjuvant (Titer-max; CytRx Co., Norcross) and used to immunize New Zealand rabbits subcutaneously.

Cloning and expression of MDV UL9

MDV UL9 gene was amplified from total cellular DNA isolated from MDV-GA-infected CEF cells by polymerase chain reaction (PCR) reaction. The upstream PCR primer (5'-GGGGTACCCCGGTTTGACGCATTCGGTTCC-3') was located 27 bp upstream of the ATG codon of MDV UL9 gene and the downstream primer (5'-GGGGTACCCCAGGGCGACTTTCTTCCGT-3') was located 5 bp downstream of the termination codon. Primers were designed to contain *Kpn*I sites at the 5'-ends (Fig. 2). PCR reactions were performed in 100-uL volume using a GeneAmp® PCR System 9600 DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT). Each reaction contained 50mM KCl, 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 0.001% gelatin (W/V), 5.0 pmole of each primer, 200uM of each deoxyribonuceloside triphosphate (dNTP), 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer) and 1ug total cellular DNA isolated from CEF infected with MDV GA strain. Cycling conditions were 20 cycles of: 95°C for 1 min; 65°C for 1 min; and 72°C for 1 min.

PCR fragments were purified by low-melting-point agarose gel electrophoresis and digested with *Kpn*I. Digested PCR fragment were cloned into the *Kpn*I site of the pBKCMV vector (Stratagene, Ja Jolla, CA.). The plasmid was designated pBKMDVUL9.

MDV UL9 protein was expressed in coupled transcription-translation reactions (TNT reticulocyte lysate; Promega, Inc., Madison, Wis.) from plasmid pMDVUL9. An aliquot of ^{35}S-labeled, *in-vitro* translation products were mixed with an equal amount of loading buffer containing 6M urea, 100mM Tris pH6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.1% bromophenol blue and 1.8 M 2-mercaptoethanol and separated on an SDS-polyacrylamide gel electrophoresis (PAGE). Gels were treated with an intensifying agent (Amplify; Amersham Life Science, Arlington Height, IL), dried and exposed to autoradiography film (XAR-5; Kodak, Mass.).

Immunoprecipitation

An aliquot of transcription-translation lysate prepared from pBKMDVUL9 was incubated with preimmune or antiserum against MDV UL9 on ice for 1 hr. A aliquot of transcription-translation lysate prepared without plasmid DNA was also incubated with antiserum against MDV UL9. The immunocomplexes were collected by 10% protein A-agarose bead suspension. After formation of immunocomplexes, the protein A-agarose bead was added to the reticulocyte lysate. The suspension was incubated at 4°C for 1 hr with rocking and then centrifuged. The pellet was washed with lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5 mM PMSF) three times, resuspended in protein sample loading buffer containing, 6M urea, 100mM Tris pH6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.1% bromophenol blue and 1.8 M 2-mercaptoethanol, boiled for 5 min and loaded onto a 10% SDS-PAGE.

Electrophoretic mobility shift assay (EMSA)

CEF nuclear extract (10 μg) or MDV strain GA or Md11-infected CEF nuclear extracts (10 μg) were incubated with ^{32}P-labeled oligonucleotides (1-2 ng) and 1 μg poly(dI-dC) at room temperature for 30 minutes in 20 μl of reaction buffer. Oligonucleotides were labeled with T4 polynucleotide kinase in presence of [^{32}P]γ-ATP. In experiments involving *in vitro* transcription-translation products, ^{32}P-labeled oligonucleotides were incubated with an aliquot of transcription-translation lysate prepared without or with plasmid DNA. The reaction buffer consisted of 12mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH (pH7.6), 4mM Tris-HCl (pH7.6), 100mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 60 μg of bovine serum albumin per ml, 12% glycerol, 5 μg of salmon sperm DNA per ml and a cocktail of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.24 trypsin inhibitor units of aprotinin per ml). DNA-protein complexes were separated on 5% native polyacrylamide gels in 0.5x TBE at 4°C. In experiments involving antibody, the proteins were incubated with antibody for 30 minutes followed by incubation with the labeled probe for an additional 30 minutes. The competition experiments were performed as described above, with premixing of specific probe and competitor DNAs prior to the addition of proteins in DNA-binding buffer. The dried gels were dried for quantitation on a phosphor-imager (Molecular Dynamics, Sunnyvale, CA).

Results

Detection of MDV UL9 gene product.

The GST-MDVUL9 fusion protein was purified as described in Materials and Methods and as expected, a 43 kDa fusion protein was detected (data not shown). Antiserum was produced by immunization of New Zealand white rabbits with purified GST-MDV UL9 fusion protein. To determine if MDV UL9 protein was expressed in the lytically infected cells, western blot analysis was performed using MDV-infected nuclear extracts and GST-BK antiserum as a primary antibody. A 95 kDa protein was detected in both MDV-GA- and Md11-infected CEF cells but not in CEF cells. The size of this polypeptide is consistent with the predicted size of MDV UL9 protein (95 kDa) (Fig. 1). In Figure 1 of Chapter 2, three virus-specific proteins could be detected. Compared to the results of western blot analysis with anti-MDV UL9 antibody, the protein with an apparent molecular size, 95 kDa, should be the MDV UL9 protein while the other two proteins were likely the degradation products.

Cloning and expression of MDV UL9 gene.

In order to examine binding activity of MDV UL9 independent of other viral proteins, the MDV UL9 gene was cloned into a pBKCMV expression vector. Based on our previously determined DNA sequence of the MDV UL9 gene, PCR primers were designed with *Kpn*I restriction sites engineered at their 5'-ends. The MDV UL9 gene was amplified by PCR using total cellular DNA isolated from MDV-GA-infected CEF cells (Fig. 2). A

Figure 1. Detection of MDV UL9 in MDV-infected cells. Western blot hybridization was performed as described in Material and Methods. Mock-infected CEF nuclear extract (CEF) was used as negative control. Position of the putative MDV UL9 protein identified in extracts of CEF cells infected with MDV strain GAp14 (CEF/GAp14) and Md11p89 (CEF/Md11p89) is indicated by an arrow to the right. Protein sizes were determined by comparison to standards of known size.

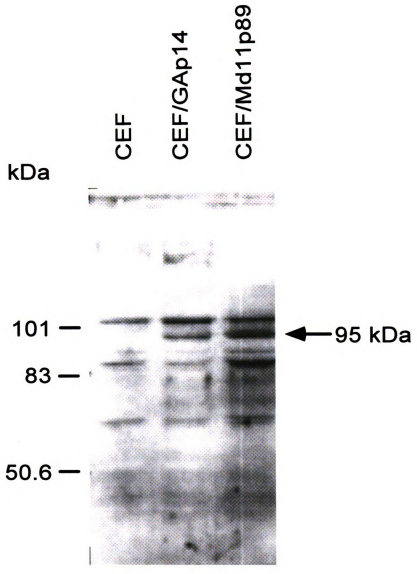

CEF
CEF/GAp14
CEF/Md11p89
kDa
101
83
50.6
95 kDa

Figure 2. Cloning of MDV UL9 gene. Two primers for PCR reactions contain KpnI sites at their 5'-ends. PCR reaction was performed as described in Material and Methods. PCR fragment was digested with KpnI and cloned into the KpnI site of pBKCMV vector (Stratagene, Ja Jolla, CA.). The schematic shows the orientation of MDV gene relative to MDV genome, the resultant fragment, the restriction enzyme used and the final ligation into pBKCMV.

