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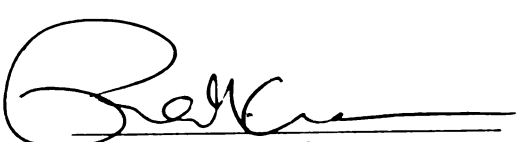

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IDENTIFICATION OF A MAREK'S DISEASE VIRUS GENE HOMOLOGOUS TO ICP27 OF HERPES SIMPLEX VIRUS TYPE 1, AND INVESTIGATION OF MDV ICP27 GENE REGULATORY FUNCTIONS

By

Delin Ren

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ABSTRACT

IDENTIFICATION OF A MAREK'S DISEASE VIRUS GENE HOMOLOGOUS TO ICP27 OF HERPES SIMPLEX VIRUS TYPE 1, AND INVESTIGATION OF MDV ICP27 GENE REGULATORY FUNCTIONS

By

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Marek's disease virus (MDV) gene expression, like other α-herpesviruses, is regulated in a cascade fashion with gene expression classified into immediate-early, early, and late groups. Compared to other α-herpesviruses, such as herpes simplex virus type 1 (HSV-1), MDV immediate-early genes have not been as extensive studied. HSV-1 ICP27, an immediate-early protein, acts as a multifunctional factor and is highly conserved in the α-, β-, and γ-herpesvirus subfamilies. In these studies, we identified an MDV immediate-early protein, MDV ICP27, which is a homolog to HSV-1 ICP27. The MDV ICP27 gene is located within the *Eco*RI-B fragment of the MDV genome and encodes 473 amino acids. The predicted amino acid sequence of MDV ICP27 suggests this protein shares structural domains highly conserved to HSV-1 ICP27. Detection of MDV ICP27 gene transcripts in MDV infected cells treated with cycloheximide suggested that MDV ICP27 gene is transcribed as an immediate-early gene. Bacterial *trp*E- and GST-fusion proteins of MDV ICP27 were expressed for production of polyclonal antisera. Two specific polypeptides of 55 kDa and 52 kDa were detected in MDV serotype 1 infected cells using anti GST-ICP27 antiserum. MDV ICP27 is a phosphoprotein and is predominantly located in the nuclei of CEF cells infected with

serotype-1 MDV or with a fowlpox virus recombinant expressing MDV ICP27. Transient expression assays indicated that MDV ICP27 possesses both intrinsic trans-activation and trans-repression activities. MDV ICP27 is able to transactivate an MDV pp14/pp38 bi-directional promoter. In contrast, MDV ICP27 strongly represses the MDV thymidine kinase (TK) early gene promoter. In addition, a heterologous RSV-LTR is also stimulated by MDV ICP27. By deletion mutant analysis, we further demonstrated that amino acids 207 to 378 are critical for MDV ICP27-mediated transrepression activity. Substitution of 3' RNA processing signals in the RSV-LTR constructs does not obviously affect MDV ICP27-mediated transactivation activity. In contrast, the MDV TK promoter shows a negative response to MDV ICP27 when the SV40 small T 3'splicing site and the early poly(A) signal in the TK promoter construct are replaced with the MDV ICP27 gene poly(A) signals. These results suggest that MDV ICP27-mediated transactivation and transrepression activities may be mediated through different transcriptional mechanisms. In contrast to HSV-1 ICP27, MDV IVCP27 does not display any co-operative activity with MDV ICP4.

To my wife, Chongyang Huang,

for her love, support, and patience,

and to my great kids,

Nan-Nan Ren and Charley Huang Ren.

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LIST OF ABBREVIATIONS

AGP	Agar gel precipitation
BMLF1	(EBV) *Bam*HI M leftward reading frame 1
BRLF1	(EBV) *Bam*HI R leftward reading frame 1
BZLF1	(EBV) B*am*HI Z leftward reading frame 1
bp	base pairs
CAT	chloramphenicol transferase
cDNA	complementary DNA
CEF	chicken embryo fibroblast
C/EBP	CCAAT/enhancer binding protein
CHX	cycloheximide
CIP	calf intestinal phosphotase
CMV	cytomegaloviurs
HCMV	human cytomegaloviurs
CsCl	cesium chloride
DEF	duck embryo fibroblast
DR	direct repeat
E	early
EBV	Epstein-Barr virus
EBNA-1	EBV nuclear antigen-1
EHV	equine herpes virus

EPA	(SV40) early polyadenylation signal
FFE	feather follicle epithelium
FITC	fluorescein-5'isothiocyanate
FPV	fowlpox virus
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
gK	glycoprotein K
GST	glutathione-S-transferase
HCF	host cell factor
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HSV-1	herpes simplex virus type 1
HVT	herpesvirus of turkeys
ICP0	(HSV) infected cell protein No.1
ICP4	(HSV) infected cell protein No.4
ICP22	(HSV) infected cell protein No.22
ICP27	(HSV) infected cell protein No.27
ICP47	(HSV) infected cell protein No.47
IE	immediate-early
IFA	indirect immunofluorescence assay
IgG	immunoglobulin G

IR_L	internal repeat long
IR_S	internal repeat short
kb	kilobase
kDa	kilodalton
IUdR	5'-iodo-2'deoxyuridine
L	late
LAT	latency-associated transcripts
LT_R	long terminal repeat
MD	Marek's disease
MDV	Marek's disease virus
mMDV	mild Marek's disease virus
meq	Marek's *Eco*RI Q
Mta	(EBV) M transactivator
NC	nitrocellulose membrane
NLS	nuclear localization signal
NuLS	nucleolar localization signal
Oct-1	octamer binding factor-1
ORF	open reading frame
ORF4	(VZV) open reading frame 4
OTF-2	octamer binding factor-2
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
PI	post inoculation
RE	restriction enzyme
Rta	(EBV) R transactivator
SDS	sodium dodecyl sulfate
TBP	TATA-binding protein
TFIIB	transcription factor IIB
TFIID	transcription factor IID
TK	thymidine kinase
TR_L	terminal repeat long
TR_S	terminal repeat short
ts	temperature-sensitive
U_L	unique long
Us	unique short
VP16	(HSV) virion protein No.16
vMDV	virulent Marek's disease virus
vvMDV	very virulent Marek's disease virus
VZV	varicella-zoster virus
Zta	(EBV) Z transactivator

Chapter I

Literature Review

Part I. Marek's disease and Marek's disease virus

1. History

Marek's disease virus (MDV) is a cell-associated avian herpesvirus that causes Marek's disease (MD) in chickens, resulting in T-cell lymphoma and mononuclear nerve demyelination (Churchill and Biggs, 1967; Nazerian *et al.,* 1968; reviewed by Calnek, 1985). MD was first reported by Joseph Marek, a Hungarian veterinarian in 1907 (Payne, 1985), but it was not until 1967, that a herpesvirus was identified as the etiological agent. At the same time, MDV was successfully propagated in tissue culture (Churchill and Biggs, 1967; Solomon *et al.*, 1968; Nazerian *et al.*, 1968).

Prior to use of vaccines, MD constituted a serious economic threat to the worldwide poultry industry because of heavy annual losses, due to death and condemnation. MD has been effectively controlled by vaccination since early 1970's with attenuated MDV serotype 1 derivatives or with an antigenically-related but apathogenic herpesvirus of turkeys (HVT). Thus, economic losses due to MD are no longer as serious as prior to vaccine development (reviewed by Witter, 1985). Vaccination, however, is not completed effective against infection by certain strains of very virulent MDV (vvMDV). Thus, development of a new generation vaccines using molecular biology approaches has been promoted (Yanagida *et al.,* 1992; Nazerian *et al.*, 1992).

MDV is one of several related herpesviruses that can induce neoplastic diseases in their natural hosts, other such related herpesvirus is Epstein-Barr virus (EBV). MD vaccines provide the first example of controlling a natural neoplastic disease by vaccination. MD, therefore, provides an excellent animal model for understanding the

oncogenicity of other herpesviruses, such as EBV, and may significantly contribute to comparative medical science.

2. Biology of Marek's disease virus

A. Virion structure

MDV virions consist of 162 hollow-centered capsomeres with icosahedral symmetry (Schat, 1985). In MDV-infected tissue culture, most virions are hexagonal naked particles or nucleocapsids, 85-100 nm in diameter, and are usually found in nuclei but occasionally in the cytoplasm of infected cells. Enveloped MDV particles, 150-160 nm in diameter, are rarely seen and are principally associated only with nuclear membranes (Nazerian *et al.*, 1971). Large numbers of cytoplasmic enveloped virions (273-400 in diameter) are only observed in feather follicle epithelium (FFE) of infected chickens.

B. Serotypes of MDV

Based on agar gel precipitation (AGP) and immunofluorescence assay (IFA), MDV has been classified into three serotypes (Bulow and Biggs, 1975). This serotype classification has been confirmed through use of monoclonal antibodies (Lee *et al.*, 1983). Oncogenic MDV is only associated with serotype 1. However, there is a wide variation in pathologic potential within strains of serotype 1. Therefore, MDV serotype 1 is further classified as mild MDV (mMDV), virulent MDV (vMDV), and very virulent MDV (vvMDV), according to pathogenesis and oncogenicity. Serotype 2 MDVs are naturally occurring non-oncogenic viruses. An apathogenic herpesvirus of turkey (HVT) is classified as serotype 3. Serial passage *in vitro* of vvMDV or vMDV will lead to

attenuation with concurrent loss of oncogenicity and pathogenesis. HVT, Serotype 2, and attenuated serotype 1 MDVs are widely used as vaccines against to MDV (Witter, 1985).

C. MDV isolation and cultivation

MDV can be isolated from chickens 1 or 2 days post-inoculation or 5 days after contact exposure and throughout the life span of infected chickens. Because MDV is highly cell-associated, intact viable cells are usually used for inoculation. Primary isolates of MDV can be propagated on chicken embryo fibroblast (CEF), duck embryo fibroblast (DEF), and chicken embryo kidney (CEK) cells. DEF and kidney cell cultures are commonly used for MDV serotype 1 infection, whereas CEF cells are suitable for propagating serotype 2 and serotype 3 (Calnek and Witter, 1991). Recently, a chicken embryo fibroblast cell line, OU2, was used for MDV infection. MDV OU2 cell lines infected with MDV serotype 1 strain MD11 were established (Abujoub and Coussens, 1995). MDV OU2 cell lines are similar to certain lymphoblastoid cell lines, and are capable of transferring MDV infection to primary CEF monolayer cultures. However, MDV OU2 cell lines are also capable of supporting a cytolytic infection of MDV (Abujoub and Coussens, 1995).

3. Pathology and immunity of MDV

A. MDV infection

MDV infections consist of productive infection (also known as lytic infection), latent infection, and transformation. Productive infection can be further divided into fully

productive and semi-productive infection. Fully productive infection, resulting in development of large numbers of enveloped and fully infectious virions, is only observed in feather follicle epithelium (FFE) of infected chickens. Semi-productive infection (or restrictive infection) mostly occurs in all other tissues and in cell culture (Calnek *et al.*, 1970). In semi-productive infection, most virions are not enveloped. During semi-productive infection, virions are not released in an infectious form, instead of virus spreads from cell to cell. Both latent infection and transformation are not productive and only a few genes are expressed in latent and/or transformation infection.

MD is horizontally transmitted by direct or indirect contact with infected birds. The infection period, clinical signs, and gross lesions of MDV can be induced experimentally in chickens. Chicks inoculated at 1 day of age, start secreting virus at 2 wk post-inoculation (PI). Clinical signs and gross lesions appear about 3-4 wk PI. The major clinical signs and gross lesions of MD can be described as either classical or acute MD. Classical MD predominantly affects peripheral nerves, but spinal roots or root ganglia are sometimes involved. Asymmetric progressive paresis, characterized by drooping wings is commonly observed in both natural or experimental diseases. A particular characteristic of infected birds is one leg stretched forward and the other backward (Biggs, 1967; Calnek and Witter, 1991). Lymphoid tumors can be seen in a variety of organs but frequently, only one or a few organs are grossly affected in classical MD. In contrast, multiple lymphoid tumors are observed more frequently in acute MD, affected tissues including the gonads, lungs, kidneys, liver, heart, spleen, bursa and skin.

B. Pathogenesis of MDV

MDV gains entry via the respiratory tract where it is most likely internalized by phagocytes. B cells appear to be the primary target cells, although some T cells may also be involved (Shek et al., 1983; Calnek, 1985). Primary degenerative changes are the major features in this early stage of lytic infection. At about 6-7 days PI, infection switches to a latent infection coincident with the development of immune responses. T cell-mediated immune responses play a central role in this switch, resulting in T cell activation (Payne, 1985; Calnek, 1985). $CD4^+$ T helper cells are the principal targets at this stage, although a few B cells may still be involved (Schat *et al.*, 1991). Latent infection is persistent and can last for the lifetime of the birds. Following latent infection, susceptible birds (but not genetically resistant birds) can develop a second lytic infection. This phase of infection usually is coincident with permanent immunosuppression. Lymphoproliferation and development of T cell tumors are commonly seen at this stage (Buscaglia *et al.*, 1988). Massive lymphomas can be observed in visceral organs, skin, muscle, and neural tissues. Lymphoproliferative changes are only observed in birds infected with virulent serotype 1 MDV.

The composition of MDV-induced lymphomas is complex, consisting of a mixture of neoplastic, inflammatory, and immune cells. Both T and B cells are present, but T cells predominate (Hudson and Payne, 1973). Neoplastic cells contain 5-15 copies of viral DNA and can be grown as continuous lymphoblastoid cell lines *in vitro* (Ross, 1985). It is believed that MDV transformed cell lines are latently infected and provide a useful system for studying both MDV latency and transformation.

MDV transformed cell lines can be distinguished as either producer or non-producer cell lines. Producer cell lines are those cells can be rescued after *in vitro* co-cultivation or

following inoculation into susceptible chickens. In non-producer cell lines, viral antigens are not detectable and virus cannot be rescued by co-cultivation (Schat *et al.*, 1989; Calnek and Witter, 1991).

Both humoral and cell-mediated immunity are involved in MDV infection. However, cell-mediated immune responses are critical for immunity. Functional T cells are required for both genetic resistance and vaccinal immunity (Sharma *et al.*, 1975).

4. Molecular biology of MDV

A. Genome structure and physical map of MDV

The genome of MDV is a linear double-stranded DNA molecule with a density of 1.705 g/cm^3 in CsCl, and a base composition of 46% guanine plus cytosine. The molecular weight of MDV DNA is 108-120 x 10^6 daltons, equivalent to 180 kb (Lee *et al.*, 1971; Cebrian *et al.*, 1982; Fukuchi *et al.*, 1984; Hirai *et al.*, 1979).

The structure of MDV DNA consists of unique long and unique short regions (U_L, U_S), flanked by inverted repeat regions (IR_L/TR_L, IR_S/TR_S, respectively) (Figure 1.1) (Cebrian *et al.*, 1982; Fukuchi *et al.*, 1984). MDV was originally classified as a γ-herpesvirus because its lymphotropism is similar to that of Epstein-Barr virus (EBV), a prototype of the γ-herpesvirus family (Roizman *et al.*, 1981). However, the genome structure and gene arrangement of MDV are more similar to that of α-herpesviruses, such as herpes simplex virus type 1 (HSV-1), and varicella-zoster virus (VZV) (Buckmaster *et al.*, 1988; Roizman *et al.*, 1992; Karlin *et al.*, 1994). Furthermore, MDV genes, particularly those identified in U_L and U_S regions, display significant homology to those

of HSV-1 and VZV (Zelnik *et al.*, 1993; Ren *et al.*, 1994; Brunovskis and Velicer, 1995; Lee *et al.*, 1995).

In addition to the inverted repeats, several direct repeats (DR) have been identified in MDV genomes (Hirai, 1988). These DR sequences consist of more than 100 bp repeats and are mostly located within the internal or terminal repeat regions (Hirai, 1988). DR1 is a tandem direct repeat of 132 bp repeat units located within TR_L and IR_L. Serial *in vitro* passage of virulent MDV in primary CEF cells results in a loss of MDV oncogenicity. The loss of oncogenicity has been found to correlated with an expansion of the 132 bp DR1 (Fukuchi *et al.*, 1985; Maotani *et al.*, 1986). Recently, a 1.8 kb transcript family and several gene products have been identified within this region (Bradley *et al.*, 1989; Cui *et al.*, 1991; Chen and Velicer, 1992; Hong and Coussens, 1994; Peng *et al.*, 1994).

The development of a *Bam*HI restriction enzyme (RE) map and associated clones was central to studies of MDV (Figure 1.1) (Fukuchi *et al.*, 1985). The MDV *Bam*HI map has been the basis for most gene identification and localization studies. RE maps of all three serotypes of MDV have been constructed and are also useful for comparative studies between MDV serotype genomes (Igarashi *et al.*, 1987; Ono *et al.*, 1992).