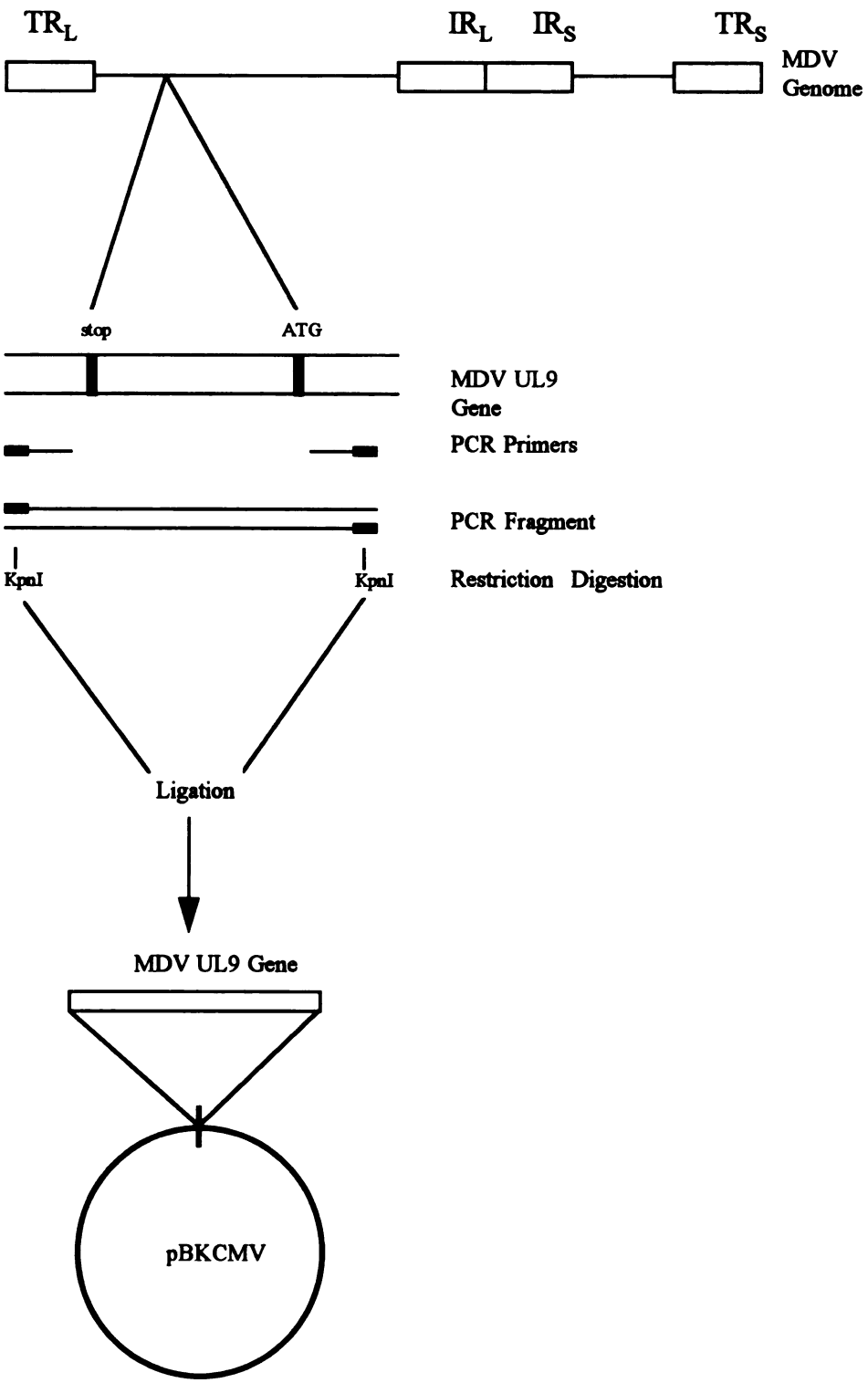
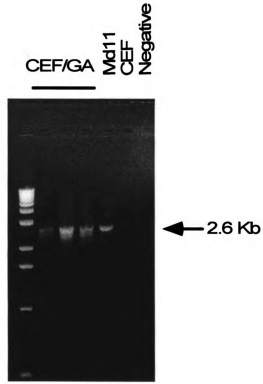

TR_L
IR_L
IR_S
TR_S
MDV Genome
stop
ATG
MDV UL9 Gene
PCR Primers
PCR Fragment
KpnI
KpnI
Restriction Digestion
Ligation
MDV UL9 Gene
pBKCMV

Figure 3. PCR results of amplification of MDV UL9 gene from total cellular DNA isolated from CEF cells infected with MDV strain GA. Maker used was 1 Kbp ladder marker. PCR reactions were performed as described in Material and Methods. Lanes 2-4 were PCR reactions performed with total cellular DNA isolated from MDV-GA-infected CEF cells (CEF/GA). The positive control (CEF/Md11) was PCR reactions performed with contour-clamped homogeneous electric field (CHEF) isolated Md11 DNA. Negative controls were PCR reactions performed with total cellular DNA isolated from mock-infected CEF cells (CEF) and without DNA template (negative).


CEF/GA
Md11
CEF
Negative
2.6 Kb

2.6 Kbp fragment was amplified from total cellular DNA isolated from MDV-GA-infected CEF cells (Fig. 3, lanes 2, 3 and 4) but no amplification was detected when mock infected CEF cellular DNA used as the template (Fig. 3, lane 6). PCR fragments were purified and cloned into the *Kpn* I site of pBKCMV vector (Fig. 2).

The wild type MDV UL9 gene construct was used to express the protein *in vitro* with the TNT system (Progema). An aliquot of the ^{35}S-labeled protein was run on an 10% SDS-polyacrylamide gel. A predominant 95 kDa polypeptide was present in the wild-type lane (Fig. 4A, lane 2) but not detected in the control reaction (Fig. 4A, lane 1). The 95 kDa protein was immunoprecipitated by antiserum against MDV UL9 (Fig. 4B, lane 4), indicating that the MDV UL9 protein was expressed. The *in-vitro* expressed MDV UL9 protein will be utilized in EMSA.

Binding of MDV OBP to the HSV-1 UL9 binding site I.

The high degree of similarity between MDV UL9 and HSV-1 UL9 implies that MDV UL9 may bind to HSV-1 UL9 binding site I. In order to test the binding activity of MDV UL9 gene product, electrophoretic mobility gel shift assays (EMSA) were performed with MDV UL9 gene product expressed *in vitro* using a 26-mer oligonucleotide probe (HSV-1 UL9 site I DNA) which contains a 11-bp motif (CGTTCGCACTT) identified as an HSV-1 UL9 binding site I (Fig. 5B). Following incubation of the plasmid programmed-lysate with HSV-1 UL9 site I DNA probe, three protein-DNA complexes, which ran with electrophoretic mobilities indistinguishable from those of complexes I, II and C' identified in EMSA with the mock lysate, were detected (Fig. 6, lane 3). However, complexes I and II identified in EMSA with the programmed-lysate became much weaker when compared

Figure 4. SDS-polyacrylamide gel analysis and Immunoprecipitation of L-[^{35}S]metthionine-labeled MDV UL9 gene product synthesized *in vitro* by using a reticulocyte lysate. MDV UL9 gene was expressed with TNT lysate system (Promega) as described in Material and Methods. An aliquot of ^{35}S-labeled protein was run on an SDS-10% polyacrylamide gel and visualized by autoradiography. (A) Lane 1, mock-programmed control; lane 2, full length product of MDV UL9 gene. (B) lane 1, mock programmed lysate; lane 2, full length product of MDV UL9 gene; lane 3, immunoprecipitation of full length MDV UL9 gene product by preimmune antiserum; lane 4, immunoprecipitation of full length MDV UL9 gene product by anti-MDV UL9 antiserum; lane 5, immunoprecipitation of mock programmed lysate by anti-MDV UL9 antiserum; The synthetic MDV UL9 protein is shown by the arrow to the right. Molecular mass marker is shown to the left.

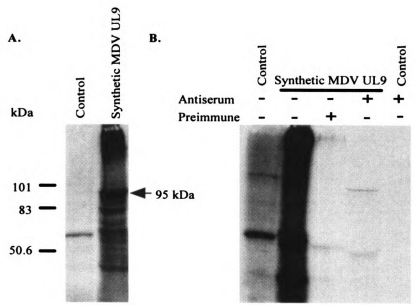
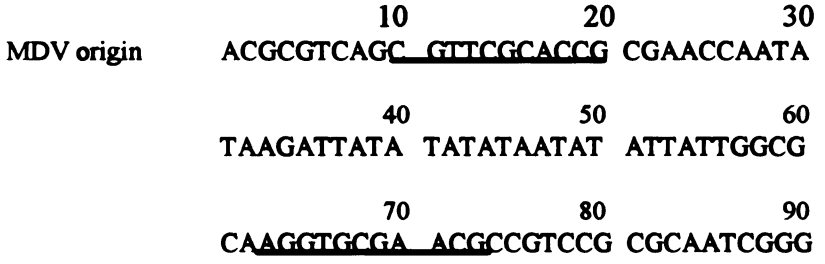

A.
Control
Synthetic MDV UL9
kDa
101
83
50.6
95 kDa
B.
Control
Synthetic MDV UL9
Control
Antiserum - - - + +
Preimmune - - + - -

Figure 5. MDV origin and comparison of OBP binding sites of HSV-1 and MDV. (A) MDV serotype 2 origin. The potential OBP binding sites are indicated by the bar under the sequence. The 9 bp sequence is conserved in the origins of HSV-1, VZV and EHV-1. (B) HSV-1 UL9 binding site I. The 11 bp conserved sequence is indicated by the sequence within the square. (C) Comparison of HSV-1 UL9 binding site I and the MDV UL9 binding site I. Ori 22 is indicated by the sequence above the bracket.

A.


10 20 30
MDV origin ACGCGTCAGC GTTCGCACCG CGAACCAATA
40 50 60
TAAGATTATA TATATAATAT ATTATTGGCG
70 80 90
CAAGGTGCGA ACGCCGTCCG CGCAATCGGG

B.


11-bp element
HSV-1 UL9 binding site I GCGAAGCGTTCGCACTTCGTCCCAAT

C.

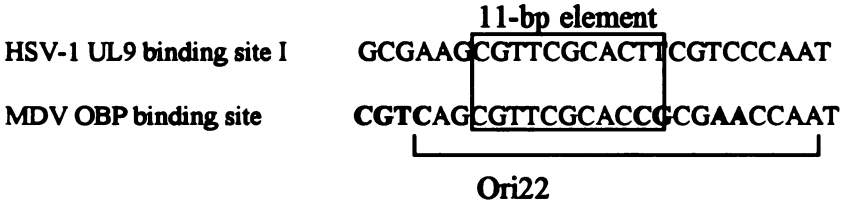
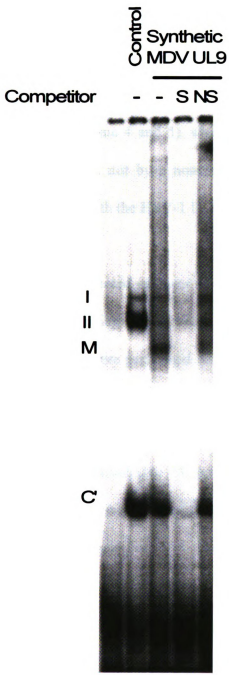

11-bp element
HSV-1 UL9 binding site I GCGAAGCGTTCGCACTTCGTCCCAAT
MDV OBP binding site CGTCAGCGTTCGCACCGCGAACCAAT
Ori22

Figure 6. Binding of *in-vitro* synthesized MDV UL9 gene product to HSV-1 UL9 site I DNA. EMSAs of mock lysates (control) or MDV UL9 gene product (synthetic MDV UL9) on HSV-1 UL9 site I DNA were performed as described in Material and Methods. Lane 1, EMSA was performed without addition of the proteins. See Fig. 5 for the sequence of site I DNA. s: specific competitor. ns : nonspecific competitor. The complexes formed are indicated by letter to the left.


Control
Synthetic MDV UL9
Competitor
- - S NS
I
II
M
C'

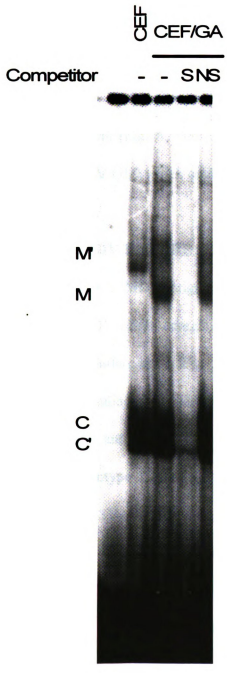
to those identified in EMSA with the mock lysate (Fig. 6, lane 2 and 3). An additional complex (M), which was not present in EMSA with the mock lysate, was detected in EMSA with the programmed-lysate (Fig 6, lane 3). This complex presumably resulted from the binding of synthetic MDV UL9 protein to the lone OBP binding site on HSV-1 UL9 site I DNA. The complex between synthetic MDV UL9 protein and the HSV-1 UL9 site I DNA was specific (Fig. 6, lane 4 and 5), since it could be inhibited by a specific competitor (nonlabeled probe) but not by a nonspecific competitor. Thus, MDV UL9 protein forms a specific complex with the HSV-1 UL9 binding site I.

Binding activities from MDV-infected extracts on HSV-1 UL9 binding site I.

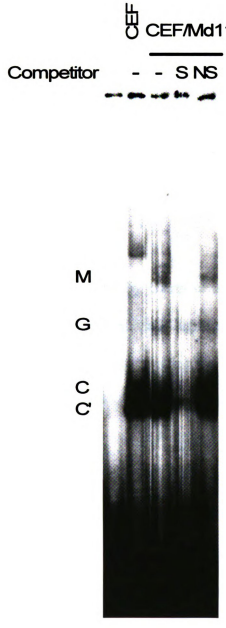
In order to test for origin-binding activity from MDV-infected cells, electrophoretic mobility gel shift assays (EMSA) were performed with the nuclear extracts prepared from MDV-GA- or Md11-infected CEF cells or uninfected CEF cells using the HSV-1 UL9 site I DNA probe. In EMSAs with MDV-GA- or Md11-infected extracts, three protein-DNA complexes (M, C and C') were identified (Fig 7A, lane 3 and 7B, lane 3) and an additional complex (G) was occasionally seen in EMSA with MDV Md11-infected-cell extract (Fig 7B, lane 3). Formation of all three complexes (M, C and C') identified in EMSAs with the infected extracts were specific (Fig. 7A, lane 4 and 5, Fig. 7B, lane 4 and 5), as they could be inhibited by a specific oligonucleotide competitor but not by a nonspecific competitor. In EMSAs with the mock extracts, two complexes with electrophoretic mobilities indistinguishable from those (C and C') identified in EMSAs with the infected extracts were detected (Fig 7A, lane 2 and 7B, lane 2). Thus, the complex M, which was identified in EMSAs with the infected extracts and not present in EMSAs with the mock extracts

Figure 7. Detection of origin-binding activities from MDV-infected cells on HSV-1 UL9 site I DNA. (A) Binding of MDV-GA-infected nuclear extract proteins. EMSAs of uninfected extracts (CEF) or MDV-GA-infected extracts (CEF/GA) on HSV-1 UL9 site I DNA were performed as described in Material and Methods. Lane 1, EMSA was performed without addition of the extracts. The complexes formed are indicated by the letters to the left. s: specific competitor. ns: nonspecific competitor. (B) Binding of MDV-GA-infected nuclear extract proteins. Legends are the same those in Fig. 7A.

A.


CEF
CEF/GA
Competitor
- - S NS
M'
M
C
C'

B.

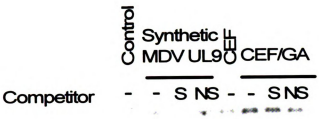

CEF
CEF/Md11
Competitor
- - S NS
M
G
C
C'

(Fig. 7A, lane 3 and Fig. 7B, lane 3), was a virus-specific complex. It presumably resulted from the interaction between MDV UL9 protein present in the infected extract and HSV-1 UL9 site I DNA. The virus-specific complex (M) identified in EMSAs with the infected extracts and the MDV UL9-specific complex (M) identified in EMSA with the *in-vitro* expressed MDV UL9 were shown to migrate at the similar rates when the *in-vitro* translated MDV UL9 gene product was used in EMSA and run in parallel with the infected extracts (Fig. 8, lane 3 and 7). This result indicated that MDV-infected cells contained MDV UL9 which could bind to HSV-1 UL9 binding site I.

Binding of MDV UL9 OBP to MDV serotype 2 origin.