B. Genome sequences and gene identification

MDV sequencing and gene identification started in the late 1980's. The first gene, encoding **A** antigen (homolog to herpes simplex virus gC), was identified by Isfort *et al.* in 1987, and sequenced by Coussens and Velicer in 1988. Using random sequence

analysis, 35 MDV and 24 HVT genes have been compared to both γ- and α-herpesviruses (Buckmaster *et al.*, 1988). The results indicated that MDV and HVT bear greater genetic similarity to α- than to γ- herpesviruses. In the following few years, MDV DNA sequencing and gene identification has progressed due to advances in molecular biological techniques. The unique short regions of MDV and HVT have been completely sequenced (Brunovskis and Velicer, 1995; Zelnik *et al.*, 1993). Although the U_L regions of MDV and HVT have not yet been completely sequenced, many α-herpesvirus gene homologs have been identified (reviewed by Velicer and Brunovskis, 1992; Lee *et al.*, 1995). These results have provided the basis for the reclassification of MDV to the α-herpesvirus family (Roizman *et al.*, 1992).

C. MDV gene expression

Like other herpesviruses, MDV gene expression is temporally regulated in a cascade fashion (Maray *et al.*, 1988; Schat *et al.*, 1989). Generally, herpesvirus genes have been divided into three kinetic families: immediate-early gene (IE or α), early (E or β), and late (L or γ) genes, based on the requirement for viral protein synthesis and/or viral DNA replication (Honess and Roizman, 1974).

a. MDV IE gene expression

IE genes are expressed immediately upon infection with no requirement for *de novo* protein synthesis. Compared with other herpesviruses, studies on gene expression and regulation of MDV are far behind. This is mostly due to the highly cell-associated

properties of MDV. Cycloheximide, a metabolic inhibitor, can block protein synthesis and accumulate immediate-early transcripts in virus-infected cells. By cycloheximide treatment, numerous IE transcripts have been detected in MDV lytically infected cells and lymphoblastoid cell lines (Maray *et al.*,1988; Schat *et al.*, 1989). However, all these reports are based only on Northern hybridization analysis, without exact gene and gene product identification.

Recently, several MDV IE genes have been reported and three MDV IE genes are homologous to HSV-1 ICP4, ICP27 and ICP22 (Anderson *et al.*, 1992; Ren *et al.*, 1994; Hong and Coussens, 1994; Brunovskis and Velicer, 1995). Anderson *et al.* (1992) reported an ICP4 gene homolog that is the major transcriptional activator in HSV-1 infection (Roizman and Sears, 1995). MDV ICP4 is located in *Bam*HI-A fragment within the MDV inverted repeats. MDV ICP4 is 4,245-nucleotide long and encodes 1415 amino acids. Based on sequence analysis, MDV ICP4 predicted polypeptide sequence can be divided into five regions in which region 2 and region 4 have higher amino acid conservation than others. A very highly conserved serine-rich domain is also found in region 1 (Anderson *et al.*, 1992). Several potential *cis*-acting elements are observed in the MDV ICP4 control region or within the MDV ICP4 coding region, including an ICP4 consensus biding site, Oct-1 site, and TAATGARAT motif that is a consensus element recognized by VP16, a virion transactivator (Anderson *et al.*, 1992; Roizman and Sears, 1995).

Another essential α-herpesvirus IE gene, ICP27, has been mapped to the *Eco*RI-B fragment of MDV DNA (Ren *et al.*, 1994). MDV ICP27 is a 473-amino-acid polypeptide

and shows 26% amino acid identity with HSV-1 ICP27. Interestingly, MDV ICP27, by comparison to other α-herpesvirus homologs, shares a higher amino acid conservation with a cysteine-rich domain and a potential zinc-binding motif within the C-terminal region (Figure 1.2A) (Ren *et al.*, 1994). A basic and arginine-rich domain has also been found between amino acids 150 to 200. Using MDV ICP27 specific antiserum, two polypeptides with molecular weights 55 and 52 kDa can be detected in MDV serotype 1 infected cells (Ren *et al.*, 1996). Functions of both MDV ICP4 and MDV ICP27 are less well understood and will be discussed in more detail in **Part II.**

Recently, a 1.6 kb IE transcript has been mapped to the MDV *Bam*HI-I2 region (Hong and Coussens, 1994). By cDNA cloning and sequence analysis, two cDNAs (C1 and C2) have been identified as spliced transcripts from this 1.6 kb transcript. C1 (1.4 kb) and C2 (1.35 kb) share identical splice acceptors and 3' ends, but differ in their 5' end and in the splicing donor site (Hong and Coussens,1994). Both C1 and C2 encode an identical highly phosphorylated protein, pp14, which is predominantly found in the cytoplasmic. Interestingly, MDV pp14 can be detected not only in MDV serotype 1 infected cells, but also in an MDV transformed lymphoma cell line, MSB-1 (Hong *et al.*, 1995). However, function of MDV pp14 is not clear. MDV ICP22 homolog is mapped to MDV U_S region, but no more information is available at this time (Brunovskis and Velicer, 1995).

b. MDV early gene expression

Early genes are expressed after onset of immediate-early gene expression. Early gene

expression is regulated by IE gene products (Roizman and Sears, 1995). Most early gene products are involved in nucleotide precursor metabolism and viral DNA synthesis. Therefore, in the presence of drugs that block viral DNA synthesis, such as phosphonoacetic acid (PAA), early gene expression is enhanced rather than reduced. Thymidine kinase (TK) is a typical early gene and has been extensively studied in the α-herpesvirus family (Roizman and Sears, 1995). A TK gene homolog has been reported in MDV (Scott *et al.*, 1989). Other α-herpesvirus early gene homologs such as DNA polymerase and ribonucleotide reductase have also been identified (Sui *et al.*, 1995; Lee *et al.*, 1995). Importantly, an MDV unique phosphoprotein, termed pp38, has been reported by several labs (Silva and Lee, 1984; Cui *et al.*,1991; Chen and Velicer 1992). Expression of MDV pp38 is relatively insensitive to PAA, indicating that pp38 may belong to the early gene family (Chen and Velicer, 1992). Initially, pp38 expression was thought to be limited to serotype 1 MDV specific and was abundantly expressed in an MDV transformed lymphoid cell line, MSB-1. These observations promoted several labs to investigate the relationship between pp38 and MDV oncogenicity (Cui *et al.*, 1991; Chen and Velicer 1992). More recently, however, pp38 homologs have also been found in both MDV serotype 2 and HVT (Ono, *et al.*, 1994; Smith *et al.*, 1995). Interestingly, both pp38 and pp14 genes share a common control region that has been defined as a bi-directional promoter (Chen and Velicer, 1991; Cui *et al.*, 1991; Hong and Coussens, 1994). There are several *cis*-acting elements including two TATA boxes, two Sp1 sites, two CAAT sites and one octamer motif within this control region (Cui *et al.*, 1991).

c. MDV late gene expression

Late (or γ) genes comprise the largest kinetic class of genes in most herpesviruses. Most late gene products are structural proteins for virion capsid, tegument, attachment, cell fusion, and envelope. A key feature of late gene transcription is the requirement for viral DNA replication. Based on dependence for viral DNA replication, late genes can be further divided into γ1 and γ2. Transcription of γ1 genes occurs prior to initiation of viral DNA synthesis and does not depend stringently on viral DNA replication. As viral DNA synthesis begins, γ1 genes are expressed abundantly and γ2 gene expression begins. Protein synthesis of γ1 and γ2 continues throughout the remaining replication cycle (Wagner, 1991; Roizman and Sears, 1995). Glycoproteins are a major component of this kinetic class and have been extensively studied. Most of the glycoprotein gene homologs have been identified in MDV, including gB, gC, gD, gE, gH, gI, gK, and gL (Ross *et al.*, 1989; Isfort *et al.*, 1987; Ren *et al.*, 1994, Yoshida *et al.*, 1994; Brunovskis and Velicer 1995). Two of these glycoproteins, gB and gC, have been extensively studied in MDV because of their unique biological properties. The MDV B-antigen, a major immunogen for inducing neutralizing antibodies, is a complex of three glycoproteins consisting of gp100, gp60, and gp49. MDV B-antigen has been confirmed as a HSV-1 gB homolog (Ross *et al.*, 1989; Chen and Velicer, 1992; Yanagida *et al.*, 1992). Expression of MDV gB gene by fowlpox virus and baculovirus recombinants followed by inoculation into genetic susceptible birds suggest that MDV gB is a valuable candidate for recombinant vaccine (Yanagida *et al.*, 1992; Nazerian *et al.*, 1992; Niikura *et al.*, 1992). MDV gC is another interesting glycoprotein and was originally termed

MDV A-antigen (Isfort *et al.*, 1987; Coussens *et al.*, 1988). MDV gC is a secreted protein and can be readily detected in the supernatant of infected cell cultures. However, expression of MDV gC is significantly reduced in attenuated MDV strains (Bulow and Biggs 1975). Compared with low-passage MDV, no DNA sequence alterations have been observed within the gC coding regions or promoter regions in attenuated MDV. These results indicate that reduced expression of MDV gC may be due to alteration of viral or cellular proteins that regulate gC promoter activity in attenuated MDV (Wilson *et al.*, 1994).

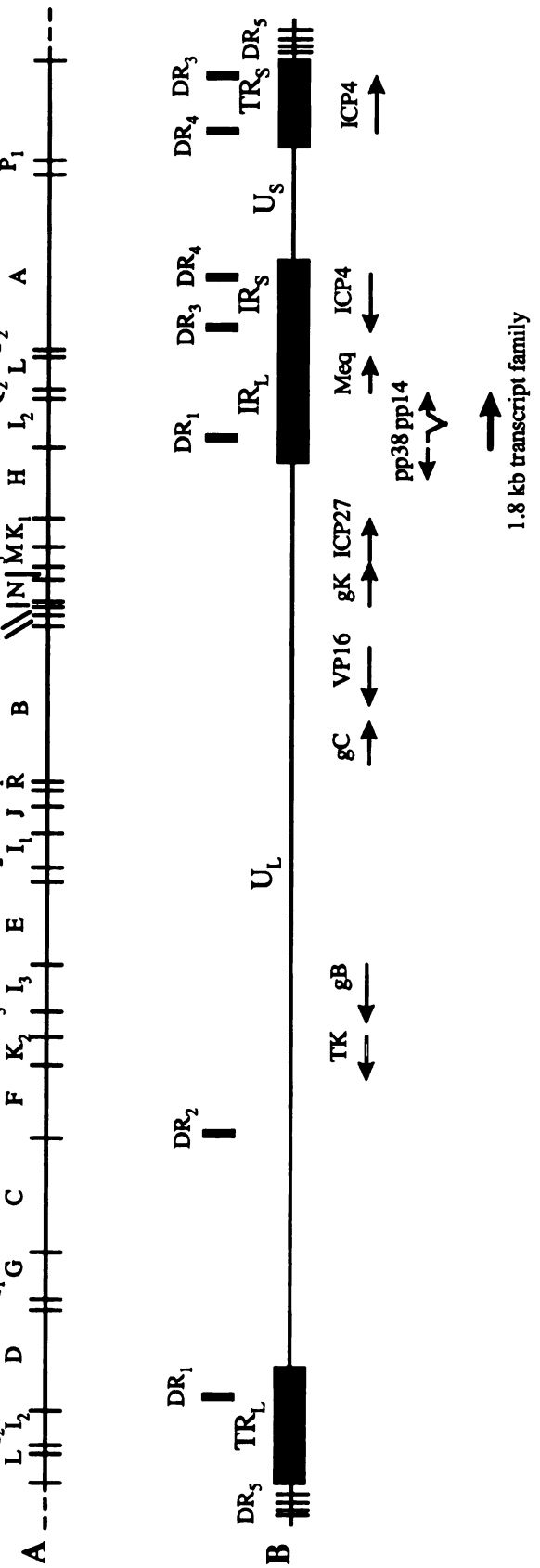

Figure 1.1 (A) The *Bam*HI restriction endonuclease map of MDV serotype 1 DNA; (B) the genome structure of MDV and location of MDV genes.

PART II. Herpesvirus immediate-early gene products and their functions

As described above, herpesvirus genes are expressed in a temporal regulation pattern during the lytic infection cycles. Much of our knowledge regarding gene expression regulation in herpesvirus has been derived from a prototype α-herpesvirus, HSV-1. Thus, in this section, review will be mostly focused on HSV-1 IE genes, however the comparative aspects in those of β- and γ- herpesviruses will also be discussed in some detail.

1. Activation of HSV-1 IE genes by VP16

HSV-1 contains five IE genes, ICP0, ICP4, ICP22, ICP27, and ICP47. Two of these IE genes, ICP4 and ICP27, are essential for viral replication and viral gene expression during lytic infection. Although HSV-1 IE gene expression does not require *de novo* protein synthesis, both viral proteins and cellular factors are involved in the IE gene activation. A virion tegument protein, named VP16 (Vmw65, α-TIF), plays a central role in IE gene transactivation (reviewed by Roizman and Sears, 1995). HSV-1 VP16 contains 490 amino acids, has a molecular weight of 54 kDa, and is synthesized during the late phase of lytic infection. During virion assembly, VP16 is incorporated into the tegument between the capsid and virion envelope. VP16 is subsequently released during infection and specifically *trans*-induces the IE gene transcription. Apparently, transactivation of IE genes requires at least one *cis*-acting sequence element, TAATGARAT (R = purine), within the IE promoter region (Mackem and Roizman,

1982; Gaffney *et al.*, 1985). Further studies has indicated that VP16 has no substantial affinity for double-stranded DNA (Marsden *et al.*, 1987), but functions by forming a multi-component complex on TAATGARAT sites, together with the cellular POU domain protein Oct-1 and host cell factor (HCF) (also called CFF or C1 factor) (Triezenberg *et al.*, 1988; Katan *et al.*, 1990; Kristie and Sap, 1995).

2. ICP27 gene families

A. HSV-1 ICP27

a. Basic protein properties of HSV-1 ICP27

HSV-1 ICP27 is located in the unique long region of the HSV-1 genome between coordinates 0.745 and 0.761. The open reading frame is 1536 nt long and encodes 512 amino acids. HSV-1 ICP27 is localized to the nuclei of infected cells and is phosphorylated, resulting in different forms of proteins being detected on SDS-PAGE. It appears that HSV-1 ICP27 contains both stable phosphate groups and phosphate groups that cycle on and off during infection. It is speculated that the different phosphorylated forms of ICP27 may specify its different regulatory activities, however, this aspect has not been thoroughly investigated (Ackerman *et al.*, 1984; Wilcox *et al.*, 1980; Sandri-Goldin, 1991). An assessment of the predicted amino acid sequence of HSV-1 ICP27 reveals that the primary structure can be divided into two halves with a hydrophilic amino terminal half and a relatively hydrophobic carboxyl terminal half (Sandri-Goldin, 1991). The first 64 amino acids from the N-terminus consist of 38% acidic residues and a serine-rich segment. The second striking feature of the N-terminal region is a highly

basic and arginine-rich domain between amino acid residues 110 and 175. This basic and arginine-rich domain contributes to nuclear localization and will be discussed in some detail below. The carboxyl terminal half of ICP27 plays the major functional role in HSV-1 ICP27-mediated transactivation or transrepression. A potential "zinc-finger" motif has been found within the last 60 amino acid residues of C-terminal region (Sandri-Goldin, 1991). Interestingly, this zinc finger motif is highly conserved in most ICP27 homologs including those of β- and γ- herpesviruses (Peara *et al.*, 1994; Zhao *et al.*, 1992; Winkkler *et al.*, 1994; Wong and Levine, 1986). Studies with ICP27 temperature-sensitive (*ts*) and deletion mutants indicate that HSV-1 ICP27 is essential for viral replication, especially for viral DNA synthesis and early and late gene expression (Sacks *et al.*, 1985; McMahan *et al.*, 1990).

HSV-1 ICP27 is localized to the cell nuclei in both viral infected cells and cells transfected with ICP27 expression plasmids (Ackermann *et al.*, 1984). HSV-1 ICP27 possesses a strong nuclear localization signal (NLS), between amino acid residues 110-137, which bears similarity to the bipartite NLSs found in *Xenopus laevis* nucleoplasmin and other nuclear proteins. The sequence of HSV-1 ICP27 between residues 110 and 152 can function as a nuleolar localization signal (NuLS) (Mears *et al.*, 1995; Robbins *et al.*, 1991). The sequence of NuLS includes ICP27's strong NLS and 15 contiguous residues consisting entirely of arginine and glycine. This arginine and glycine rich element is very similar to an RGG box, a putative RNA-binding motif found in a number of cellular proteins involved in nuclear RNA processing (Kiledjian and Greyfuss, 1992). Interestingly, Hibbard and Sandri-Goldin (1995) further demonstrated that two arginine-

rich regions located within the NuLS are required but not efficient for wild-type nuclear localization of ICP27. More importantly, when the NLS and R-rich regions are substituted by the heterologous NSL (SV40) or by the RNA binding domain of HIV-1 Tat individually, ICP27 mutants are defective in modulating late gene expression. Therefore, arginine-rich regions may be required for efficient nuclear localization and for the regulatory activity of ICP27 involved in co-operative activation of viral late gene expression (Hibbard and Sandri-Goldin, 1995).

b. Regulatory functions of HSV-1 ICP27

Studies from *ts* mutants with lesions within ICP27 and from deletion mutants have shown that HSV-1 ICP27 acts as a transactivator and a transrepressor (McCarthy *et al.*, 1989; Sacks *et al.*, 1985). The immediate-early genes (ICP4 or ICP0) and early genes are over-expressed in *ts* mutant infected cells, while late genes are poorly expressed. Phenotype analysis of these mutants indicates that ICP27 is required for the switch from early to late gene expression. Evidence from transient experiments also indicated that ICP27 acts either as a repressor or as an activator depending on the target genes examined (Hardwicke *et al.*, 1989; Rice *et al.*, 1990; Su and Knipe, 1989). In these transient expression assays, ICP27 appears to have little or no affect on expression of a wide range of target promoter constructs. However, when a plasmid encoding ICP27 is combined with effector plasmids encoding ICP4 or ICP0, both positive and negative effects can be observed (Hardwicke *et al.*, 1989; Su and Knipe, 1989; Rice *et al.*, 1989).