A serotype 2 MDV origin had been functionally identified by Camp et al (1991). serotype 2 MDV origin contains two 11-bp sequences each of which is highly similar to HSV-1 UL9 binding site I (Fig. 5A). Thus, MDV serotype 2 origin contains two potential MDV OBP binding sites. These two 11-bp elements are present at the left (MDV UL9 binding site I) and right (MDV UL9 binding site II) arms of the palindrome sequence within the serotype 2 MDV origin. The sequence of MDV UL9 binding site I is different from that of HSV-1 UL9 binding site I in two positions while that of MDV UL9 binding site II is different only in one position (Fig. 5). Using a 22-mer oligonucleotide (MDV UL9 site I DNA) probe containing MDV UL9 binding site I, no viral or cellular binding activities were detected in EMSAs with MDV GA-infected-cell nuclear extracts (data not shown). However, using a 26-mer oligonucleotide (MDV UL9 site II DNA) probe containing MDV UL9 binding site II, two virus-specific complexes (M and M') were

Figure 8. Comparison of binding of MDV-GA-infected extract proteins and *in-vitro* synthesized MDV UL9 gene product to HSV-1 UL9 site I DNA. EMSAs of uninfected extracts (CEF) or MDV-GA-infected extracts (CEF/GA) and mock lysates (control) or *in-vitro* synthesized MDV UL9 gene product (synthetic MDV UL9) on HSV-1 UL9 site I DNA were performed as described in Material and Methods. Lane 1, EMSA was performed without addition of the proteins. s: specific competitor. ns: nonspecific competitor. The complexes formed are indicated by the letters to the left.


Control
Synthetic MDV UL9
CEF
CEF/GA
Competitor
- - S NS - - S NS

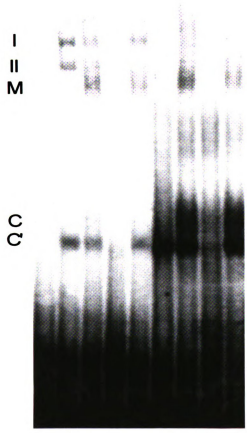
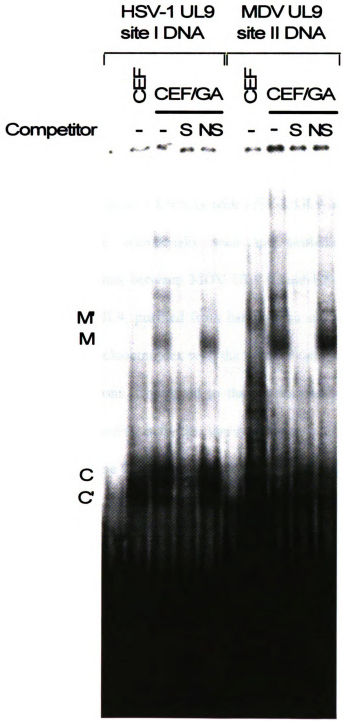

I
II
M
C
C'

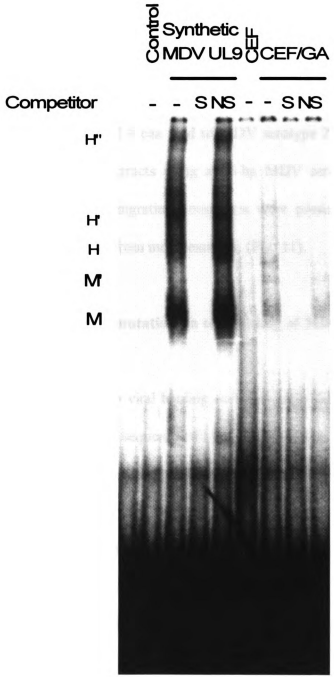
Figure 9. Detection of binding activities from MDV-GA-infected cells on MDV UL9 site II DNA. Lane 1-5, EMSAs were performed using HSV-1 UL9 site I probe; lane 6-10, EMSAs were performed using MDV UL9 site II probe. EMSAs of uninfected extracts (CEF) or MDV-GA-infected nuclear extracts (CEF/GA) on HSV-1 UL9 site I DNA or MDV UL9 site II DNA were performed as described in Material and Methods. Lane 1 and 6, EMSAs were performed without addition of the extracts. s: specific competitor. ns : nonspecific competitor. The complexes formed are indicated by the letters to the left.


HSV-1 UL9
site I DNA
MDV UL9
site II DNA
CEF
CEF/GA
CEF
CEF/GA
Competitor
-
-
S
NS
-
-
S
NS
M'
M
C
C'

identified (Fig. 9, lane 8). These two complexes were specific and not present in EMSAs with the mock extracts (Fig. 9, lane 9 and 10). These two complexes (M and M') probably resulted from the interaction between MDV UL9 present in the infected extract and MDV UL9 site II DNA. The two complexes identified in EMSAs with MDV UL9 site II DNA probe migrated at rates similar to those of two complexes identified in EMSA with HSV-1 UL9 site I DNA probe (Fig. 9, lane 3 and 8). The slowly migrating virus-specific complex (M') detected both in EMSAs with HSV-1 UL9 site I DNA and MDV UL9 site II DNA probes were occasionally seen and probably resulted from intra- and intermolecular interactions between MDV UL9 bound-DNA. Rabkin and Hanlon (1991) reported that HSV-1 UL9, purified from baculovirus vector-infected insects cells, could form a high order of nucleocomplex with the plasmid containing HSV-1 ori_S via inter- and intramolecular interactions. Compared to the results of EMSAs with HSV-1 UL9 site I DNA probe, cellular binding activities were different in EMSAs with MDV UL9 site II DNA probe (Fig. 9, lane 3 and 8). Super-EMSA were also performed to detect to the MDV UL9 protein present in the complex M. The complex M was reduced a little in presence of MDV UL9-specific antibody (data not shown).

When EMSA was performed with the *in-vitro* expressed MDV UL9 by using MDV UL9 site II DNA probe, four specific-binding complexes not present in the control reaction were detected (Fig. 10, lane 3). The fastest migrating complex (M) migrated at a rate similar to that of complex M identified in EMSAs with MDV GA-infected nuclear extracts, while the other three complexes (H, H' and H") migrated at a slower rate (Fig. 10, lane 3 and 7). The three slowly migrating complexes detected in EMSA with the *in-*

Figure 10. Binding of *in-vitro* synthesized MDV UL9 gene product to MDV UL9 site II DNA. Lane 1-5, EMSA were performed with mock lysate (control) or *in-vitro* synthesized MDV UL9 gene product (synthetic MDV UL9); lane 6-10, EMSAs were performed with uninfected extracts (CEF) or MDV-GA-infected nuclear extracts (CEF/GA). EMSAs of *in-vitro* synthesized MDV UL9 gene product or MDV-GA-infected nuclear extracts on MDV UL9 site II DNA were performed described in Material and Methods. Lane 1, EMSA was performed without addition of the proteins.


Control
Synthetic MDV UL9 OBE
CEF/GA
Competitor
- - S NS - - S NS
H''
H'
H
M'
M

vitro expressed MDV UL9 were also likely due to the intra- and intermolecular interactions between MDV UL9 bound-DNA. The result of EMSA with the *in-vitro* expressed MDV UL9 suggested that MDV-infected cells contain MDV UL9 which can bind to MDV UL9 binding site II.

Since MDV UL9 can be shown to bind to one of the potential OBP binding sites, it is not surprising that MDV UL9 can bind to MDV serotype 2 origin. The result of EMSA with MDV-GA infected extracts using a 96-bp MDV serotype 2 origin as the probe showed that two closely migrating complexes were present in EMSAs from infected extracts but not in EMSAs from mock extracts. (Fig. 11).

Effect of single-base-pair mutation on the binding of MDV UL9 to MDV UL9 site II DNA.

As mentioned above, no viral binding activities were detected in EMSA using MDV UL9 site I DNA probe. The sequence of MDV UL9 binding site I is different from that of MDV UL9 binding site II in one position (Fig. 12A). The last nucleotide of MDV UL9 binding site I is a G while that of MDV UL9 binding site II is a T. In order to investigate if nucleotide T within the MDV UL9 binding site II is important for the viral binding activities of MDV UL9 binding site II *in vitro*, a mutant MDV UL9 site II DNA was made and tested in competitive EMSAs. The 11-bp motif within the mutant MDV UL9 site II DNA was made to have the same sequence as that of 11-bp motif within MDV UL9 site I DNA; that is, nucleotide T within MDV UL9 site II DNA was mutated to nucleotide G (Fig. 12A). The results of competitive EMSAs analyzed in a phosphor

Figure 11. Detection of binding activities from MDV-GA-infected cells on MDV serotype 2 origin. EMSAs of uninfected extracts (CEF) or MDV-GA-infected extracts (CEF/GA) on MDV serotype 2 origin were performed as described in Material and Methods. Lane 1, EMSA was performed without addition of the extracts.

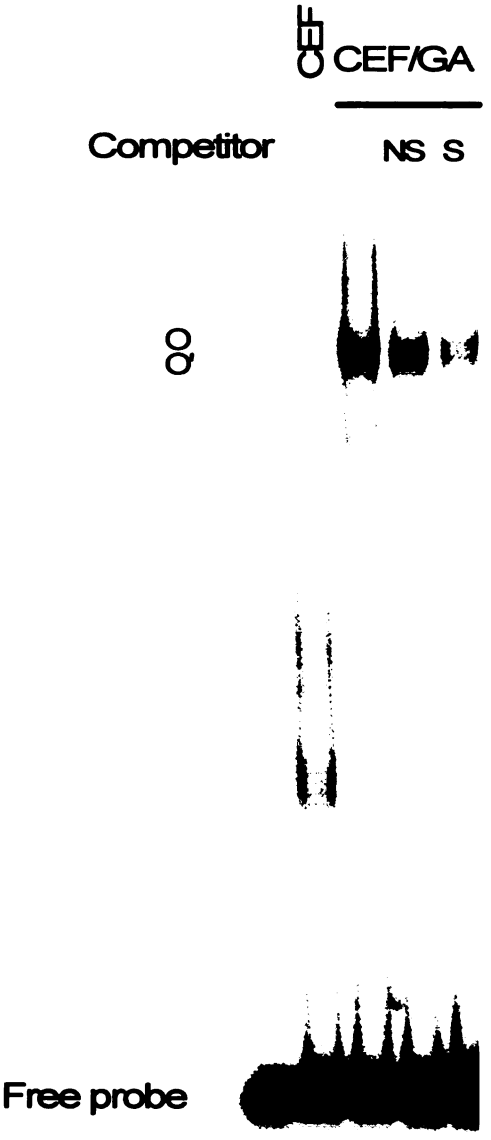

CEF
CEF/GA
Competitor
NS S
OC
Free probe

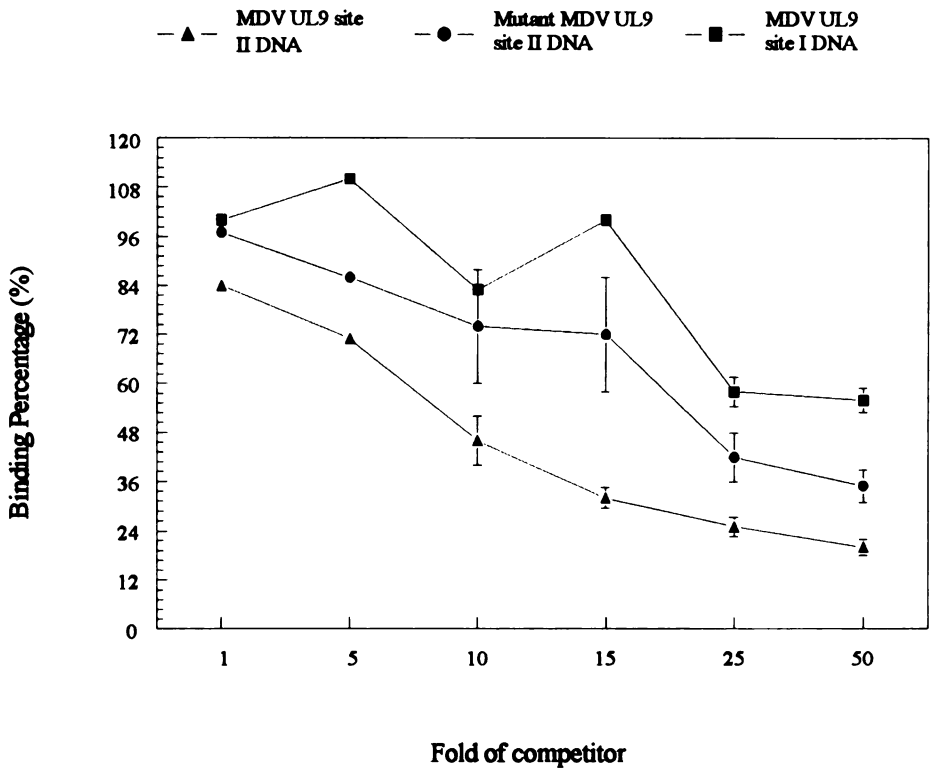
Figure 12. Mutation analysis of MDV UL9 binding site II. (A) The position of point mutation. The point mutation is indicated by the letter within square. (B) Graph of the interpolated phosphor-imager analysis of competition analyses of the M complexes. Competitive EMSAs were performed with MDV-GA-infected extracts using MDV UL9 site II DNA probe as described in Material and Methods. Each band was scanned with the phosphor-imager and results were compared (as a percentage) with those obtained without competitor (100%). The binding percentage was plotted against the molar ratio of unlabeled competitor. Each value represents the average of 2 to 3 experiments.

A.

MDV UL9 site II DNA GGACGGCGTTCGCACCTTGCGCCAAT

Mutant MDV UL9 site II DNA GGACGGCGTTCGCACCGTGCGCCAAT

B.



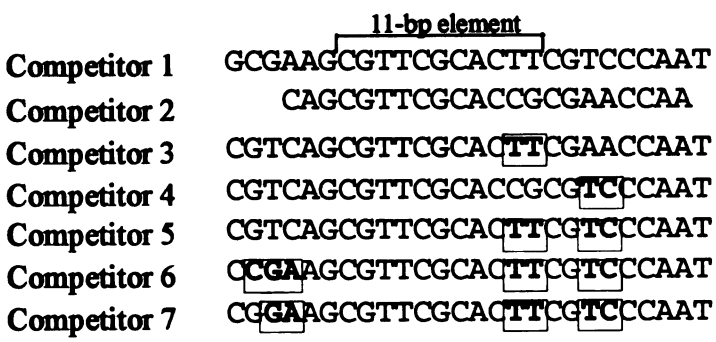
imager (Molecular Dynamics, Sunnyvale, CA) are shown in Fig. 12B. Since complex M is the major viral complex, the results were derived from quantitation of complex M. The wild type MDV UL9 site II DNA gave the predicted reduction in binding whereas the mutant MDV UL9 site II DNA had much less competition ability than that of wild type MDV UL9 site II DNA. MDV UL9 site I DNA has the least ability to compete and was comparable to that of mutant MDV UL9 site II DNA. This result suggested that the last nucleotide T of MDV UL9 binding site II is essential for the viral binding activities of MDV UL9 binding site II *in vitro.*

Binding activities from MDV-infected cells on MDV UL9 site I DNA.