With regard to the distinct regulatory activities of ICP27, various approaches have been employed to map functional domains within HSV-1 ICP27. Rice *et al.* (1989)

reported that mutated polypeptides that possessed either 406 or 504 amino acids of ICP27 failed to activate gene expression but retained full transrepression activity. A polypeptide containing the amino-terminal 263 amino acids retained partial transactivation ability, but it was unable to transrepress target gene expression. Therefore, HSV-1 ICP27 possesses two genetically separable activities which can modulate gene expression in transfected cells. One activity positively affects gene expression, while the other inhibits gene expression (Rice *et al.*, 1989). In contrast, using in frame insertion or deletion strategies, Hardwick *et al.* reported that mutants with insertions between position 262 and 406 lost their transactivation activity but retained transrepression function, whereas those with insertions between position 434 and 504 lost both activities (Hardwick *et al.*, 1989). Thus, they concluded that the C-terminal half of ICP27 is required for the transactivation activity of ICP27 but only the C-terminal 78 amino acids are critical for transrepression activity (Hardwick *et al.*, 1989; Sandri-Goldin, 1991). Smith *et al.* (1991) sought to determine whether any of the ICP27 insertion mutants would display a dominant phenotype, in other words, to determine whether these mutants would interfere with wild-type ICP27 activities. By both transient and stable expression analysis, they found that mutations in the activation regions of ICP27 between residues 262 and 406 were dominant to wild-type ICP27 and specifically interfered with its transactivation function. In contrast, those mutants defective in repressor function, residues 434 to 505, cannot compete with wild-type ICP27 (Figure 1.2B) (Smith *et al.*, 1991).

Regulatory activity of HSV-1 ICP27 is independent of target promoter sequences but depends on the presence of different mRNA processing signals (Sandri-Goldin and Mendoza, 1992). The ICP27 activation function correlates with different

polyadenylation sites, whereas repressor function correlates with the presence of introns either 5' or 3' to the target gene-coding sequences. This hypothesis is supported by the following observations: First, HSV-1 ICP0 and ICP4-mediated transactivation of TK promoter can be repressed 12-fold by HSV-1 ICP27, when the TK reporter plasmid contains an SV40 early polyadenylation signal (EPA) and a small T intron. When the SV40 EPA and small T intron are replaced with a synthetic poly A hexanucleotide (AATAAA), HSV-1 ICP27 is able to stimulate (but not repress) the TK promoter. Splicing studies have also suggested that ICP27 directly inhibits splicing by sequestering snRNPs (Sandri-Goldin and Mendoza, 1992). Second, ICP27 affects the accumulation of CAT poly(A) mRNA in transfection assays with CAT expression plasmids containing different 3'RNA processing signals. ICP27 determines which polyadenylation signal is used where more than one signal is present. Generally, late gene polyadenylation signals were used more efficiently than those of early gene (McLauchla *et al.*, 1989; Sandri-Goldin and Mendoza, 1992). HSV-1 ICP27 also affects host mRNA accumulation. In infection with viral mutants defective in ICP27, the accumulated levels of three spliced host mRNAs are much higher than those seen with wild-type HSV-1. Thus, it appears that HSV-1ICP27 contributes to the decrease in cellular mRNA levels during infection by inhibiting splicing (Hardwick and Sandri-Goldin, 1994). More recent studies support the hypothesis that ICP27 is involved in stabilizing mRNA and binding to 3' ends (Brown *et al.*, 1995). However, it is not clear whether binding involves specific poly(A) signals or the long AU-rich instability-associated motifs presenting in these transcripts. The RNA binding motifs within HSV-1 ICP27 polypeptide remain to be identified. There are two potential domains that may be related to RNA binding. The repetitive

RGGRRGRRRGRGRGG motif between amino acid residues 138 and 152 is very similar to a well-defined RNA-binding motif, the RGG box (Mears *et al.*, 1995; Kiledjian and Dreyfuss, 1992). In addition, a putative zinc-finger motif which is highly conserved in most ICP27 homolog families could also function as a RNA-binding motif (Brown *et al.*, 1995; Sandri-Goldin, 1991). The polypeptide structure and functional domains of HSV-1 ICP27 are summarized in Figure 2.2B.

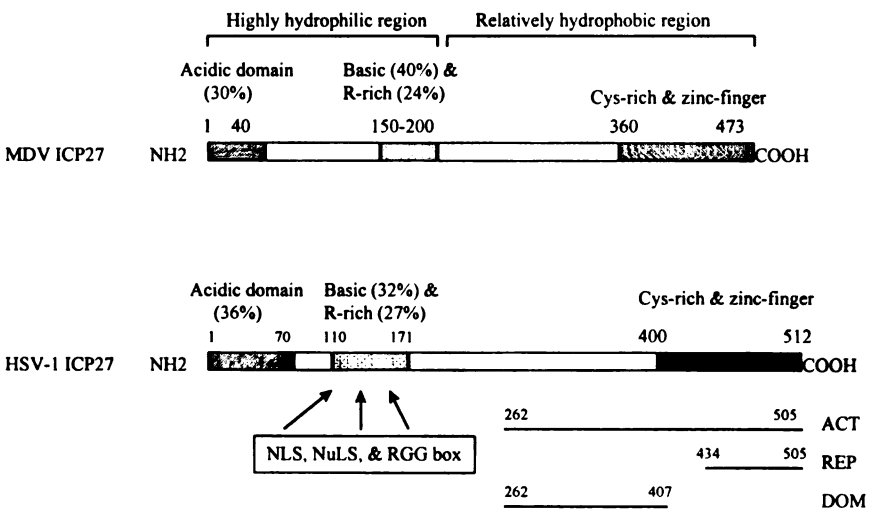

Figure1. 2 Schematic structures of the predicted polypeptides of MDV ICP27 (A), and HSV-1 ICP27 (B). ACT, activation region; REP, repression region; DOM, dominant region; NLS, nuclear localization signal; NuLS, nucleolar localization signal

B. Varicella-zoster virus (VZV) ORF4

Varicella-zoster virus (VZV) open reading frame 4 (ORF4) encodes a 452-amino acid polypeptide which is the VZV homolog to HSV-1 ICP27 (Inchaupe and Ostrove, 1989;

Perera *et al.*, 1994). The protein properties and functions of ORF4 can be summarized as below: **(1)** ORF4 protein shares an overall amino acid identity of 27% with HSV-1 ICP27, while certain regions, especially in the C-terminal portion, can be as high as 45%. A cys-rich domain and a putative zinc-finger motif are also conserved in the C-terminal region. **(2)** ORF4 protein acts as a transcriptional activator that can effectively activate certain VZV gene promoters such as ORF62 (ICP4 homolog), TK gene promoter, and a heterologous HIV-LTR (Inchaupe and Ostrove, 1989; Defechereux *et al.*, 1993). **(3)** ORF4 has little or no effect on late gene promoters. **(4)** ORF4-mediated induction of gene expression occurs primarily at the level of transcription since 3'RNA-processing signals are not dominant determinants for the ORF4-mediated transactivation activity (Perera *et al.*, 1994). **(5)** Oligonucleotide-directed site-specific mutagenesis indicates that of 10 cysteine residues in the ORF4 polypeptide, only C-421 and C-426 are essential for transactivation function. Both C-421 and C-426 are located within the putative zinc-finger motif conserved in numerous IVP27 homologs. Perera *et al.* (1994) further suggests that protein-protein interactions may be essential for ORF4 inducibility and that amino acids C-421, C-426, and H-417 may play a critical role in these interactions.

C. ICP27 homolog in β-herpesvirus

The UL69 open reading frame of human cytomegalovirus (HCMV) is homologous to the immediate-early protein ICP27 of HSV-1. Unlike HSV-1 ICP27 which is transcribed as an immediate-early gene, HCMV UL69 belongs to the early gene family and is detected approximately 7 hours infection infection (Winkler *et al.*, 1994). Protein of

HCMV UL69 localizes within intranuclear inclusions that are characteristic for HCMV infection. Cotransfection assays have shown that HCMV UL69 is able to transactivate an HCMV early promoter, UL112, as well as several heterologous promoters, whereas HCMV late promoters could not be activated by UL69 (Winkler *et al.*, 1994). In addition, UL69 protein cannot substitute for HSV-1 ICP27 in the context of HSV-1 infection, suggesting functional differences between the two proteins (Winkler *et al.*, 1994).

D. ICP27 homolog in γ-herpesvirus

ICP27 gene homolog is not only conserved in α- and β- herpesvirus, but also in γ-herpesviruses. These include BMLF1 of EBV and the IE-52 gene of herpesvirus saimiri (Kenney *et al.*, 1989; Nicholas *et al.*, 1988). They will be described in some detail in a later section.

3. Other HSV-1 IE genes

A. HSV-1 ICP4

As a major transcriptional activator, ICP4 is one of the most extensively studied immediate-early proteins in HSV-1 (Reviewed by Roizman and Sears, 1995). ICP4 is encoded by the IE175 gene located within the short unique repeats. Therefore, there are two copies of ICP4 in each HSV-1 genome. ICP4 is a highly phosphorylated protein and predominantly localizes in nucleus of infected cells. ICP4 is 1,298 amino acids in length with a predicted protein molecular weight of 133 kDa. However, there are at least three

modified polypeptides in denaturing SDS-PAGE with molecular weights of 160 kDa, 163 kDa, and 170 kDa (Pereira *et al.*, 1977). Although all α-proteins are phosphorylated in HSV-1, ICP4 appears to be the only poly(ADP) ribosylated α-protein (Preston and Notarianni, 1983). A more recent report indicates that ICP4 is also guanylated and adenylated (Blaho and Roizman, 1991).

Through studies involving viral mutant analysis, transient expression assays, and biochemical analysis, it has been established that ICP4 is essential for viral growth and for viral gene expression (Dixon and Schaffer, 1980; DeLuca, and Schaffer, 1985; Roizman and Sears, 1995). The regulatory functions of ICP4 can be summarized as two major activities: **(i)** transactivation of early and late gene expression; **(ii)** transrepression of expression of ICP4 gene promoter and possibly other immediate-early gene promoters. The most striking feature of ICP4 is its DNA binding preference. Faber and Wilcox (1986) initially identified a strong binding site with a consensus sequences of ATCGTCNNNNYCGRC where R=purine, Y=pyrimidine, and N=any base. Subsequent studies have reported numerous ICP4 binding sites that did not correspond to this consensus sequence (Michael *et al.*, 1988). Thus, HSV-1 ICP4 can bind to both consensus and non-consensus sites (Michael *et al.*, 1988). Although it is clear that ICP4 is essential for induction of early gene and some late gene expression, exhaustive studies have failed to reveal any evidence for the existence of ICP4-specifc induction sequences in target promoters (Coen *et al.*,1986). It has been suggested that the binding of ICP4 to specific sites is not required for ICP4-mediated transactivation (Gu and DeLuca, 1994). However, specific ICP4-binding sites are required for ICP4-mediated repression activity

(Gu and DeLuca, 1994; Smith *et al.*, 1995; Roizman and Sears 1995). Studies have shown that ICP4 can transactivate a minimal promoters in which the only recognizable *cis*-element is a TATA box (Imbalzano and DeLuca, 1991). This finding suggests that ICP4 operates through the basal transcriptional machinery acting on the TATA box. This hypothesis was supported by a subsequent study that was reported by Smith *et al* (1993). By gel retardation and footprinting assays, they found that ICP4 forms a tripartite complex with TFIIB and either TATA-binding protein (TBP) or TFIID. Formation of this complex was not result of the simple tripartite occupancy of DNA but the consequence of protein-protein interactions (Smith *et al.*, 1993). Therefore, it was speculated that formation of the TPB-TFIIB-ICP4-DNA complex was involved in the mechanism of ICP4 function, particularly including its repression activity. A more recent report has provided a strong correlation between tripartite complex formation and repression activity of ICP4 (Kudus *et al.*, 1995). Both tripartite-complex formation and transcriptional repression are efficient when the ICP4-binding site is downstream of the TATA box, with a short distance (less than 40 bp) and in a proper orientation (Kudus *et al,* 1995). In contrast, when the TATA box and the ICP4-binding site was separated by more than 50 bp, both tripartite-complex formation and repression were concomitantly reduced. This observation strongly suggests that ICP4-mediated repression activity is strikingly related to the degree of tripartite-complex formation on the ICP4 promoter. In addition, when the ICP4-binding site was in its natural orientation, threefold greater tripartite-complex formation was observed. It also suggested that ICP4 predominantly represses transcription in a direction-dependent manner and not simply by blocking transcription (Kudus *et al,* 1995).

B. HSV-1 ICP0

HSV-1 IC0 (or Vmw 110) is encoded by a spliced IE gene that lies in TR_L and IR_L, therefore like ICP4, there are two copies in the viral genome (Preston *et al.*, 1978). DNA sequence and S1 nuclease mapping analysis showed that theHSV-1 ICP0 gene contains three exons separated by two introns. Like other IE gene products, ICP0 is predicted to be 775 amino acids with a molecular weight of 80 kDa, however, in denaturing SDS-PAGE gel it has a molecular weight of 110 kDa. ICP0 is highly phosphorylated and predominantly located in nuclei of infected cells (Wilcox *et al.*, 1980; Perry *et al.*, 1986). Primary structure of ICP0 exhibits some striking features that, at least partially, reflect the protein activities. The first 71 amino acid residues of the ICP0 N-terminus are highly acidic, adjust to two zinc-finger motifs that have been described as functional DNA-binding domains in several transcriptional factors (Berg, 1986; Evans and Hollenberg, 1988). This potential zinc-finger region is critical for the effects of ICP0 both *in vitro* and *in vivo* (Evertt *et al.*, 1991). By site-directed mutagenesis, it has been found that substitution of Cys or His residues in the zinc-finger domain abolishes ICP0-mediated transactivation (Moriuch *et al.* 1992). Two proline-rich regions are present in exon 3, but the function of these proline-rich domains is unclear. ICP0 may dimerize or oligomerize, based on biochemical properties of the purified intact ICP0 protein (Chen, *et al.*, 1992; Everett *et al.*, 1991).

Transient expression assays indicate that ICP0 has unusually powerful and promiscuous activities. The list of promoters that can be transactivated by ICP0 includes promoters from all three kinetic classes of HSV-1 genes, heterologous promoters such as

HIV-LTR and SV40 early promoter, and host gene promoters (reviewed by Everett *et al.*, 1991). ICP0 response elements have not been clearly identified, partially due to its unusually broad range of induction on target promoters. Promiscuous activity of ICP0 suggests that ICP0 may nonspecifically bind to target promoters and interact with some transcription factors, in which ICP0 functions as a bridge molecule (Chen *et al.*, 1992; Everett, 1991). However, the mechanism of how ICP0 mediates gene activation is not clear. In some cases, ICP0 shows synergistic activity with ICP4. By both *in vitro* and *in vivo* assays, ICP0 exhibits much stronger transactivation activity in synergy with ICP4 than in absence of ICP4. By a serial in-frame insertion mutant analysis, the carboxyl region (between amino acids 633 and 723) and a Cys-rich domain in the N-terminus were identified as essential for ICP0 synergy with ICP4 (Everett *et al.*, 1991; Everett, 1988).

ICP0 localizes in a very unusual punctate pattern consisting of several dozen phase-dense granules in DNA transfected cells. In contrast, ICP0 normally localizes in a much smaller micro-punctate pattern at early times in virus infected cells (Chen *et al.*, 1991; Everett *et al.*, 1988; Giufo *et al.*, 1994). More recently, a short basic amino acid motif VRPRKRR mapped to amino acid sequences 500-506 has been identified as a functional motif for ICP0 nuclear localization (Mullen *et al.* 1994). A similar motif with a sequence of GRKRKSP has also been identified in HSV-1 ICP4 protein between amino acids 726-732 (Mullen *et al.*, 1994). By analysis of deletion mutants and analysis of ICP0/ICP4 hybrid protein, it has been suggested that this nuclear localization motif does not contribute to ICP0 unusual punctate pattern. In contrast, amino acid residues from 105 to 244 are critical for conferring the punctate localization feature (Mullen *et al.*,

1994; Giufo *et al.*, 1994). ICP4 and ICP27 can inhibit ICP0 nuclear localization both in transiently transfected cells and in virally infected cells (Zhu *et al.*, 1994). These negative effects may contribute to the functional cooperation among these three α-proteins.

Based on mutation studies *in vivo*, it appears that ICP0 is not essential for lytic infection in cell culture (Chen and Silverstein 1992; Everett 1991). Under high multiplicity infections, ICP0 function is dispensable for viral DNA replication, viral polypeptide synthesis, and virion formation (Sacks and Schaffer, 1987; Everett 1991). Importantly, ICP0 has also been suggested to play a role in the efficient establishment and reactivation of latency, where viral gene expression is limited to transcription of the latency-associated transcript (LAT) and no infectious virus can be detected (Leib *et al.*, 1989) . However, viruses with mutations in both copies of ICP0 do not reactivate from latency as efficiently as wild type viruses. The role of ICP0 in establishment of latency may increase the efficiency of virus replication in primary infections and in ganglionic neurons, the site of latent infection (Leib *et al.*, 1989). In reactivation from latency, ICP0 may boost viral gene expression at the onset of latency (Cai and Schaffer, 1992; Leib *et al.*, 1989). Therefore, an alternative pathway, independent of VP16-Oct-1, may exist for activating IE gene expression, particularly in the early stage of reactivation from latency where VP16 is absent. ICP0 has been suggested to play a back-up role for VP16 in IE gene expression (Elsshiekh *et al.*, 1991). However, the molecular mechanism by which ICP0 genes are regulated to exert reactivation of latency is not clear.

C. HSV-1 ICP22 and ICP47

HSV-1 ICP22 and ICP47 have been less studied than other HSV-1 IE genes. HSV-1 ICP22 is encoded by the US1 gene, located in the unique short region. ICP22 is a dispensable gene since deletion of ICP22 had no effects on DNA synthesis and virus infection. However, in some cell cultures, such as RAT-1 cell lines and human embryonic lung cells, plating efficiency of virus with ICP22-mutant was reduced and the yield of virus was dependent on the multiplicity of infection (Sears *et al.*, 1985; Roizman and Sears, 1995). It has been reported that HSV-1 infection results in a rapid alteration of phosphorylation on the large subunit of cellular RNA polymerase II. This modification generates a novel form of the large subunit, designated IIi (Rice *et al.*, 1994). Further studies suggest that 22/n99, an HSV-1 mutant containing a nonsense mutation in ICP22, is significantly deficient in IIi induction. In 22/n99 infected cells, late gene transcription is less efficient, and antisense transcription through out the genome is diminished compared with that of wild type infection (Rice *et al.*, 1995). These results suggest that HSV-1 ICP22 may be necessary for virus-induced aberrant phosphorylation of RNAP II and for normal patterns of viral gene transcription in certain cell lines (Rice *et al.*, 1995). HSV-1 ICP47 is the only IE protein that does not show obvious regulatory functions in HSV-1 infection. Thus, the function of this gene is unclear.

4. The β-herpesvirus IE genes and their function

Human cytomegalovirus (HCMV) is a prototype virus in the β-herpesvirus subfamily. HCMV is highly species specific and has the largest genome (229 kb) (Marcarski,

1995). As with α-herpesviruses, gene expression of HCMV in infected cells occurs in a cascade fashion. At the immediate-early time, gene expression is restricted to four loci of the genome. The most abundantly expressed IE region, termed the major IE gene region, is located in the large unique (U_L) component between 0.732 and 0.751 map units. Two transcripts from the major IE gene region, designated IE1 and IE2, have been extensively studied. A striking feature is that both IE1 and IE2 share a common regulatory region defined as an enhancer-containing promoter-regulatory region. This promoter-regulatory region is unusually strong and contains multiple sets of highly conserved repetitive elements. (Reviewed by Stinski *et al.*, 1991). The IE1 gene, immediately down stream of the major IE promoter, encodes a spliced mRNA consisting of 4 exons, designated exons 1, 2, 3, and 4 respectively. The translation initiation codon of IE1 is located in exon 2. IE1 encodes a highly phosphorylated protein with a molecular mass ranging from 68 to 72 kDa. The largest polypeptide, designated 72kDa-E1, has been extensively investigated and will be discussed in more detail. The three different sized transcripts derived from IE2 region share a common 3' region, but differ in their 5' regions, due to alternative splicing. Three polypeptides derived from these transcripts are termed 86-kDa IE2, 54-kDa IE2, and 28-kDa IE2, based on their molecular weights. Functions of these IE proteins are dramatically different, although they share some common domains. 72-kDa IE1 and 86-kDa IE2 are the best characterized proteins. Briefly, 86-kDa IE2 independently transactivates viral early and weak late gene (γ_1 gene) promoters, as well as heterologous early promoters from adenovirus, while it negatively regulates the major IE promoter. In contrast, 72-kDa IE1 positively regulates both IE1 and IE2 gene

expression in HCMV infected cells, but has no significant effect on early and late gene expression (Hermiston *et al.*, 1987; Malon *et al.*, 1990; Stinski *et al.*, 1991). Furthermore, 86-kDa IE2-mediated transactivation is augmented in the presence of 72-kDa IE1. However, both IE1 and IE2 gene proteins fail to stimulate true late (γ_2) gene expression. This suggests that additional regulatory proteins encoded by HCMV may play a role (Depto and Stenberg, 1992; Puchtler and Stamminger, 1991), but these factors remain to be identified.

5. The γ-herpesvirus IE genes and their functions

Epstein-Barr virus (EBV) is a prototype of γ-herpesviruses that can infect human B lymphocytes and induce cellular proliferation or transformation. Latently infected lymphocytes persist for life and can be established as continuous lymphoblastoid cell lines. EBV transcription has been analyzed for the most part in lymphoblast cell lines. Most of the infected lymphocytes are latently infected and only limited number of viral transcripts can be detected. It is difficult to study gene regulation in EBV since EBV lacks a lytic tissue culture system. B cells latently infected with EBV can be induced to express lytic cycle genes by treatment with variety of agents including phorbol esters, butyrate, and anti-immunoglobulin antibiotics (Hamper *et al.*, 1974; Raab-Traub and Gilligan, 1991; Hayward and Hardwick, 1991).

There are three immediate-early genes in cells lyticly infected with EBV, including the **Z** transactivator (Zta), the **M** transactivator (Mta), and the **R** transactivator (Rta).

Zta, also termed BZLF1, is a major transactivator for induction of EBV lytic gene

expression. Zta gene has been mapped within the *Bam*HI-Z fragment. The Zta proteins are encoded by two spliced mRNAs transcribed from two different promoters. A 0.9 kb mRNA is initiated from an unusual TATA box (TTTAAA) and encodes Zta protein, whereas a 2.8 kb bicistronic mRNA is transcribed from an upstream promoter of BRLF1 and encodes both Zta and Rta proteins. Zta is a highly phosphorylated protein and is localized to the nucleus (Daibata *et al.*, 1992). By cotransfection assays, several EBV promoters have been identified as Zta responsive promoters in which Zta can recognize and bind to a highly conserved seven-bp sequence, ZRE (Zta response elements). The interesting thing is that Zta not only binds to homologous promoters but also is capable of binding to a sequence TGACTCTA, the AP-1 recognition site for c-*jun/fos* transcriptional activation. Exon 2 of Zta has a high homology to a basic DNA binding domain conserved in c-*jun/fos* family (Lieberman *et al.*, 1990; Hayward and Hardwick, 1991). Expression of the Zta triggers disruption of latency in EBV-infected cells.

Rta, also termed BRLF1, is encoded within a multi-spliced 2.8 kb mRNA that is bicistronic. This bicistronic transcript also contains the complete Zta coding sequences downstream of the Rta open reading frame. Rta is predominantly localized in the nuclei. Three EBV promoters have been identified as Rta responsive promoters, including the duplicate sequences DS_L, DS_R, and the Mta promoter. The Rta responsive elements have been mapped in all three promoters. These consensus elements function as enhancer elements and behave in a position and orientation independent manner (Kenney *et al.*, 1989; Hayward and Hardwick, 1991). The heterologous promoter, HIV-1 LTR is also stimulated by Rta. However, Rta transactivation of the HIV-1 promoter does not require the HIV-1 enhancer. Thus, Rta may transactivate by at least two different mechanisms,

one mechanism involving certain enhancer elements and another mechanism being enhancer independent (Quinlivan *et al.*, 1990).

Mta, also known as BMLF1, is the product of the BSLF2/ BMLF1 open reading frames that consist of two alternatively spliced mRNAs. Mta consists of three major polypeptides with molecular weights of 45, 50, and 60 kDa. The 50-and 60-kDa Mta polypeptides are phosphorylated. Mta is homologous to HSV-1 ICP27. Thus, Mta is the only α-herpesvirus IE gene homolog that has been identified in EBV. In cotransfection assays, Mta can transactivate CAT reporter constructs derived from both homologous and heterologous promoters. This Mat-induced activation of promoters is reporter-gene dependent and functions at the post-transcriptional level (Kenney *e al.*, 1989). However, the mechanism of Mta-mediated transactivation is not clear.

As described above, ICP27 gene families play some critical roles for herpesvirus growth and for viral gene expression. The specific aims of this project are 1) to identify the ICP27 homolog in MDV and further characterize the gene product; 2) and, to investigate and evaluate the regulatory functions of the MDV ICP27 homolog. We will focus on in vitro studies of the transcriptional regulatory mechanism of the MDV ICP27 homolog.

Chapter II

Identification and Characterization of Marek's Disease Virus Genes Homologous to ICP27 and Glycoprotein K of Herpes Simplex Virus-1

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ABSTRACT

We have identified two Marek's disease virus (MDV) genes within the *Eco*RI-B fragment of MDV-GA genomic DNA. *Eco*RI-B is 11.3 kb long and maps within the long unique (U_L) region of MDV genome. A 3.2 kb fragment of *Eco*RI-B has been sequenced and contains two open reading frames, ORF53 and ORF54. ORF53 (MDV gK), a homolog to HSV-1 glycoprotein K (gK), is 1,062 nucleotides (nt) long and encodes 354 amino acids (39.5 kDa). ORF54, designated MDV ICP27, based on significant similarity to HSV-1 ICP27, is 1,419 nucleotides long and encodes 473 amino acids (54.5kDa). In Northern blot hybridization, two overlapping transcripts (2.9kb and 1.6kb) were detected in MDV-infected DEF cells treated with cycloheximide, suggesting that both transcripts belong to the immediate-early gene family. Amino acid sequence analysis of MDV gK shows some common glycoprotein features, including a putative N-terminal signal sequence, four N-linked glycosylation sites, and four potential transmembrane domains. Comparison of the predicted amino acid sequence of MDV ICP27 with that of HSV-1 ICP27 and VZV ORF4 shows a high degree of conservation within the C-terminus. The C-terminal region of HSV-1 ICP27 has been demonstrated to be critical to its function. A conserved zinc finger metal-binding motif $C_{(442)}$-X_4-$C_{(447)}$-X_{13}-$H_{(461)}$-$C_{(467)}$ was also found in the C-terminus of MDV ICP27. Furthermore, MDV ICP27 upstream sequences contain four copies of consensus sequence elements similar to the tegument protein target sequence TAATGARAT. TrpE-ICP27 fusion protein was expressed in *E.coli,* and rabbit antisera were generated using purified fusion protein. A 55 kDa protein has been detected in both MDV GA and Md11 infected cells using immunoblot analysis.

INTRODUCTION

Marek's disease virus (MDV) is a cell-associated herpesvirus that induces T-cell lymphomas and peripheral nerve demyelination in susceptible chickens (Calnek, 1985). MDV was originally classified as a gamma herpesvirus on the basis of its lymphotropism. However, MDV genomic structure closely resembles members of the alpha herpesvirus group (e.g. herpes simplex virus, and varicella-zoster virus) (Buckmaster *et al.*, 1988; Roizman *et al.*, 1992).

Herpesvirus genes are classified into three kinetic classes, immediate-early (IE), early (E) and late (L) genes, based on requirements for viral protein synthesis and DNA replication (Honess *et al.*, 1974). To date, five immediate-early (IE) gene products have been identified and mapped in herpes simplex virus type-1 (HSV-1), including ICP0, ICP4, ICP22, ICP27, and ICP47 (Honess *et al.*, 1974; Sacks *et al.*, 1985). ICP4, as a major regulatory protein, plays an essential role throughout the viral replication cycle (DeLuca *et al.*, 19850). ICP0 has been shown as a potent transcriptional activator (Chen *et al.*, 1992).

HSV-1 ICP27 is a 63 kDa phosphoprotein localized in the nucleus of infected cells (McCarthy *et al.*, 1989). A series of temperature-sensitive (*ts*) mutants of ICP27 have been isolated and provided powerful tools to study gene function (Sacks *et al.*, 1985). ICP27 is an essential protein which is required for virus replication and for modulation of early and late gene expression at transcriptional and post-transcriptional levels (McCarthy *et al.*, 1989; Smith *et al.*, 1991; Sandri-Goldin *et al.*, 1992). Evidence from transfection studies has shown that ICP27, in the presence of ICP4 and ICP0, can

repress some early genes (e.g. thymidine kinase gene) and enhance expression of some late genes (e.g. VP5 and glycoprotein B) (Smith *et al.*, 1992), but it has little or no trans-regulatory effect on target genes by itself (Sekulovich *et al.*, 1988).

Genes encoding ICP27 homologues have been identified for other alpha hepesviruses, including varicella-zoster virus (VZV) (Davison *et al.*, 1986) and equine herpesvirus type 1 (EHV-1) (Zhao *et al.*, 1992). Though similar in amino acid sequence (28% identity), VZV ORF4 has been shown to be functionally distinct from HSV-1 ICP27 (Moriuchi *et al.*, 1994; Perera *et al.*, 1994). Unlike its HSV-1 counterpart, VZV ORF4 efficiently activates heterologous promoters in the absence of other virus proteins (Defechereux *et al.*, 1993).

To date, little is known about MDV immediate-early genes and the gene products they may encode. A gene encoding MDV ICP4, a homolog to HSV-1 ICP4, has been identified and mapped within BamHI fragment A of the MDV genome (Anderson *et al.*, 1992). However, the function of MDV ICP4 remains to be determined. Recently, a 1.6 kb spliced immediate-early gene has been reported and mapped to BamHI-I2 fragment, within the MDV inverted repeat region (IR_L). Protein products encoded by this gene appear to be MDV specific (Hong and Coussens, 1994).

In this study, we report the DNA sequence of the two open reading frames within the MDV genome which encode proteins homologous to HSV-1 glycoprotein K and ICP27. Two overlapping transcripts, corresponding to MDV gK and ICP27 genes, were identified in Northern blot analysis. Based on expression in the absence of protein synthesis, both transcripts may belong to the immediate-early gene family. MDV gK shows critical homology to HSV-1 gK and has some common features of glycoproteins.

Because our laboratory is primarily concerned with understanding MDV gene regulation, further attention was focused on MDV ICP27. By comparing to other alpha herpesvirus counterparts, MDV ICP27 exhibits a significant conservation in its C-terminal region. Rabbit antiserum specific against MDV ICP27 was produced using trpE-ICP27 fusion protein in order to detect ICP27 gene products in MDV infected cells.

MATERIALS AND METHODS

Cells and viruses

Duck embryo fibroblast (DEF) cells were prepared, maintained and infected with MDV according to previously described procedures (Glaubiger *et al*, 1983). Two low passage cell-associated MDV serotype 1 strains, GA (passage10) and Md11 (passage12), were used for this study. DEF cultures were grown in Leibovitz-McCoy medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% calf serum at 37° C, in a humidified atmosphere containing 5% CO2. Calf serum concentration was reduced to 1% following infection.

DNA sequencing

An *Eco*RI-B fragment (11.3kb) from the genomic library of MDV-GA was kindly provided by Dr. Robert Silva (USDA-Agricultural Research Service, Avian Disease and Oncology Laboratory, East Lansing, MI). A series of overlapping subclones, representing the entire 11.3kb of *Eco*RI-B, were constructed in pUC18. DNA sequencing was performed on double stranded plasmids by dideoxy chain termination (Sanger *et al.*, 1977) using [α-^{35}S]ATP (NEN Research Products) and TAQuence DNA sequencing kits (United States Biochemical Corp., Cleveland, Ohio). DNA sequences were analyzed using MacVector 3.5 (International Biotechnologies), programs GAP and PILEUP of the University of Wisconsin Genetics Computer Group (GCG), GenBank release 80.0 (IntelicGenetics), and the Protein Identification Resource, Version 38.0 (National

Biomedical Research Foundation).

Total cellular RNA isolation and northern blot analysis

Total cellular RNA was isolated from mock-infected and MDV-infected DEF cells using the guanidinium-phenol:chloroform method as described by Chomczynski *et al.*,(1987). Immediate-early (IE) and early RNAs were obtained by adding cycloheximide (CHX,100ug/ml) and phosphonoacetic acid (PAA, 100 ug/ml) at the time of infection, respectively. IE RNA was extracted 12 hours post-infection and CHX treatment. Early RNA was extracted 24 hours post-infection and PAA treatment. Total RNA (10ug) was loaded onto 1.2% agarose gels containing 6% formaldehyde and electrophoresed for 12 hours at 30V. RNA was transferred onto Hybond-N membrane (Amersham Corp., Heights, IL) as described by Sambrook *et al.*, (1989). Two DNA fragments, *Cla*I-*Cla*I (CC, Figure 2.1) and *Bam*HI-*Kpn*I (BK, Figure 2.1) were used as probes and labeled using [α^{32}P]dCTP (NEN Research Products). Northern blot hybridization was performed using standard procedures (Sambrook *et al.*, 1989). Transcript size was determined by comparison to an RNA ladder marker (BRL, Gaithersburg, MD).