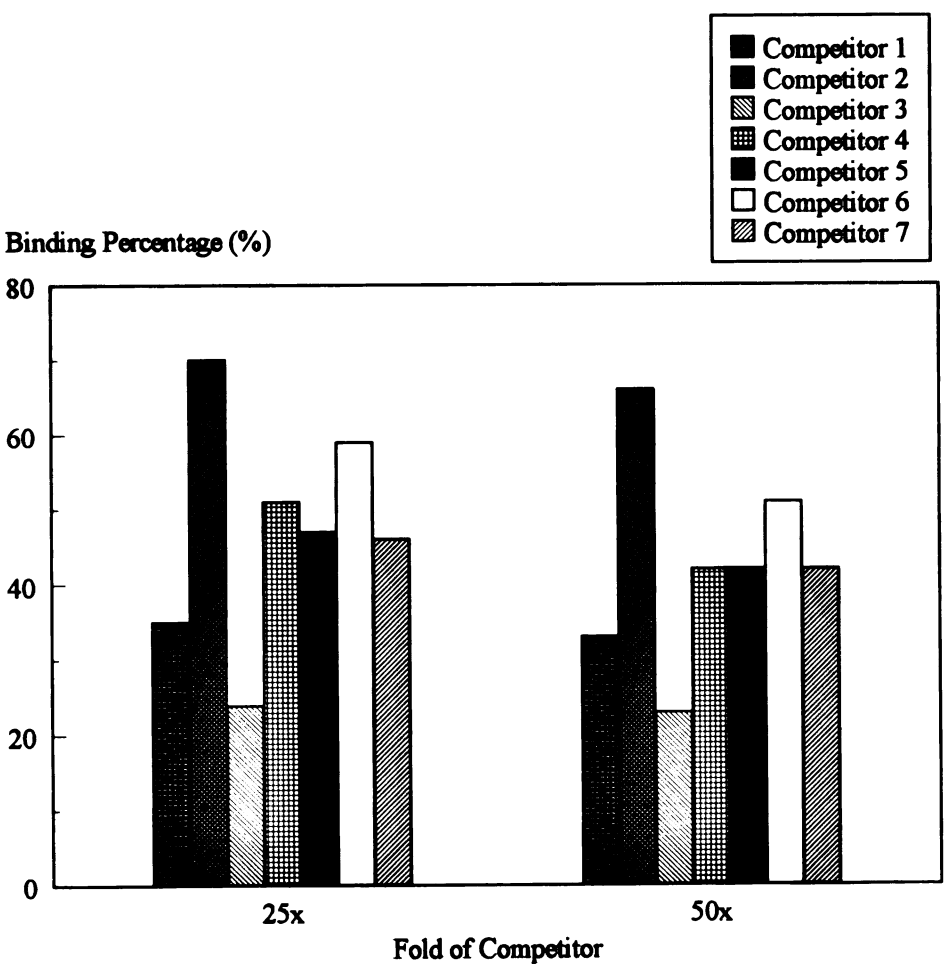
Our previous studies showed that no viral or cellular binding activities were detected in EMSAs with MDV GA-infected extracts using MDV UL9 site I DNA probe (data not shown). Since the sequence differences between MDV UL9 site I DNA and HSV-1 UL9 site I DNA are primarily within the flanking sequences (Fig. 5C), we investigated the effects mutations within the flanking sequence may have on the viral binding activities. To investigate the effects of mutations, five 26-mer oligonucleotides each of which contained point mutations within MDV UL9 site I DNA were synthesized and tested in competitive EMSAs by using HSV-1 UL9 site I DNA probe. The mutant oligonucleotides were designed by mutating wild type MDV UL9 site I DNA to become more HSV-1 UL9 site I DNA-like. Figure 13A shows the positions of mutations. The results of assays are shown in Fig 13B. HSV-1 UL9 site I DNA (competitor 1) gave the predicted reduction in binding. While all competitors had the effects on the binding, competitor 3 containing the

Figure 13. Mutation analysis of MDV UL9 binding site I. Competitive EMSAs were performed with MDV-GA-infected extracts using HSV-1 UL9 site I DNA probe as described in Material and Methods. (A) Sequences of the point mutants. Competitor 1 indicates the sequence of HSV-1 UL9 site I DNA with the 11-bp UL9 recognition sequence indicated by the bracket on top of the sequence. Competitor 2 indicates the sequence of wild type MDV UL9 site I DNA. Competitors 3-7 indicate the sequences of a series of point mutants. The point mutations are indicated by the letters within squares. (B) Graphic display of the phosphor-imager analysis of competitive analyses of the M complexes. Each band was scanned with the phosphor-imager and results were compared (as a percentage) with those obtained without competitor (100%). The bars indicate binding in the presence of indicated amounts of excess competitor expressed as percentage of binding with no competitor.

A.


11-bp element
Competitor 1 GCGAAGCGTTCGCACTTCGTCCCAAT
Competitor 2 CAGCGTTCGCACCGCGAACCAA
Competitor 3 CGTCAGCGTTCGCACTTCGAACCAAT
Competitor 4 CGTCAGCGTTCGCACCGCGTCCCAAT
Competitor 5 CGTCAGCGTTCGCACTTCGTCCCAAT
Competitor 6 CCGAAGCGTTCGCACTTCGTCCCAAT
Competitor 7 CGGAAGCGTTCGCACTTCGTCCCAAT

B.


Competitor 1
Competitor 2
Competitor 3
Competitor 4
Competitor 5
Competitor 6
Competitor 7
Binding Percentage (%)
80
60
40
20
0
25x
50x
Fold of Competitor

perfect HSV-1 11-bp element within the context of MDV UL9 site I DNA had the most profound effect. Although competitors 5, 6 and 7 contained the perfect HSV-1 11-bp element and the flanking sequence was more HSV-1 UL9 site I DNA-like, they had much less competitive abilities than competitor 3 and had competition abilities comparable to competitor 2 and competitor 4 both of which contained the MDV UL9 binding site I. This result suggested that the last two nucleotides within the 11-bp motif of HSV-1 UL9 site I DNA are essential for the binding activity of MDV UL9 protein *in vitro*. This result was consistent with the result of previous section in which the last nucleotide (T) of MDV UL9 binding site II was important for the binding activitiy of MDV UL9 *in vitro*. The results of competitive EMSAs also indicated that mutations changing the flanking sequences to become more HSV-1 UL9 site I DNA-like had a detrimental effect on the MDV OBP binding.

Discussion

Virus-specific OBPs have been identified and characterized extensively in HSV-1 and other systems, including large T antigens of simian virus 40 (SV40) and polyomavirus (Borowiec et al, 1990; Gaudray et al, 1981). In SV40, large T antigen plays an essential role in initiation of viral DNA replication. It is required to initiate the protein-DNA interactions which result in maturation of the replication machinery and subsequent unwinding of the origin. The precise role of HSV-1 OBP in HSV-1 DNA replication has not been established but it has been suggested that it plays a role in initiation of HSV-1 DNA replication similar to the role of SV 40 large T antigen. Less is known about the requirements for MDV DNA replication.

In our previous studies, we determined the sequence of MDV gene homolog to the HSV-1 UL9 gene. Based on predicted amino acid sequences, HSV-1 UL9 and MDV UL9 share 49% identity and 66% similarity while those of VZV gene 51 (VZV UL9 homolog) and MDV UL9 share 46% identity and 63% similarity. MDV UL9 also shares numerous motifs with both HSV-1 UL9 and VZV gene 51. Conserved motifs include several helicase moieties and a leucine zipper in the N-terminus, a C-terminal pseudo-leucine zipper, and a putative helix-turn-helix structure. Our previous data suggested that MDV UL9 may have a function similar to that of HSV-1 UL9.

In this report, we detected a MDV UL9 protein in infected-cell nuclear extracts, with an apparent size consistent with the predicted size for MDV UL9 (95 kDa) Futhermore, a predominant 95 kDa protein was expressed in the *in-vitro* coupled transcription-translation programmed with MDV UL9 expression plasmid. Alignment of HSV-1 UL9,

VZV gene 51 and MDV UL9 protein sequences suggests that MDV UL9 may have biochemical activities similar to HSV-1 UL9, including the origin-binding activity. Electrophoretic mobility gel shift assays (EMSAs) showed that synthetic MDV UL9 not only bound to HSV-1 UL9 binding site I but also to MDV UL9 binding site II. In addition, MDV UL9 binding site II- and HSV-1 UL9 binding site I-binding activities were present in the infected-cell nuclear extracts. This activity generated two complexes in which the faster migrating complex migrating at a rate similar to the fastest migrating complex identified in EMSA with the *in-vitro* expressed MDV UL9. The slower migrating complex probably resulted from inter- and intramolecular interaction between MDV UL9 site II DNA-bound MDV UL9 protein. The EMSAs indicated that MDV UL9 gene encodes an origin-binding protein.

Although viral binding activities could be detected for MDV UL9 binding site II, no viral binding activities were detected on MDV UL9 site I DNA. By using a mutant oligonucleotide mutated from MDV UL9 site II DNA as the competitor, competitive EMSA indicated that nucleotide T in the last position of MDV UL9 binding site II was essential for the viral binding activities of MDV UL9 bindng site II *in vitro*. Elias *et al.* (1990) reported that the 11-bp sequence (CGTTCGCACTT) was required for high-affinity binding of HSV-1 UL9. However, Hazuda *et al.* (1990) refined the UL9 recognition sequence to 10 bp (CGTTCGCACT), suggesting that nucleotide T in the last position of 11-bp motif was not important. Compared to our results, nucleotide T in the last position is essential for the binding. This difference may be because the sequence in the 11-bp element (CGTTCGCACCT) within MDV UL9 site II DNA is different from

that within HSV-1 UL9 site I DNA in one position, or that the spatial arrangement of conserved motifs within C-terminal domain of MDV UL9 is different from that of C-terminal of HSV-1 UL9 (data not shown).