Expression of trpE fusion protein

The pATH vector systems, which encode a 37 kDa bacteria trpE ORF under control of an inducible *trp* promoter, were used to express trpE-MDV fusion proteins (Chen *et al,* 1992). A 688 bp BamHI-*Cla*I fragment (BC), encoding MDV-ICP27 amino

acid residues 24 to 252, was cloned between *Bam*HI and ClaI sites of pATH11 (Figure 2.1). A 520 bp *Kpn*I-BglII fragment (KBg) which represents the ICP27 ORF coding region between amino acids 205 and 378 was cloned into pUC18 to generate pUC18KBg. A second fusion protein was constructed by cloning an *Eco*RI and *Hind*III fragment from pUC18KBg into pATH3 (Figure 2.1). TrpE-ICP27 ORF fusion proteins were analyzed by 8% SDS-PAGE and partially purified as described previously (Chen *et al.*, 1992). New Zealand white rabbits were injected with 200mg of fusion protein emulsified in Freund's complete adjuvant (Life Technologies, Inc., Gaithersburg, MD). Rabbits were boosted with the same amount of protein in Freund's incomplete adjuvant after 4 weeks interval and bled ten days following the last injection.

Western blot analysis

Mock infected and MDV infected DEF cell lysates were prepared with triple-detergent lysis buffer (Sambrook *et al.*, 1989). Lysates were separated on 12% SDS-PAGE Minigels (Bio-Rad), and transferred to Nitrocellulose membrane (NC) (Scheicher & Schuel). NC membranes were blocked using 5% dry milk. Rabbit antiserum against fusion protein was used at a 1:100 dilution, and donkey anti-rabbit IgG conjugated with horseradish peroxidase was used as second antibody. Amersham ECLTM was used as substrate according to the manufacturer's specification.

Protein translation in vitro and immunoprecipitation analysis

The MDV-ICP27 gene was modified by the polymerase chain reaction (PCR) to create a *Bgl*II restriction site in front of the ATG codon. Primer sequences were

5'-CCCCAGATCTAAAAATGTCTGTAGATGCATTCT3' and 5'-CATGGTCATTCCA-CATCGAA3'. A 628 bp *Bgl*II-*Kpn*I fragment obtained from PCR products was ligated with a 1,077 bp *Kpn*I-XhoI fragment which contains C-terminal coding regions of the ICP27 gene (Figure 2.1) and cloned between BamHI and XhoI sites of pBluescript KSII+/- vector (Figure 2.1, pBlue-ICP27). A 549 bp *Bam*HI-*Kpn*I fragment of ICP27 was cloned into prokaryotic expression vector pRSET-A, directly downstream of a T7 promoter using histidine-tagged protein ATG as the translation start codon. The TNT™ T7 Coupled Reticulocyte Lysate System (Promega) was used for coupled in vitro transcription/translation. Translation products were labeled using [^{35}S]methionine (NEN Research Products). Immunoprecipitation analysis of labeled translation products was carried out as described previously (Silva and Lee, 1984). Rabbit antiserum against trpE-ICP27 fusion protein was used in this experiment.

RESULTS

DNA sequence analysis of MDV ICP27 and gK genes

The MDV *Eco*RI-B fragment (11.3 kb), located in the long unique (U_L) region, spans *Bam*HI-H, K1, M, P3, N, and T fragments. *Eco*RI-B was chosen to initiate a search for immediate-early gene ICP27, based on structure similarity between HSV-1 and MDV (Fukuchi *et al.*, 1984; McGeoch *et al.*, 1988). A partial restriction enzyme map of the MDV EcoRI-B fragment is shown in Fig.1. A 3.2 kb DNA fragment of EcoRI-B suspected to contain the ICP27 has been sequenced in both directions (Figure 2.2). Analysis of nucleotide sequences of this fragment revealed two open reading frames (ORFs) (Figure 2.1 and Figure 2.2). ORF53 is 1,062 nucleotides long and encodes 354 amino acids with a calculated molecular weight of 39.5 kDa. ORF54 is 1,419 nucleotides long and encodes 473 amino acid residues with a calculated molecular weight of 54.5 kDa. ORF54, designated MDV ICP27, has homology with HSV-1 ICP27 and VZV ORF4 (Figure 2.3). ORF53, designated MDV gK, is homologous to HSV-1 glycoprotein K (gK) (Data not shown). The collinear relationship of gK and ICP27 genes is consistent with their positions in other alpha herpesviruses (Davison *et al.*, 1986; McGeoch *et al.*, 1988; Zhao *et al.*, 1992).

Translational start codons (ATG) of each ORF have been assigned at nt 427 for MDV gK and nt 1,639 for MDV ICP27, based on comparison with HSV-1 homologs and on similarity to Kozak's consensus sequence (Kozak *et al.*, 1989). A number of potential transcriptional elements are present both upstream and downstream of gK and ICP27. Two TATA boxes are located 269 bp and 294 bp upstream from the putative ATG of

MDV gK. There are three potential TATA boxes located 24 bp, 28 bp, and 87 bp upstream of the putative ATG of MDV ICP27. Three poly(A) signals were found at positions 3,132, 3,142, 3,146. No potential poly(A) signals were identified within the ORF53-ORF54 junction region. Interestingly, four copies of sequence elements, which are very similar to the IE-specific element TAATGARAT responsive to presence of the virion-associated a gene activator VP16 in HSV-1 IE genes, have been found within the putative promoter region of MDV ICP27 (Figure 2.2).

Analysis of MDV ICP27 and gK gene Transcripts

Northern blot hybridization was performed to detect the gene transcripts of both MDV gK and ICP27. Two DNA fragments, which map within ORF53 and ORF54 were used as probes for detection of transcripts. Probe BK which maps within ORF54 (Figure 2.1), hybridized to two transcripts (1.6kb and 2.9kb) in both MDV GA infected DEF cells and CHX-treated cells (Figure 2.4 A, lane 2 and lane 3). No transcript was detected in mock-infected cells or GA infected cells treated with phosphonoacetate (PAA) (Figure 2.4 A, lane 1 and lane 4). Probe CC which maps within ORF53 (Figure 2.1), only hybridized to the 2.9 kb transcript in GA-infected cells and infected cells treated with CHX (Figure 2.4 B, lane 2 and lane 3). These data, taken together, suggest that MDV gK and MDV ICP27 transcripts overlap, sharing a common transcriptional terminus, most likely due to read through transcription of the MDV gK gene. MDV ICP27 is also transcribed independently of the MDV gK gene. This result is consistent with DNA sequence analysis in that there are multiple poly(A) signals in the region downstream of the ICP27 gene, but not between gK and ICP27 (Figure 2.2). Both

transcripts were abundantly expressed in untreated MDV infected cells and in MDV infected cells treated with CHX. Neither transcript was detected in MDV infected cells treated with PAA. Accumulation of transcripts in CHX treated cells but not in PAA treated cells is characteristic of MDV IE genes (Lee *et al.*, 1974; Hong and Coussens, 1994).

Comparative analysis of MDV ICP27 and gK predicted amino acid sequences

Our laboratory is primarily concerned with investigating MDV gene regulation. Given the importance of HSV-1 ICP27 in early and late gene regulation in HSV-1 infected cells, we opted to focus additional attention on MDV ICP27. To further define the ORF54 gene as a homolog of HSV-1 ICP27, the deduced amino acid sequence of ORF54 was compared with ICP27 homologs from other alpha herpesviruses. The average amino acid sequence identity and similarity between MDV ICP27 and HSV-1 ICP27 are 25.8% and 42.5%, respectively; between MDV ICP27 and VZV ORF4 are 29.5% and 49.4%, respectively. A partial amino acid sequence is shown in Figure 2.3. Interestingly, significant amino acid conservation exists in the C-terminal region of all ICP27 homologues. We find 37.3% identity between MDV and HSV-1 ICP27 and 32.7% identity between MDV ICP27 and VZV ORF4 within the C-terminal region. In contrast, the N-terminal half of MDV ICP27 has poor homology with both HSV-1 and VZV, even though it shares a similar hydrophilic feature (data not shown). In addition, we note a conserved potential zinc finger metal-binding motif $C_{(442)}$-X_4-$C_{(447)}$-X_{13}-$H_{(461)}$-$C_{(467)}$ within the MDV ICP27 C-terminus (Figure 2.3).

Based on computer analysis, MDV gK shows a significant similarity to other

alpha herpesvirus counterparts, including HSV-1 glycoprotein K, and VZV ORF5. The average amino acid sequence identity and similarity between MDV gK and HSV-1 gK are 26.6% and 49.7%; between MDV gK and VZV ORF5 are 29.4% and 56.1%, respectively (Data not shown). Several common features existed between HSV-1 gK and MDV gK: i) a possible N-terminal signal sequence (amino acids 1-30 in HSV-1 gK and 1-29 in MDV gK); ii) several potential N-linked glycosylation sites present in N-terminal regions (two N-glycosylation sites in HSV-1 gK; four N-glycosylation sites in MDV gK); iii) the similar location of four potential hydrophobic transmembrane domains (Figure 2.2).

Analysis of MDV ICP27 gene products

Two DNA fragments, BC and KBg (Figure 2.1) were cloned into pATH bacteria expression vectors (Koener *et al.*, 1991) to generate in frame trpE-ICP27 fusion proteins. Clone KBg, encoding amino acids 205 to 308 of MDV ICP27, produced a 57 kDa fusion protein in great abundance (Figure 2.5). On the contrary, clone BC, which encodes amino acids 24 to 252 of ICP27 ORF and has a significant hydrophilic characteristic, failed to express in trpE expression vectors (Figure 2.5). Antisera were produced by immunization of New Zealand white rabbits with trpE-KBg fusion proteins, as described in Materials and Methods. To demonstrate specificity of antiserum against trpE-KBg fusion protein, *in vitro* translation and immunoprecipitation studies of MDV ICP27 were performed as described in Materials and Methods. A trpE-KBg antiserum immunoprecipitated a 55 kDa polypeptide from pBlue-ICP27 primed *in vitro* transcription/translation products. As expected, no product was detected with a

pRSET-BK primed reaction (Figure 2.6A). These results indicate that trpE-BKg antisera can specifically detect a MDV ICP27 gene product.

In HSV-1, ICP27 is essential for replication and growth of the virus (Sacks *et al.*, 1985). To determine if MDV ICP27 is expressed in lytically infected cells, western blot analysis was performed using MDV-infected cell extracts and our trpE-KBg antisera. A 55 kDa protein was detected in both MDV GA and Md11 infected DEF cells, but not found in mock-infected DEF cells (Figure 2.6B). The size of this polypeptide is consistent with that predicted from translation of the MDV ICP27 ORF.

DISCUSSION

We have identified and sequenced two genes located within the *Eco*RI-B fragment of the MDV genome. These correspond to coding units for HSV-1 UL53 and ICP27, based on gene arrangement and amino acid sequence similarity. MDV UL53 is similar to HSV-1 UL53 encoding glycoprotein K (gK) (DebRoy *et al.*, 1985). Several syncytial mutations of HSV-1 have been mapped to the UL53 gene (Pogue-Guile and Spear, 1987). The recent reports indicate that the natural HSV-1 UL53 gene product is involved in cell fusion of HSV-1 infected cells (Hutchinson *et al.*, 1992; Ramaswamy and Holland, 1992). Although MDV gK shows some common glycoprotein features and critical similarity to HSV-1 gK, such as N-terminal signal sequence, N-linked glycosylation sites, and similar location of hydrophobic transmembrane domains, the precise function of MDV gK remains to be determined.

HSV-1 ICP27 was reported to act as a transactivator of late gene promoters, when expressed in combination with ICP4 and ICP0, but has little or no effect by itself (Evert *et al.*, 1987; Smith *et al.*, 1991). HSV-1 ICP27 can also act as a transrepressor on several immediate-early and early gene promoters (Hardwicke *et al.*, 1989, Rice *et al.*, 1989). The positive and negative regulatory activities of HSV-1 ICP27 are separable (Rice *et al.*, 1989). Recent studies define the activator region as those sequences which lie between residues 260 and 434. The repressor region has been localized to the C-terminal 78 amino acids (Smith *et al.*, 1991). In contrast, VZV ORF4 directly transactivates plasmids containing homologous or heterologous promoters and has no apparent transrepressing activities (Moriuchi *et al.*, 1994). Interestingly, the C-terminal region of MDV ICP27,

HSV-1 ICP27, and VZV ORF4 are strikingly similar (37.3% identity between MDV ICP27 and HSV-1 ICP27; 32.7% identity between MDV ICP27 and VZV ORF4, see Figure 2.3). A putative zinc finger metal-binding domain within the C-terminal repressor region of HSV-1 ICP27 is conserved in MDV ICP27. HSV-1 ICP27 binds zinc in vitro (Smith *et al.*, 1991) and the zinc finger motif was shown to be involved in binding DNA, RNA and protein-protein interactions (Breg *et al.*, 1986). The importance of this element in ICP27 function is underscored by conservation in many alpha herpesviruses, such as VZV (ORF4) and EHV-1 (ORF3) (Perera *et al.*, 1994; Zhao *et al.*, 1992). Based on structure similarities, we speculate that MDV ICP27 may have similar functions in MDV gene regulation.

Four DNA elements were found within the MDV ICP27 putative transcriptional control region with striking similarity to the sequence TAATGARAT, an essential element of HSV-1 immediate-early gene promoters (Greves *et al.*, 1990). A virion tegument protein, VP16, interacts with cellular factor Oct-1 (octamer-binding protein), and forms a protein-DNA complex with the target sequence TAATGARAT in HSV-1 IE promoter regions (Gelman *et al.*, 1987; Moriuchi *et al.*, 1993). Recently, the gene encoding MDV VP16 has been identified (Yanagida *et al.*, 1992). Experiments to explore the possible activation of ICP27 expression by MDV VP16 are in progress.

Two significant features of MDV ICP27 were demonstrated in this report: i) Two different size transcripts are highly expressed in MDV infected cells treated with cycloheximide. Herpesvirus immediate-early (IE) genes usually express immediately upon infection and do not require de novo protein synthesis. Thus IE transcripts are the only viral RNA transcribed when infected cells are treated with a protein synthesis

inhibitor, such as cycloheximide. Our results suggest that both transcripts observed in northern blots with ICP27 probes belong to the immediate-early family; ii) Two transcripts were detected using probe BC (which maps within the ICP27 gene ORF). However, only the larger transcript (2.9kb) was detected using the probe CC (which maps within the gK ORF). Read-through transcription of the ORF53-54 gene region is likely due to absence of polyA signals between MDV gK and ICP27. The transcriptional pattern of MDV ICP27 and gK is similar to that of VZV and EHV-1 (Inchauspe *et al.*, 1989, Zhao *et al.*, 1992); but different from that of HSV-1 which contains two independent polyA signals (DebRoy *et al.*, 1985). It will be of considerable interest to learn whether MDV gK is translated as an immediate-early glycoprotein or if gK translation is repressed until later in infection. HSV-1 ICP27 is a phosphorylated protein located predominately in the infected cell nuclei, where it can be detected with monoclonal antibodies (Ackermann *et al.*, 1984). HSV-1 ICP27 is a multifunctional transcription/translation regulator. The importance of ICP27 is underscored by lethality of ICP27 deletions. It will also be of considerable interest to determine if MDV ICP27 displays the same multifunctional role as HSV-1 ICP27.

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Research Grant program administered by U.S. Department of Agriculture.

Figure 2.1 Diagram of MDV-GA structure and location of MDV gK and MDV ICP27. Genes are indicated by arrows. DNA fragments or plasmid clones are indicated by unbroken lines. Clone names are shown below the appropriate paragon. Restriction enzymes used are: B, *BamHI*; Bg, *Bgl*II; C, *Cla*I; E, *EcoR*I; K, *Kpn*I; Xh, *Xho*I.

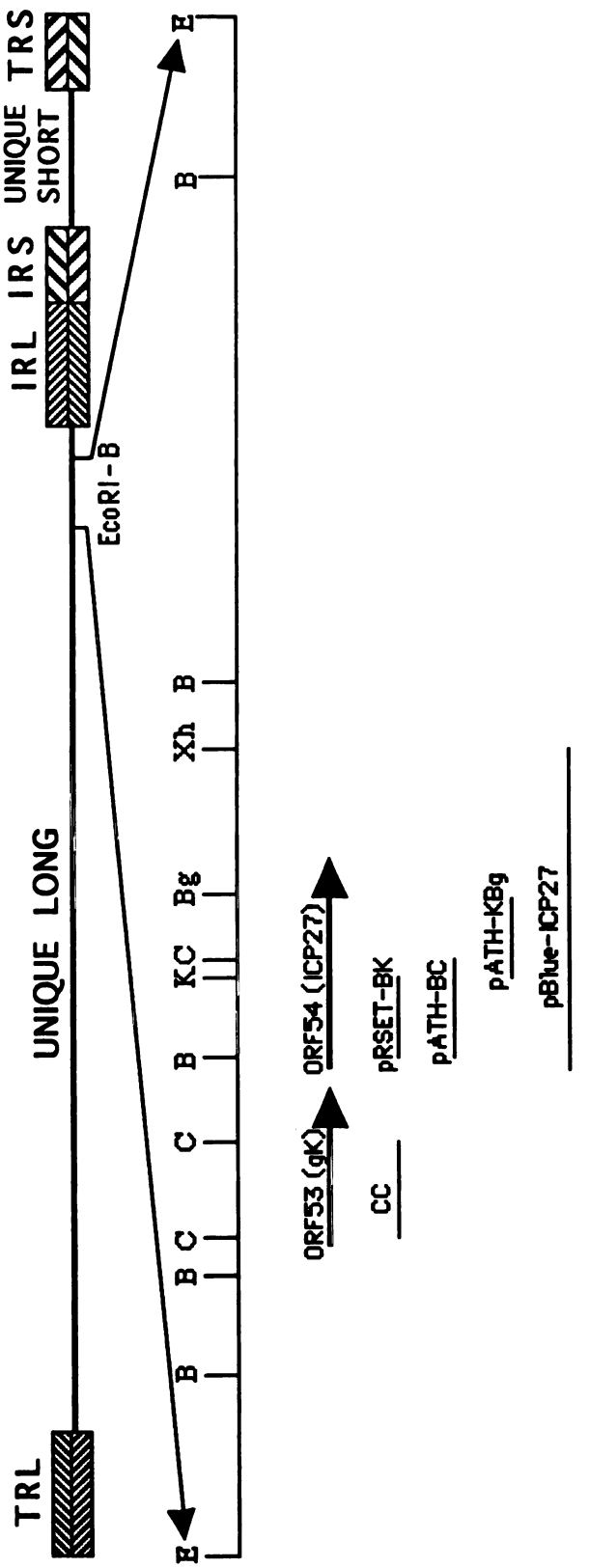

TRL
UNIQUE LONG
IRL IRS UNIQUE SHORT TRS
EcoRI-B
E
B
B C
C
B
KC
Bg
Xh
B
E
ORF53 (gK)
ORF54 (ICP27)
CC
pRSET-BK
pATH-BC
pATH-KBg
pBlue-ICP27

Figure 2.2 Nucleotide sequence of a 3,200 bp fragment of MDV GA from *EcoR*I-B. Predicted amino acid sequence of gK and ICP27 are indicated below the DNA sequence. Potentially important sequence motifs are indicated as follows: ^, TATA box; *, polyA signal. The four potential IE-specific regulatory elements (TAATGARAT) are underlined. Glycoprotein signal sequence, N-linked glycosylation sites and potential transmembrane domains are shown and underlined.

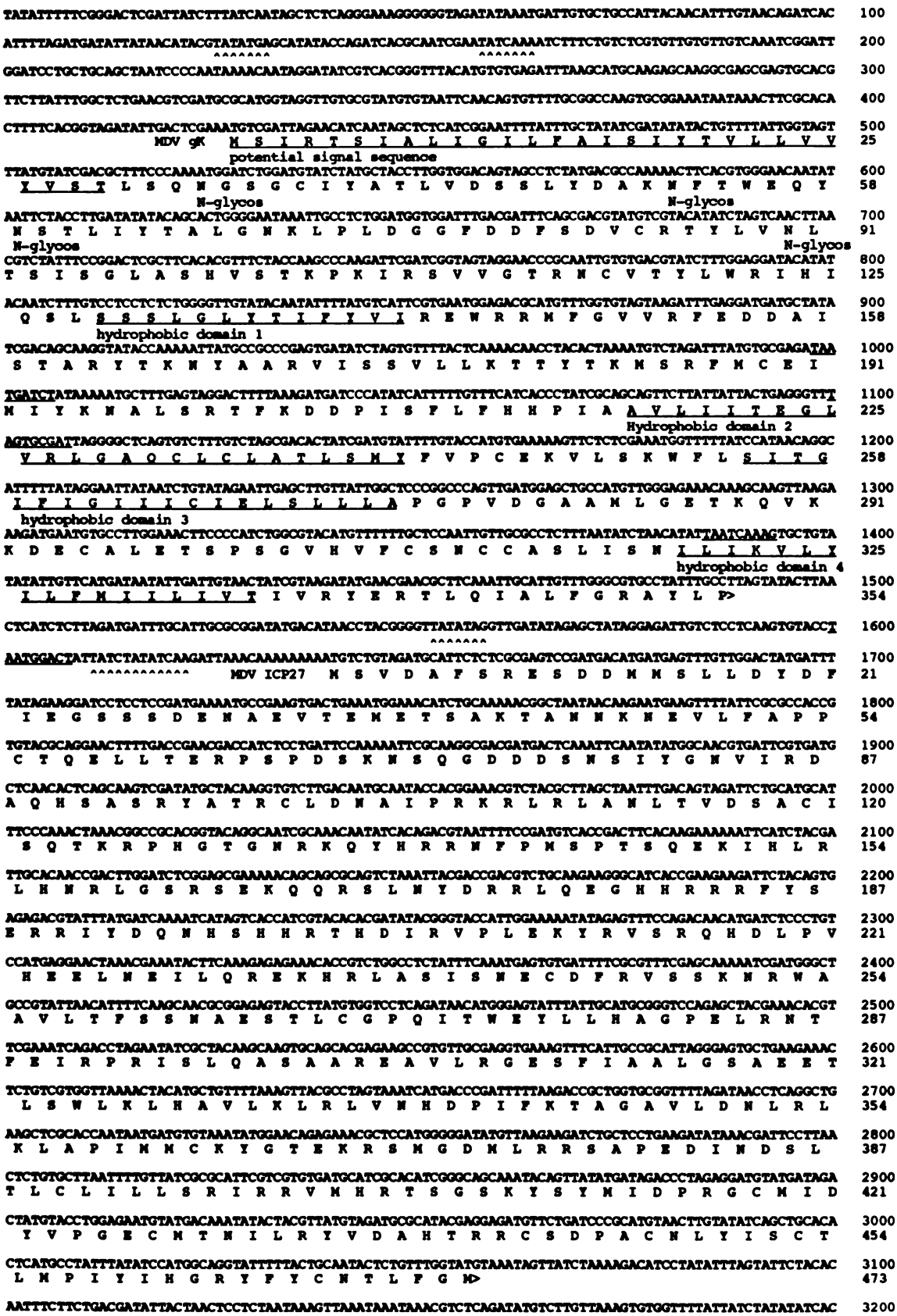

MDV gK
potential signal sequence
N-glycos
N-glycos
N-glycos
N-glycos
hydrophobic domain 1
Hydrophobic domain 2
hydrophobic domain 3
hydrophobic domain 4
MDV ICP27

Figure 2.3 Alignment of three herpesvirus C-terminal ICP27 amino acid sequences. Amino acids identical to those found in MDV ICP27 are boxed. A $C\text{-}X_4\text{-}C\text{-}X_{13}\text{-}H\text{-}X_5\text{-}C$ conserved zinc finger metal-binding motif is boxed and shaded.

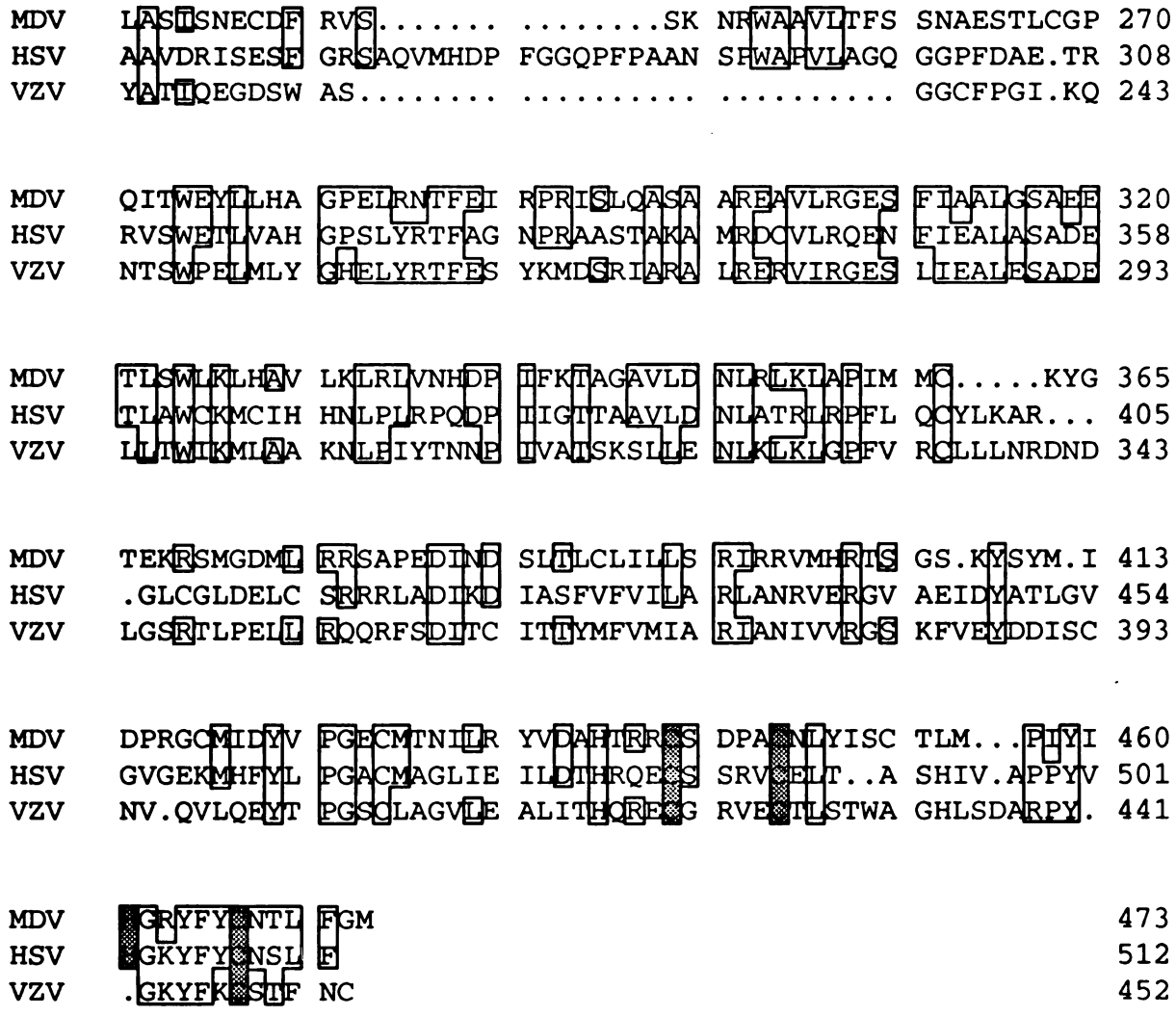

MDV LASTSNECDF RVS..... .....SK NRWAAVLTFS SNAESTLCGP 270
HSV AAVDRISESF GRSAQVMHDP FGGQPFPAAN SPWAPVLAGQ GGPFDAE.TR 308
VZV YATTQEGDSW AS..... .... GGCFPGI.KQ 243
MDV QITWEYLLHA GPELRNTFEI RPRISLQASA AREAVLRGES FIAALGSAEE 320
HSV RVSWETLVAH GPSLYRTFAG NPRAASTAKA MRDCVLRQEN FIEALASADE 358
VZV NTSWPELMLY GHELYRTFES YKMDSRIARA LREFVIRGES LIEALESADE 293
MDV TLSWLKLHAV LKLRLVNHDP IFKTAGAVLD NLRLKLAPIM MC.....KYG 365
HSV TLAWCKMCIH HNLPLRPQDP IIGTTAAVLD NLATRLRPFL QCYLKAR... 405
VZV LLIWIKMLAA KNLFIYTNNP IVATSKSLLE NLKLKLGPFV RCLLLNRDND 343
MDV TEKRSMGDML RRSAPEDIND SLTLCLILLS RIRRVMHRTS GS.KYSYM.I 413
HSV .GLCGLDELC SRRRLADIKD IASFVFVILA RLANRVERGV AEIDYATLGV 454
VZV LGSRTLPELL RQQRFSDITC ITTYMFVMIA RIANIVVRGS KFVEYDDISC 393
MDV DPRGCMIDYV PGECMTNILR YVDAHIRRSS DPAANLYISC TLM...PIYI 460
HSV GVGEKMHFYL PGACMAGLIE ILDTHRQEKS SRVFELT..A SHIV.APPYV 501
VZV NV.QVLQEYT PGSCLAGVLE ALITHQREKG RVEATLSTWA GHLSDARPY. 441
MDV GRYFYNTL FGM 473
HSV GKYFYNSL F 512
VZV .GKYFKSTF NC 452

Figure 2.4 Northern blot analysis of MDV ICP27 and gK. Total cellular RNA was isolated from infected or uninfected cells and 10ug RNA was loaded per well as described in Materials and Methods. Panel A: DNA fragment BK (see Figure 2.1) was used as probe; Panel B: DNA fragment CC (see Figure 2.1) was used as probe. Lane 1, mock-infected RNA; Lane 2, MDV GA infected-DEF RNA; Lane 3, MDV GA infected-DEF treated with cycloheximide; Lane 4, MDV GA infected DEF treated with PAA.

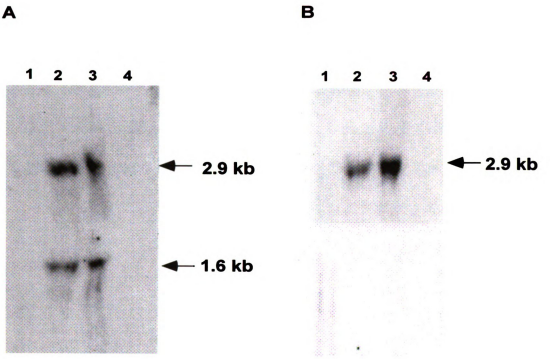

A
1 2 3 4
2.9 kb
1.6 kb
B
1 2 3 4
2.9 kb

Figure 2.5 TrpE-ICP27 fusion protein expressed in *E.coli*. Two DNA fragments of the MDV ICP27 coding region were cloned into pATH vectors to express trpE-ICP27 fusion proteins (see Materials and Methods). Lanes 1, pATH-KBg (see Figure 2.1); Lanes 2, pATH-BC (see Figure 2.1); Lanes 3-4, pATH3 vector control; Lane 1, 2, 3 were induced using IAA. TrpE-KBg fusion protein (57 kDa) and trpE protein (37 kDa) are indicated by the arrows.

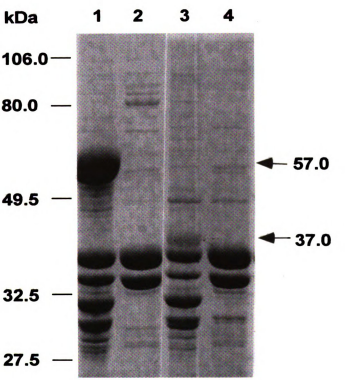
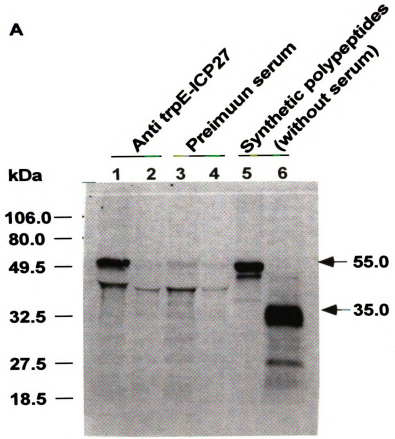
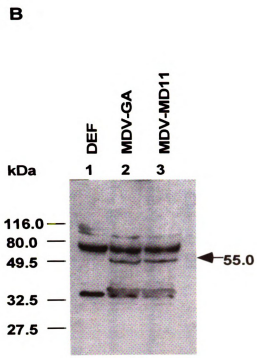

kDa
1
2
3
4
106.0
80.0
49.5
32.5
27.5
57.0
37.0

Figure 2.6 (A) Immunoprecipitation analysis of MDV ICP27 translation products in vitro. Lane 1, 3 and 5, pBlue-ICP27; Lane 2, 4 and 6, pRSET-BK; Lane 1 and 2, translation products were precipitated using rabbit antisera against trpE-KBg; Lane 3 and 4, normal rabbit serum; Lane 5 and 6, no serum control.

(B) Detection of MDV ICP27 in MDV-infected cells. West blot analysis was performed as described in Materials and Methods. Mock-infected DEF cell lysate (lane1) is used as the negative control. MDV GA strain (lane 2) and Md11 strain (lane 3) infected DEF cell lysates were pre-absorbed using normal rabbit serum and precipitated by Protein A Sepherose^{R} CL-4B (Pharmacia). MDV ICP27 was indicated by an arrow. Protein molecular weight was calculated by comparison to prestained protein standards (Bio Rad).