The sequence of 11-bp element (CGTTCGCACCG) within MDV UL9 site I DNA is different from that within HSV-1 UL9 site I DNA (CGTTCGCACTT) in two positions. By using five mutant MDV UL9 site I DNA as competitors, which were designed by mutation of wild type MDV UL9 site I DNA to become more HSV-1 UL9 site I DNA-like, competitive EMSAs indicated that the last two nucleotides (TT) of 11-bp element within HSV-1 UL9 site I DNA was important for the binding activities of MDV UL9 protein *in vitro*.

No binding has yet been demonstrated for HSV-1 UL9 binding site III *in vitro* but HSV-1 UL9 site III is required for viral replication *in vivo*. This is also probably true for MDV as well. Although MDV UL9 was shown not to bind to MDV UL9 binding site I *in vitro*, it is likely to be important for the viral replication *in vivo*. Several explanations can be offered to account for the inabilities to determine the binding on MDV UL9 site I DNA *in vitro*. First, it is possible that binding of the MDV UL9 protein to MDV UL9 binding site I needs the cooperation from the MDV UL9 binding site II-bound MDV UL9 proteins. If this is the case, it is not surprising that MDV UL9 protein can not bind to MDV UL9 site I DNA containing the lone MDV UL9 binding site I in EMSAs. DNase I footprinting on the whole serotype 2 MDV origin should solve the question. Second, it is postulated that MDV UL9 actually binds to MDV UL9 binding site I in an *in vivo* environment but that other cellular or viral factors need to bind to the site in order to

change the overall structure making the site more accessible for MDV UL9 binding. Thus, the binding of MDV UL9 to MDV UL9 binding site I which may occur in *in vivo*, can not be detected under the current conditions used for *in-vitro* DNA-binding studies. Third, MDV UL9 binding site I may provide sites for other factors to act upon as the replicon is formed. Fourth, certain nucleotides within MDV UL9 binding site I may be important for maintaining secondary structure of the origin for the recruitment of replication machinery.

Acknowledgements

We thank Jean Wilms-Robertson, Carolyn Cook, and Ronald Southwick for excellent technical assistance. We also thank Dr. Richard Schwartz for suggestions for electrophoretic mobility gel shift assays.

Summary and Conclusion

In many of the DNA replication systems that have been studied *in vitro*, the first step in initiation is the recognition of a specific DNA sequence at the origin of DNA replication by an origin-binding protein (OBP). The OBP is required to initiate the protein-DNA interactions which result in the formation of mature replication complexes and subsequent destabilization of the origin. Using transient replication assays, seven genes were identified, which are both necessary and sufficient for origin-dependent DNA replication (Wu et al, 1988). HSV-1 UL9 gene, one of these seven essential genes, encodes an origin-specific binding protein. It binds to specific sequence elements within the viral DNA replication origin. The biochemical activities of HSV-1 UL9 protein suggests that it may play a role in initiation of viral DNA replication similar to SV40 large T antigen (Bruckner *et al.*, 1991; Koff *et al.*, 1991; Rabkin and Hanlon, 1991; Fierer and Challberg, 1992; Boehmer *et al.*, 1993; Dodson and Lehman, 1993).

An origin of serotype 2 MDV DNA replication has been functionally identified using transient replication assays (Camp et al, 1991). The serotype 2 MDV replication origin is located in the inverted repeats flanking the unique long region (Camp et al, 1991), suggesting that there are at least two copies of the origin. The origin of serotype 2 MDV DNA replication is located within a 90-bp region. It contains an imperfect palindrome with 30 bp of alternating AT sequence located at the center. The structure and sequence of serotype 2 MDV DNA replication origin is very similar to the HSV-1 ori_L and ori_S, VZV origin, and equine herpes virus type 1 (EHV-1) origin (Camp et al, 1991). In addition, the serotype 2 MDV DNA replication origin contains a 9-bp motif which is located both at the left and right arms of the palindrome sequence and this 9-bp motif is

highly conserved among alphaherpesviruses (Camp *et al.*, 1991). The 9-bp motif is a subset of a 11-bp motif that is recognized by the HSV-1 origin binding protein (UL9). The presence of two copies of the 9-bp motif suggests that there are two potential binding sites for the MDV origin-binding protein. Based on gene colinearity between MDV and other alphaherpesviruses, as well as origin similarities, it seems likely that MDV encodes a UL9 homolog which is critical for initiation of DNA replication.

Conservation of potential UL9 binding sites within the MDV core origin of replication suggests that MDV UL9 may be highly similar to the HSV-1 UL9 protein. To analyze this possibility, western blot analyses of MDV-infected and uninfected cell extracts were performed using a polyclonal anti-HSV-1 UL9 protein antibody. A protein with an apparent molecular size similar to HSV-1 UL9 protein was specifically detected in extracts from CEF infected with MDV, strain Md11 but not detected in mock-infected CEF.

Based on gene colinearity and random sequencing analysis of *BamH*I G fragment of MDV, strain GA genome, it was likely that the major portion of the putative MDV UL9 gene was located within *BamH*I C fragment of MDV, strain GA genome. Terminal sequencing of five internal *EcoR*I subclones and comparison of deduced amino acid sequences with HSV-1 UL9 revealed that one internal *EcoR*I subclone with a 3.1 kb insert contains one end which is very similar to the N-terminal portion of HSV-1 UL9. Using this end as a starting point, the whole putative MDV UL9 gene was identified. The MDV UL9 gene is located within *BamH*I G and C fragment of MDV, strain GA genome. It is transcribed from right to left relative to MDV genome. The orientation and location are

similar to HSV-1 UL9. The upstream region of MDV UL9 gene contains several transcription factor binding motifs, including CAAT boxes, GC boxes and four putative TATA boxes. The MDV UL9 gene encodes an 841 amino acid polypeptide which is similar to HSV-1 UL9 (851 amino acids) and VZV gene 51 (835 amino acids).

The predicted amino acid sequences of HSV-1 and MDV UL9 share 49% identity and 66% similarity while those of VZV gene 51 and MDV UL9 share 46% identity and 63% similarity. Alignment of MDV UL9, HSV-1 UL9, and VZV gene 51 protein predicted amino acid sequences revealed that MDV UL9 is highly similar to HSV-1 UL9 and VZV gene 51. Based on the predicted amino acid sequences, many features which are conserved within HSV-1 UL9 and VZV gene 51 can be found within MDV UL9. For example, all putative helicase motifs found within the N-terminal portion of HSV-1 and VZV UL9 proteins are also present in MDV UL9. Furthermore, the spatial arrangement of these motifs is conserved between the three origin binding proteins. It has been shown that the conserved helicase motifs are important for the helicase activity of HSV-1 UL9 (Martinez *et al.*, 1992). Conservation of these motifs in MDV UL9 suggests that, like HSV-1 UL9, MDV UL9 possesses an intrinsic helicase activity within the N-terminal portion. Also within the N-terminal domain of MDV UL9, a leucine zipper region can be found, which is essential for dimerization and cooperativity of HSV-1 UL9 (Elias *et al.*, 1992; Hazuda *et al.*,1992)

The C-terminal domain of MDV UL9 contains two structural motifs in common with the C-terminal domains of HSV-1 UL9 and VZV gene 51, a pseudo-leucine zipper (not clearly defined in VZV UL9) and a helix-turn-helix motif. The pseudo-leucine zipper

was shown to be important for DNA-binding activity of HSV-1 UL9 (Deb and Deb, 1991; Arbuckle and Stow, 1993; Martin *et al.*, 1994). However, importance of the helix-turn-helix motif for the DNA-binding activity remains to be elucidated. In addition to these two recognizable structural motifs, a highly conserved region is found within the very C-terminal end of MDV UL9. This sequence, termed the VZV homology region, is highly similar to corresponding sequences in HSV-1 and VZV UL9 proteins. The VZV homology region has been shown to be critical in maintaining the DNA-binding activity of HSV-1 UL9 (Deb and Deb, 1991; Arbuckle and Stow, 1993; Martin *et al.*, 1994). In addition to conservation of known structural/functional motifs, there are three highly conserved regions in MDV UL9, HSV-1 UL9, and VZV gene 51 which do not show clear homology to previously identified functional domains. The high similarity between deduced amino acid sequences of MDV UL9, HSV-1 UL9 and VZV gene 51 suggests that MDV UL9 gene encodes an origin-binding protein which possesses the similar biochemical activities to HSV-1 UL9 and may play a role in initiation of MDV viral DNA replication.

To detect the MDV UL9 transcript, four DNA probes isolated from the MDV UL9 gene were used in northern blot analysis. With the *EcoR*I-*Ava*I probe from the N-terminal portion of MDV UL9, 4.4 and 2.1 kb transcripts were detected whereas with the *EcoR*I subfragment from *BamH*I C which contains the partial N-terminal domain and the upstream region, 4.4, 2.6 and 2,1 kb transcripts were detected. With the *Bgl*II-*BamH*I probe from the C-terminal domain, 4.4 and 2.0 kb transcripts were detected whereas with the *EcoR*I-*BamH*I probe which is approximately centrally located in *BamH*I C, 4.4 kb

transcript was detected. Based on gene colinearity, it is suggested that the 4.4 kb transcript may represent the primary MDV UL9 transcript and the 2.1 kb transcript may represent the MDV UL10 homolog transcript.

In order to detect the MDV UL9 protein in the MDV-infected cells, western blot analyses were performed with a polyclonal antibody against the MDV UL9 protein raised from MDV UL9-GST fusion protein. A protein with an apparent molecular size corresponding to the predicted size (95 kd) was detected in the infected nuclear extracts but not in uninfected nuclear extracts.

To examine the binding activity of MDV UL9 independent of other viral protein, the MDV UL9 gene was amplified by PCR from the total cellular DNA isolated from MDV, strain GA-infected cells. The resulting product was cloned into the pBKCMV vector. The MDV UL9 protein was expressed with the coupled transcription-translation system (Promega). A 95 kd protein could be expressed in the coupled transcription-translation lysate and was immunoprecipitated by the polyclonal antibody against the MDV UL9 protein. The *in vitro* expressed MDV UL9 protein was used in electrophoretic mobility gel shift assays (EMSA) using HSV-1 UL9 site I DNA probe. A MDV UL9-specific complex (M) could be detected in EMSAs with *in vitro* expressed MDV UL9 but not detected in EMSAs with the control lysates. Consistent with this result, a similar virus-specific complex (M) was also detected in EMSAs with MDV GA or Md11-infected nuclear extracts. These results demonstrated that MDV UL9 protein can bind to HSV-1 UL9 site I DNA.

The serotype 2 MDV origin contains two potential OBP binding sites. To detect if MDV UL9 can bind to the OBP binding sites within the serotype 2 MDV origin, EMSAs were performed with *in vitro* expressed MDV UL9 protein or MDV-infected nuclear extracts using MDV UL9 site II DNA probe. The results of EMSAs showed that *in vitro* expressed MDV UL9 protein can form a complex (M) with MDV UL9 site II DNA migrating at a rate similar to a complex resulting from MDV GA-infected nuclear extracts. Besides the complex M, high order complexes were also detected in EMSAs with *in-vitro* expressed MDV UL9 protein or MDV GA-infected nuclear extracts. Using the entire serotype 2 MDV origin as a probe, two virus-specific complexes could be detected in EMSAs with MDV GA-infected nuclear extracts. The results of EMSAs demonstrated that MDV UL9 can bind to the serotype 2 MDV origin.

Although MDV UL9 was shown to bind to one of the potential OBP binding sites (MDV UL9 binding site II) within the serotype 2 MDV origin, MDV UL9 was not detected to bind to the MDV UL9 binding site I. The sequence of MDV UL9 binding site I is different from that of MDV UL9 binding site II in one position. The last nucleotide of MDV UL9 binding site I is a G while that of MDV UL9 binding site II is a T. To examine the importance of the last nucleotide in MDV UL9 binding site II, a mutant MDV UL9 site II DNA was synthesized and tested in competitive EMSAs. The 11-bp motif within the mutant MDV UL9 site II DNA has the same sequence as MDV UL9 binding site I. The results of competitive EMSAs showed that the last nucleotide (T) of MDV UL9 binding site II is essential for the binding of MDV UL9 to MDV UL9 site II DNA *in vitro* and the flanking sequence may have a minor effect on the binding activities.

Our previous studies showed that no viral or cellular binding activities were detected in EMSAs with MDV GA-infected extracts using MDV UL9 site I DNA probe (data not shown). Since the sequence differences between MDV UL9 site I DNA and HSV-1 UL9 site I DNA are primarily within the flanking sequences, the effects mutations within the flanking sequence may have on the viral binding activities were investigated. To investigate the effects of mutations, five 26-mer oligonucleotides each of which contained point mutations within MDV UL9 site I DNA were synthesized and tested in competitive EMSA using HSV-1 UL9 site I DNA probe. The mutant oligonucleotides were designed by mutating wild type MDV UL9 site I DNA to become more HSV-1 UL9 site I DNA-like. The results of competitive EMSAs demonstrated that the last two nucleotides within the 11-bp motifs of HSV-1 UL9 site I DNA are essential for the binding activity of MDV UL9 protein *in vitro*.

Future Research Directions

Less is known about the mechanisms of MDV DNA replication compared to HSV-1. Identification of MDV UL9 gene adds important information to studies of MDV DNA replication, particularly regarding the initiation of MDV DNA replication. Alignment of the predicted amino acid sequences suggests that MDV UL9 may have the similar biochemical activities to HSV-1 UL9. However, the real functions of MDV UL9 need to be investigated. Many functions need to be examined and future research should focus on structural and functional analysis.

Structure-function studies of HSV-1 UL9 have been intensive. The N-terminal domain of HSV-1 UL9 encodes the helicase activity and responsible for cooperativity. Within the N-terminal domain of HSV-1 UL9, the conserved helicase motifs have been shown to be essential for the helicase activity as well as for viral replication and the leucine zipper region is essential for the cooperativity. MDV UL9 also contains the same conserved motifs within the N-terminal domain. The importance of helicase conserved motifs within the N-terminal domain of MDV UL9 could be investigated by point mutations or deletion of each helicase conserved motif. The wild type or mutant MDV UL9 protein could be purified from the MDV UL9-expressing recombinant baculovirus-infected insect cells. Purified wild type or mutant MDV UL9 proteins could be tested for *in vitro* helicase assays.

Although MDV UL9 protein has been shown to bind to one of two OBP binding sites, it was not detected to bind MDV UL9 site I DNA containing the lone MDV UL9 binding site I located on the left arm of palindrome sequence. It is possible that binding of

the MDV UL9 protein to MDV UL9 binding site I requires the cooperation from MDV UL9 proteins bound to MDV UL9 binding site II. This cooperativity could be examined by DNase I footprinting of the serotype 2 MDV origin. If MDV UL9 protein did cooperatively bind to the two OBP binding sites, the importance of leucine zipper region within the N-terminal portion of MDV UL9 for cooperativity should be investigated by insertion mutation of the leucine zipper region. The cooperativity could be addressed by DNase I footprinting with wild type or mutant MDV UL9 protein purified from recombinant baculovirus-infected insect cells.

The C-terminal domain of HSV-1 UL9 encodes the DNA-binding domain. A pseudoleucine zipper region and a VZV homolog region have been shown to be important for the DNA binding activity whereas the importance of helix-turn-helix motif is not clear. The C-terminal domain of MDV UL9 protein contains all conserved motifs except that the spatial arrangements of leucine zipper and helix-turn-helix motif are different. The boundaries of C-terminal domain should be determined by EMSAs with *in vitro* coupled transcription-translation expressed wild type or deletion mutant MDV UL9 proteins. The importance of leucine zipper could be addressed by insertion mutation of wild type DNA-binding domain using EMSAs while the VZV homolog region can be examined by the deletion.

In addition to the conserved motifs mentioned above, three small regions are also conserved within HSV-1 UL9 and MDV UL9. The conserved regions II and III are located within the N-terminal domain while the conserved region I is located within the C-terminal domain. The importance of these three regions could be investigated by the

deletion mutation. Conserved region II and III will be tested for cooperativity and conserved region III will be tested for DNA binding activity.

Studies on the structure-function of MDV UL9 will give insight to possible mechanisms for initiation of MDV DNA replication and may help elucidate mechanisms in other related alphaherpesviruses.

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