A
Anti trpE-ICP27
Preimuun serum
Synthetic polypeptides (without serum)
kDa
1 2 3 4 5 6
106.0
80.0
49.5
32.5
27.5
18.5
55.0
35.0


B
DEF
MDV-GA
MDV-MD11
kDa
1 2 3
116.0
80.0
49.5
32.5
27.5
55.0

Chapter III

A Marek's Disease Virus (MDV) Immediate-early Protein, MDV ICP27, Has Both Positive and Negative Regulatory Activities *in vitro*

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ABSTRACT

We previously reported a Marek's disease virus (MDV) immediate-early protein, MDV ICP27, which is a homolog to the HSV-1 *trans*-regulatory protein, ICP27. Here, we report that MDV ICP27 is a phosphoprotein and dominantly localizes to nuclei of CEF cells infected with serotype-1 MDV or with a fowlpox virus recombinant expressing MDV ICP27. In HSV-1 infection, ICP27 acts as a multi-functional nuclear protein and contains separable positive and negative functional domains. Independently, HSV-1 ICP27 has little or no effect on target promoters, but can enhance or repress target gene promoters in the presence of transcriptional activators ICP4 or ICP0. The regulatory activities of HSV-1 ICP27 are independent of target gene promoter sequences but, instead depend on presence of different mRNA processing signals. In this report, we present evidence that MDV ICP27 possesses both intrinsic trans-activation and trans-repression activities. MDV ICP27 can transactivates MDV pp14 and pp38 bi-directional promoters, but strongly represses the MDV thymidine kinase (TK) early gene promoter. In addition, a heterologous RSV-LTR U3 promoter is also stimulated by MDV ICP27. We further demonstrate that the region from amino acids 207 to 378 are critical for MDV ICP27-mediated repression activity. The substitution of 3' RNA signals in RSV-LTR promoter construct does not obviously affect MDV ICP27-mediated transactivation activity. In contrast, MDV TK promoter shown a negative response to MDV ICP27 when the SV40 small T 3'splicing site and the early poly(A) signal were replaced with the MDV ICP27 gene poly(A) signals in the TK promoter construct. These results suggest that MDV ICP27-mediated transactivation and transrepression activities may be mediated through

different transcriptional mechanisms. In contrast to HSV-1 ICP27, MDV IVCP27 dose not display the functional co-operative activity with MDV ICP4. No measurable activation or repression effects of MDV ICP27 on MDV immediate-early gene promoters (MDV ICP4 and ICP27) as well as a late gene promoter (gB) were observed. Thus, MDV ICP27 may play a minimal role in expression of these genes.

INTRODUCTION

Marek's disease virus (MDV) is a cell-associated avian herpesvirus that induces a T-cell lymphoma and nerve demyelination in susceptible chickens (Reviewed by Calnek, 1985). Originally classified as a γ-herpesvirus on the basis of its lymphotropism. MDV has a genome structure and gene content with more similarity to members of the α-herpesvirus group (Buckmaster *et al.*, 1988; Roizman *et al.*, 1992; Karlin *et al.*, 1994). Herpesvirus gene expression is typically regulated in a cascade fashion with genes classified into three classes, depending on expression kinetics. Immediate-early (IE, or α), early (E, or β), and late (L, or γ) gene classification depends upon requirements for viral protein synthesis and/or viral DNA replication. In HSV-1, β and γ genes are further subdivided into β_1 and β_2, γ_1 and γ_2, respectively (Roizman and Sears, 1995). Much of our knowledge regarding gene regulation in α-herpesviruses has been derived from studies of the prototype HSV-1 system. There are five IE gene products in HSV-1, including ICP0, ICP4, ICP22, ICP27, and ICP47. With the exception of ICP47, HSV-1 IE gene proteins have demonstrable regulatory functions that affect subsequent IE, E, and L gene expression (Reviewed by Roizman and Sears, 1995). ICP4 and ICP27 play essential roles in virus infection (DeLuca *et al.*, 1985; Sacks *et al.*, 1985; Roizman and Sears 1995). ICP4, the major transcriptional regulatory protein, is the most extensively studied IE protein in HSV-1. The major function of ICP4 is to participate in HSV-1 early and late gene transactivation and IE gene repression. ICP4 acts by binding to both consensus and non-consensus sites on DNA. The repression function of HSV-1 ICP4 is

dependent upon its DNA binding capability, in which ICP4 forms a tripartite complex with TBP and TFIIB on the consensus sequence of IE gene promoter regions (Smith *et al.*, 1993). More recent studies demonstrate that both tripartite-complex formation and transcription repression were efficient when the ICP4-binding site was down stream of a TATA box, within a short distance and in proper orientation (Kuddus *et al.*, 1995).

HSV-1 ICP27 is a 63kDa phosphorylated nuclear protein (Ackerman *et al.*, 1984). Studies with ICP27 temperature-sensitive and null mutants have revealed that ICP27 is involved in the negative regulation of immediate-early and early genes, as well as host cellular genes, but positively regulates late gene expression (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice *et al.*, 1990). The regulatory activities of HSV-1 ICP27 are independent of target gene promoter sequences but depend on the presence of different mRNA processing signals. Activation functions correlated with different polyadenylation sites, whereas repression function correlated with the presence of introns either 5' or 3' to target gene-coding sequences (Sandri-Goldin and Mendoza, 1992). Both positive and negative modulation domains have been mapped in the C-terminal half of HSV-1 ICP27 (Rice *et al.*, 1989; Hardwicke *et al.*, 1989). The N-terminal half of HSV-1 ICP27 protein is critical for nuclear localization (Mears *et al.*, 1995; Hibbard and Sandri-Goldin, 1995). Interestingly, Rice *et al.* (1993) reported that the acidic amino-terminal region of HSV-1 ICP27 is also participates in its regulatory activities in both lytic infection and transient expression.

Homologs of HSV-1 ICP27 have been identified not only in other α-herpesviruses, including Varicella-zoster virus (VZV) ORF4 (Perera *et al.*, 1994) and

equine herpesvirus-1 (EHV-1) gene 3 (Zhao *et al.*, 1992; Zhao *et al.*, 1995), but also in β- and γ-herpesviruses, including the UL69 gene of human cytomegalovirus (HCMV) and BMLF1 of Epstein-Barr virus (EBV) (Winkler *et al.*, 1994; Kenney *et al.*, 1989). All of the proteins encoded by these genes modulate gene expression. Studies of reporter-gene dependent activities of BMLF1 indicate that BMLF1 acts in *trans* by a post-transcriptional mechanism (Kenney *et al.*, 1989). In contrast, VZV ORF4-mediated induction of gene expression occurs primarily at the level of transcription (Perera *et al.*, 1994). Despite their apparent functional differences, all ICP27 homologs share a high amino acid residue conservation and a cysteine-rich domain in their C-terminal region.

Similar studies of MDV IE genes have been hampered by the highly cell-associated nature of MDV *in vitro* and low level of gene expression even in fully lytic infection. Characterization of RNA transcripts isolated from MDV infected cells treated with a metabolic inhibitor (e. g. cycloheximide) indicate that MDV IE gene transcripts are mainly clustered in repeat regions, similar to locations of other α-herpesvirus IE genes (Maray *et al.*, 1988; Schat *et al.*, 1989). Recently, several MDV IE genes were reported and three of them were identified as homologs to HSV-1 ICP4, ICP22, and ICP27 (Anderson *et al.*, 1992; Ren *et al.*, 1994; Hong and Coussens, 1994; Brunovskis and Velicer, 1995). MDV ICP4 is highly homologous to HSV-1 ICP4 and contains several similar functional domains as its counterpart in HSV-1 (Anderson *et al.*, 1992). MDV ICP4 is able to enhance expression of the MDV early pp38 gene promoter by transient expression assays using MDV transformed lymphoid cell lines (Pratt *et al.*, 1994). MDV ICP4 is also able to transactivate a Rous sarcoma virus long terminal repeat

(RSV-LTR) U3 promoter (Banders and Coussens, 1994). The MDV ICP27 gene homolog has been mapped to the *EcoR*I-B fragment of MDV DNA (Ren *et al.*, 1994). MDV ICP27 is a 473 amino acid polypeptide that has 26% amino acid identity with HSV-1 ICP27. By comparison to other α-herpesvirus homologs, MDV ICP27 shares a high amino acid conservation and contains a cysteine-rich domain and a potential zinc-binding motif within it's C-terminal region (Ren *et al.*, 1994). Furthermore, a basic and arginine-rich domain has been found between amino acids 150 to 200 (Ren *et al.*, 1994). Sequence and structure similarities suggested that MDV ICP27 may have important regulatory functions in MDV gene expression.

In this report, we demonstrate that the MDV ICP27 gene product is a prominent nuclear protein and is subject to post-translation phosphorylation. To evaluate potential regulatory functions of MDV ICP27, we transiently expressed the MDV ICP27 gene in chicken embryo fibroblast (CEF). Six MDV gene promoters representing all three MDV kinetic classes, and a heterologous promoter (RSV-LTR) were selected as target promoters and transiently cotransfected with MDV ICP27 expression constructs into CEF cells. MDV ICP27 independently transactivated MDV pp14 and pp38 gene promoters, as well as RSV-LTR promoter, which contains MDV-mediated induction elements (Banders and Coussens, 1994; Sun and Coussens, manuscript in preparation). In contrast, MDV ICP27 repressed expression from the MDV TK promoter. MDV ICP27-mediated repression was mapped to amino acid residues 207 to 378. We also reported that the substitution of 3'RNA processing signals on target promoters display to controversy response to MDV ICP27. Surprisingly, two MDV IE gene promoters (ICP4 and ICP27)

and a late gene promoter (gB) were not responsive to MDV ICP27 gene transfection. Little or no cooperative effect between MDV ICP27 and MDV ICP4 was observed on any target promoter.

MATERIALS AND METHODS

Cells and viruses

Preparation, propagation, and infection of CEF cell cultures with MDV and fowlpox virus (FPV) were as described previously (Solomon, 1975; Yanagida et al., 1992). MDV serotype 1 strain Md11 (passage 23 and passage 86) and GA (passage 14) were used for this study. A fowlpox virus recombinant expressing the MDV ICP27 gene (rFPV/ICP27) and a wild type fowlpox virus were kindly provided by Dr. N. Yanagida (USDA-ADOL, East Lansing, MI) for this study.

Plasmid constructions

All recombinant plasmids were generated by standard methods (*Sambrook et al.*, 1989). All enzymes used were from Bioehringer Mannheim (Indianapolis, IN), unless otherwise noted. To generate the MDV ICP27 expression plasmid M-ICP27CMV (Figure 3.1 B), a *Spe*I-*Xho*I fragment derived from plasmid pBlue-ICP27 (Ren *et al.*, 1994) which contains the entire MDV ICP27 coding region from -10 bp upstream of the ATG codon to +274 bp downstream of a stop codon was cloned into *Spe*I and *Xho*I sites of pBK/CMV vector (Stratagene, La Jolla, CA). To increase transcription efficiency in mammalian cells, a 200bp fragment of the prokaryotic *Lac* promoter region between the CMV promoter and the MDV ICP27 gene insert was removed and the plasmid was recircularized with T4 DNA ligase. To generate the MDV ICP4 expression plasmid M-ICP4CMV (Figure 3.1 B), a *Xho*I fragment containing 227 bp of upstream sequences and the entire coding region of MDV ICP4 was cloned into a *Xho*I site of pBK/CMV with

properorientation. The prokaryotic *lac* promoter region in M-ICP4 was removed as previously described. The three MDV ICP27 deletion mutant constructs, M-ICP27D1, M-ICP27D2, and M-ICP27D3, are derivatives of plasmid M-ICP27CMV (Figure 3.7 A). To generate M-ICP27D1 (a.a. 1-378), plasmid M-ICP27CMV was digested with *Bgl*II and *Xho*I, followed by end-filling with Klenow enzyme and ligation with T4 DNA ligase. For M-ICP27D2 (a.a. 1-207), a 1.1 kb fragment was excised from plasmid M-ICP27CMV with *Kpn*I digestion and recircularized with T4 DNA ligase. To create M-ICP27D3 (a.a. 36-473), a 280 bp fragment including the *lac* promoter and 80 bp of MDV ICP27 coding sequences was removed from plasmid M-ICP27CMV by *Nhe*I and *Bam*HI digestion followed by end-filling with Klenow enzyme and recircularization with T4 DNA ligase.

Three reporter plasmids, pSph, pp38CAT, and pp14CAT, have been described (Banders and Coussens, 1994; Abujoub *et al*, 1996). Briefly, for pSph, a 137 bp fragment from the RSV-LTR U3 region containing MDV-mediated transactivation elements was fused with the chloramphenicol acetyltransferase (CAT) gene in plasmid pCAT-Basic (Promega, Madison, WI). For pp38CAT and pp14 CAT, a 700 bp fragment from the MDV pp38 gene and pp14 gene bi-directional promoter region has been placed upstream of the CAT gene in opposing orientations (Figure 3.4 A) (Abujoub and Coussens, 1996). To generate TKCAT, the oligonucleotides 5'-CACGCATGCTACAT-CTAATACCATGACC-3' and 5'-GGGTCTAGAGTTCAATGGGAGAGAA-3' were used as upstream and downstream primers respectively, to amplify the MDV TK gene promoter (-243, +1). The 250 bp fragment amplified by PCR using DNA isolated form MDV strain MD11 infected CEF cells as template was digested with *Sph*I and *Xba*I, and

fused with the CAT gene in plasmid pCAT-Basic (Figure 3.4 A). In gBCAT, the oligonucleotides 5'-GGGAAGCTTGTATTTAAATGTGGCG-3' and 5'-GGGTCTAGA-GTGAGATGATCTTAATGTGC-3' were used as primers to amplify the MDV gB gene promoter (-361, +1) by PCR, followed by *Hind*III and *Xba*I digestion and cloning into the pCAT-Basic vector (Fig. 4 A). To create M-ICP27CAT, an *Xba*I-*Bsm*I fragment corresponding to DNA sequences (-660, +17) of the MDV ICP27 gene promoter was blunt-ended with T4 DNA polymerase and cloned into pCAT-Basic in the proper orientation. M-ICP4CAT plasmid was generously provided by Dr. M. Boussaha (Boussaha *et al.*, 1996).

To create a panel of reporter plasmids with RSV-LTR U3 and TK promoter-driven CAT constructs differing only in their 3' RNA processing signals, a 250 bp *Pvu*II fragment containing three poly (A) consensus elements followed by two GT-rich elements derived from the MDV ICP27 3'RNA region was cloned into the pBluescript KSII+/- vector in a *Sma*I site to generate p27PA. Oligonucleotide 5'-GCGGATAACA-ATTTCAC-3', which is identical to the upstream sequences of the CAT gene and 31 bp from a *Hind*III site in the multiple cloning sites of the pCAT-Basic, was used as 5'primer. Oligonucleotide 5'-GGATCCGCTTATCACTTATTCA-3' carrying a *Bam*HI site was used as 3' primer to amplify the CAT open reading frame without the 3' RNA processing signals, which includes a SV40 small T 3' splicing site and the early poly A signal. The PCR amplified CAT gene fragment flanked with *Xba*I and *Bam*HI was fused with 27PA. To create pCAT27PA (Figure 3.6 A), an *Xba*I-*Bam*HI fragment from the pCAT-Basic, which contains the CAT gene and 3' RNA processing signals as described above, was replaced with an *Xba*I-*Hind*III fragment containing the CAT gene followed by

a 27PA element. The pCAT27PA plasmid was used as parent plasmid to generate RSVCAT27PA, and TKCAT27PA, where the promoters are identical to those used in pSph and TKCAT, respectively, allowing evaluation of differences in 3'RNA processing signals only (Figure 3.6 A).

Expression of GST fusion proteins and antiserum production

A *Bam*HI-*Kpn*I fragment from M-ICP27CMV corresponding to amino acids 23-206 of MDV ICP27 was cloned into a pGEX2T vector (Pharmacia, Alameda, CA) to generate in frame glutathione S-transferase (GST) fusion proteins. Expression and purification of GST-ICP27 fusion proteins were performed according to manufacturer's specification. Purified GST-ICP27 fusion proteins were used to generate rabbit polyclonal antiserum as described previously (Hong and Coussens, 1994).

Indirect immunofluorescence assay

CEF cells infected with MDV strain Md11 and with fowlpox virus recombinant expressing MDV ICP27 gene (rFPV/ICP27) were fixed with acetone/methanol (1:1). Uninfected CEF cells and cells infected with wild type fowlpox virus were used as negative control. Indirect immunofluorescence assay was performed as described by Harlow and Lane, (1988). Anti GST-ICP27 serum was used as the primary antibody at a dilution of 1:40. Goat anti-rabbit IgG conjugated with fluorescein-5'-isothiocyanate (FITC) (Sigma) was used as the second antibody at a dilution of 1:20. Cells were visualized using a Laser Scanning Confocal Microscope (Carl Zeiss, Inc.) with a 488 nm argon laser line and a 520/560 barrier filter.

Immunoprecipitation and protein dephosphorylation assay

Mock-infected CEF cells, CEF cells infected with MDV serotype 1 strains MD11 and GA, and CEF cells infected with ICP27FPVr were cultured in 100 mm plates and pre-incubated in phosphate-free Dulbecco Modified Eagle medium (Gibco-BRL, Gaithersburg, MD) for 1 hour. Cells were labeled in 500 μCi ^{32}P orthophosphate per plate for 6 hours before harvesting (DuPont, Wilmington, DE). Cell lysates were immunoprecipitated with GST-ICP27 antiserum and protein A-agarose beads (Pharmecia, Alameda, CA) as described by Hallow and Lane (1988). Immunoprecipitates were washed, resuspended with 1X SDS-PAGE loading buffer, and analyzed by electrophoresis in a 12.5% SDS-PAGE minigel (Bio-Rad, Richmond, CA).

For the protein phosphatase assay, samples were divided into two parts after final washing of immunoprecipitation reactions and one part was added with 20 IU of calf intestinal phosphatase (CIP) for 1-2 hours at 37^0C. Dephosphorylation was terminated by washing with lysis buffer (50 mM Tris, 500 mM NaCl, 1%NP40). CIP treated and control samples were analyzed by electrophoresis in 12.5% SDS-PAGE minigels.

Western blot analysis and Protein translation in vitro

MDV infected CEF cells or uninfected CEF cells were lysed, cellular proteins were separated on a 12.5% SDS-PAGE minigel and electrophoretically transferred to nitrocellulose membranes. Immune blotting was performed as previously described (Ren et al., 1994). GST-ICP27 polyclonal antiserum was used at a 1:200 dilution and donkey

anti-rabbit IgG conjugated with horseradish peroxidase (Amersham, Life Science, England) was used as second antibody at a 1: 4000 dilution. Amersham ECL Western blot kit was used for detection essentially according to the manufacturer's specification.

For protein translation *in vitro*, plasmid pBlueICP27, in which the MDV ICP27 gene was placed under the T7 promoter as described previously (Ren *et al.*, 1994), was used as transcription template. The TNT/T7 coupled reticulocyte lysate system (Promega, Madison, WI) was used for coupled *in vitro* transcription and translation as described previously (Ren *et al.*, 1994).

DNA transfection and CAT assay

All DNA transfections for transient-expression assays were performed by electroporation with a Gene-Pulser Electroporater (Bio-Rad, Richmond, CA). Primary cultured CEF cells were removed from plates by exposure to 0.05% trypsin and washed twice with PBS. After final washing, cells were resuspended with HBS 2X buffer (50 mM HEPES, pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4) at a density of $7.5x10^6$ cells/ml. 800ul of cell suspension ($6X10^6$ cells) was mixed with defined quantities of plasmid DNA and incubated on ice for 15 to 20 minutes. Cell mixtures were transferred into an electroporation cuvette and electroporated with a single pulse at 350 mV and 960 μF with a capacitance extender. After pulsing, cells were incubated at room temperature for 10 minutes and then plated equally into three 60mm tissue culture plates in 3ml of Leibovitz-McCoy medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% calf serum (Gibco BRL, Life Technologies, NY). Cells were grown for 48

hours before harvesting. Total amount of plasmid DNA used for each individual transfection was adjusted to a consistent level by adding pBK/CMV. The amount of plasmid DNA used in each particular transfection experiment noted in the text and figure legends. A CMV-β-gal plasmid (0.3 ug, Kindly provided by Drs. R. F. Silva and J. D. Reilly, USDA-ADOL, East Lansing, MI) was included as internal control for transfection efficiency in each individual experiment.

Cells were harvested 48 hours after transfection and disrupted by three cycles of freezing and thawing in 0.1M Tris-HCl (pH 7.8). Protein concentration of cell extracts was determined by Lowry Assay. CAT activity was assayed using the same amount of total protein for all samples in an individual experiment. CAT assays were performed using a simultaneous diffusion assay as described by Neumann *et al.*, (1987). All transfections and CAT assays were repeated at least three times with three replica plates per individual experiment to control for variability. CAT activity was normalized by assessing the β-gal activity present in each cell extract as described previously (Sambrook *et al.*, 1989)

RESULTS

MDV ICP27 is a nuclear phosphoprotein.

Protein subcellular localization is determined, at least partially, by the function of the target protein. To determine if MDV ICP27 is transported to infected cell nuclei, as most other herpesvirus IE proteins are, we performed indirect immunofluorescence assays. As expected, MDV ICP27 specific staining was predominantly localized to the nuclei in both CEF cells infected with MDV serotype 1 strain Md11 (Figure 3.2 A) and with rFPV/ICP27 (Fig.2 B). In wild type MDV infected cells, ICP27 displayed nearly exclusive nuclear localization with diffuse staining well-distributed within the nuclei (Figure 3.2 B). rFPV/ICP27 infected CEF cells were chosen as positive control because they offer abundant expression of MDV ICP27 that was readily detected by MDV ICP27 specific antiserum. Second, other MDV gene products expressed in PFV recombinants exhibited the same subcellular localization as in the wild type MDV infected cells (Yanagida *et al.*, 1992; Yoshida *et al.*, 1994). As shown in Figure 3.2 B, ICP27 specific staining occurred with a very strong nuclear localization in rFPV/ICP27 infected cells. However, the distribution pattern was distinguished from wild type MDV infected cells in that specific staining was granulated with defined high density regions (Figure 3.2 B). As expected, ICP27 specific staining signals were not detected in either mock infected CEF cells or CEF cells infected with wild type FPV (Figure 3.2 C and D). We previously reported that a 55 kDa polypeptide was detected by anti trpE-ICP27 specific antiserum in MDV serotype-1 infected DEF cells (Ren *et al.*, 1994). Another smaller polypeptide (52 kDa) was also detected in MDV serotype 1 infected CEF cells as well as

in rFPV/ICP27 infected cells when GST-ICP27 antiserum was used in immunoprecipitation or Western blot analysis (Figure 3.2 A). Failure to detect the smaller polypeptide with trpE-ICP27 antiserum may be due to the relative poor title of this antibody, particularly since the fragment used for production of this polypeptide was derived from the C-terminal region of MDV ICP27, a region of poor predicted antigenicity. In contrast, the fragment used for GST fusion antibody expression was from the N-terminal half of MDV ICP27, a region with very high antigenic features. The most likely explanation for presence of two distinct ICP27 species was post-translational modification of precursor polypeptide. Give the role of other ICP27 homologs in transcriptional regulation, and presence of 14 potential sites for phosphorylation of serine or threonine in the predicted MDV ICP27 amino acid sequence, it seemed most likely that the 52 kDa species represented a precursor polypeptide and 55 kDa species resulted from phosphorylation of this precursor. To determine if MDV ICP27 is indeed phosphorylated, CEF cells infected with rFPV/ICP27 were labeled with ^{32}P and immunoprecipitated with anti GST-ICP27 antiserum as described in Materials and Methods. SDS-PAGE analysis of immunoprecipitation samples revealed that only the 55 kDa polypeptide was detectable in ^{32}P labeled rFPF/ICP27 infected cell extracts. A similar phosphoprotein was not detected in mock-infected or wild type FPV infected cells (Figure 3.3 B). Significant reduction in intensity of ^{32}P labeled ICP27 bands in CIP treated samples suggested that the 55 kDa form of MDV ICP27 was sensitive to phosphatase (Figure 3.3 C).

MDV ICP27 can selectively transactivate or transrepress different target

promoters.

To evaluate if MDV ICP27 is a transcriptional activator, we undertook transient expression / reporter construct experiments. Although this method cannot fully mimic the complex regulatory interactions that occur in MDV infected cells, it provides an appropriate strategy for studying the regulatory properties of a given protein. We constructed an MDV ICP27 gene expression plasmid, M-ICP27CMV, in which the coding region of MDV ICP27 gene was placed under the strong constitutive promoter of human cytomegalovirus immediate-early promoter (CMV). Expression of MDV ICP27 from this construct in transfected CEF cells was verified by Western blot analysis (Figure 3.7 C). Seven reporter constructs where various target promoters were fused with the CAT gene were co-transfected with M-ICP27CMV plasmid as described in Materials and Methods. Basal CAT activity of reporter promoters in the absence of effector plasmid (M-ICP27CMV) was arbitrarily set as 1.0. Fold induction of CAT activity following cotransfection of reporter plasmid with M-ICP27CMV was calculated relative to basal CAT activity. As summarized in Figure 3.4 B, four reporter constructs were responsive to MDV-ICP27, including pp14CAT, pp38CAT, TKCAT, and pSph. Three reporter constructs, M-ICP4CAT, M-ICP27CAT, and gBCAT, exhibited no obvious responsiveness to MDV ICP27.

MDV pp14 and pp38 genes are controlled by a bi-directional promoter region (Chen and Velicer, 1991; Cui *et al.*, 1991; Hong and Coussens, 1994). Co-transfection studies indicated that MDV ICP27 can significantly transactivate both pp14 and pp38 promoters. CAT activities of in extracts of cells co-transfected with pp38CAT and pp14CAT were increased 12.5 fold and 3 fold, respectively, in the presence of M-

ICP27CMV. In contrast, the activity of another MDV early promoter, the TK gene promoter, was strongly depressed (12.5-fold) by co-transfection with M-ICP27CMV (Figure 3.4 B). Interestingly, MDV ICP27 can also stimulate a heterologous promoter construct, pSph, which was derived from RSV-LTR U3 sequences. The relative CAT activity of pSph was increased approximating 2.0 fold when co-transfected with M-ICP27CMV (Figure 3.4 B). By comparison to other responsive promoters, activation of pSph by MDV ICP27 appears rather weak, however, pSph exhibits a very high level of basal CAT activity. Therefore, the reduced level of induction may be partially due to enhancement beyond the liner range of our CAT assay.

To define more clearly whether MDV ICP27CMV can directly affect target promoters and if concentration of M-ICP27 plasmid was critical to reporter promoter activation, increasing amounts of M-ICP27CMV plasmid were co-transfected with a consistent amount of various reporter plasmids. As shown in Figure 3.5A, when 2 ug of pp14CAT was co-transfected with 0.25 ug of effector plasmid, CAT activity increased only 1.1 fold. Increasing effector plasmid concentration up to 2.0 ug per plate, a peak of CAT activity 4-fold above basal expression levels was observed. A dose-dependent pattern was also observed in MDV ICP27-mediated transrepression activity with the TK reporter plasmid, TKCAT, with a maximum repression of 33-fold at 1.5 ug effector plasmid per plate (Figure 3.5 B).

MDV ICP27 dose not co-operate with MDV ICP4.

In HSV-1, it is clear that ICP27 has little or no intrinsic effect on target gene expression. Instead, HSV-1 ICP27 is able to affect both positive and negative regulatory

activities when in the presence of two additional HSV-1 transcriptional activators, ICP4 or ICP0 (Sandri-Goldin *et al.*, 1992). In contrast, ICP27 homologs in VZV (ORF4) and EHV (1 UL3) independently transactivate certain target promoters (Inchauspe, *et al.*, 1989; Zhao, *et al.*, 1995). To determine if MDV ICP27 could co-operate with MDV ICP4 in promoter activation or repression, we constructed a MDV ICP4 expression plasmid, M-ICP4CMV (Figure 3.1 B), and employed transient co-transfection assays. Three MDV ICP27 responsive promoter constructs, pp14CAT, pp38CAT and pSph, were also stimulated by MDV ICP4 alone (Table 3.1). This finding is consistent with previously published reports (Pratt *et al*, 1994; Banders and Coussens, 1994). Addition of MDV ICP27 had no demonstrable effect on promoter activation by MDV ICP4 with any of the all three target promoter constructs (Table 3.1). Thus, MDV ICP27 and MDV ICP4 are both capable of independently activating homologous and heterologous gene promoters but exhibit no co-operativity This pattern of independent activation without co-operation supports the observation that MDV ICP27 and ICP4 are functionally more similar to VZV ORF4 and ORF62, respectively, than to their respective HSV-1 counterparts.

Effects of 3'RNA processing signals on MDV ICP27-mediated trans regulatory activities.

Our preliminary *in vitro* studies indicated that MDV ICP27 selectively and independently modulates certain MDV gene promoters as well as a heterologous (RSV-LTR) promoter. To determine if 3' RNA-processing signals affect MDV ICP27-mediated gene-modulation as has been described for HSV-1 ICP27, we created a panel

of reporter constructs, RSVCAT27PA and TKCAT27PA (Figure 3.6 A) which are derived from pSph and TKCAT, respectively. Each plasmid differs from it's parent only in the 3' RNA-processing signals. As summarized in Figure 3.7 B, pSph (SV40 poly A signal) and RSVCAT27PA (ICP27 poly A signal) respond similarly to MDV ICP27 expressed by co-transfection with M-ICP27CMV. This result suggests that different 3'RNA processing signals may not affect MDV ICP27-mediated transactivation activity of the RSV-LTR promoter. However, the basal activity of RSVCAT27PA activity was reduced 2-3 fold relative to pSph (Data not shown), presumably as a result of the reduced stability of an unspliced transcript. Interestingly, CAT activity in cells transfected with TKCAT decreased 10-12 fold in the presence of MDV ICP27, but no measurable fold change (increase or decrease) was observed when TKCAT27PA was co-transfected with M-ICP27CMV. We also noted that the basal promoter activity of TKCAT27PA was 2 to 3-fold less than the parental TKCAT construct (data not show). However, no obvious repression activity was observed even a over range of concentration of TKCAT37PA was cotransfected with M-ICP27CMV. These results indicated that the abolishment transrepression of MDV ICP27 on the TK promoter was not due to the reduced basal promoter activity, but due to the 3'RNA processing signal substitution of TK promoter construct.

Functional dissection of MDV ICP27.

Our studies imply that MDV ICP27 may possess complicated regulatory activities since it not only transactivates homologous (e.g. pp14 and pp38) and heterologous (RSV-LTR) gene promoters, but also strongly transrepresses the MDV

TK promoter. To further map and gain an insight into potential MDV ICP27 functional domains, we created three MDV ICP27 deletion mutants, as illustrated in Figure 3.7A and described in Materials and Methods. In M-ICP27D_1, a 95 amino acid segment containing the cysteine-rich domain and a potential zinc-finger motif was deleted from the C-terminus of MDV ICP27. In M-ICP27D_2, the entire C-terminal half of MDV ICP27 was deleted. In M-ICP27D_3, only 35 amino acids from the N-terminus were deleted. To map the MDV ICP27 domain responsible for promoter repression, 2.0 ug of TKCAT plasmid was transfected alone or in combination with 1.5 ug of MDV ICP27 mutant expression plasmids. Altered MDV ICP27 polypeptides expressed from M-ICP27D1 and M-ICP27D3 repressed MDV TK promoter activity to the same extent as wild type MDV ICP27 (Figure 3.7B). In contract, M-ICP27D_2 caused little or no repression on MDV TK promoter activity. These results suggest that amino acids from 207 to 378 contain functional domains involved in negative modulation of the MDV TK promoter. All three MDV ICP27 mutants lost the ability to transactivate RSV-LTR and pp14 gene promoters. To verify that differences between MDV ICP27 mutants were not due to altered protein expression or transfection efficiency, CEF cells were transfected with 3.0 ug of each plasmid DNA by electroporation and cell extracts were obtained after 48 h. Immunoblot assay was performed using GST-ICP27 antiserum as described in Materials and Methods. As shown in Figure 3.7C, comparable specific polypeptides were detected in cell extracts from M-ICP27CMV and the three mutants. Furthermore, all polypeptides exhibited a molecular size consistent with their respective deletion. As expected, no MDV ICP27 specific polypeptide was detected in cell extracts transfected with pBK/CMV plasmid. Immunoblot results indicate that the loss of regulatory

activities in mutated M-ICP27CMV constructs were not due simply to mutation-induced polypeptide instability.

DISCUSSION

We previously reported that a MDV immediate-early protein, MDV ICP27, has significant amino acid identity and protein structure similarity to HSV-1 ICP27 and VZV ORF4 protein (Ren *et al*, 1994). In the present study, we demonstrated that MDV ICP27, like it's HSV-1 homolog, is a phosphorylated nuclear protein. Predominant nuclear localization of MDV ICP27 in MDV serotype 1 infected cells and in CEF cells infected with rFPV/ICP27 suggest that MDV ICP27 may contain signal peptides which direct the protein to the cell nucleus during MDV infection. Recently, a strong nuclear localization signal (NLS) and a nucleolar localization signal (NuLS) have been identified and mapped within the N-terminal half of HSV-1 ICP27 (amino acid residues 110 to 137 and 110 to 153, respectively) (Mears *et al.*, 1995). NLS of HSV-1 ICP27 bears similarity to the bipartite NLSs found in *Xenopus laevis* nucleoplasmin (Robbins *et al.*, 1991), whereas the NuLS includes NLS as well as 15 contiguous residues which consist entirely of arginine and glycine residues (the RGG box), a putative mRNA binding motif. (Mears *et al.*, 1995). Although neither the bipartite NLS elements nor an RGG box has been observed in MDV ICP27, MDV ICP27 contains a region rich in basic amino acids (40% basic amino acid residues and 24% arginines) from amino acid residues 151 to 200. Despite absence of a universal consensus sequence for NLSs, most are short sequences with a high basic amino acid content (Robbins *et al.*, 1991). Conservation of basic and arginine rich in MDV ICP27 suggests that these sequences have some potential relationship with its predominant nuclear localization of MDV ICP27 property. However, more defined mutagenesis studies will be required to verify

this possibility.

MDV ICP27 can significantly and independently tansactivate MDV pp14 and pp38 gene promoters in transient transfection experiments. It is notable that pp14 and pp38 genes are located within MDV genomic repeat regions (IRL and TRL), which have been intensively investigated due to a potential relationship to MDV oncogenicity and abundant expression of immediate-early transcripts (Maray *et al.*, 1988; Schat *et al.*, 1989). MDV pp14 and pp38 genes share a common control region which has been defined as a true bi-directional promoter and both gene products can be detected in MDV oncogenic serotype 1 transformed lymphoblastic cell lines (Cui *et al.*, 1991; Chen and Velicer, 1992; Hong and Coussens 1994). It is also noteworthy that pp38 and pp14 genes are classified as belonging to different kinetic classes. MDV pp14 is classified as an immediate-early gene whereas pp38 is considered as an early gene (Hong and Coussens, 1994; Chen and Velicer, 1992). There are several *cis*-acting elements including two TATA boxes, two Sp1 sites, two CAAT sits and one Oct-1 site within the pp38/ pp14 control region (Cui *et al.*, 1991). Pratt *et al.* (1994) demonstrated that MDV ICP4, can enhance pp38 and pp14 gene expression in MDV transformed lymphoblastic cell lines. This observation was further supported by our *in vitro* studies using M-ICP4CMV expression plasmid. Our studies combined with previous reports lead us to conclude that MDV pp14 and pp38 gene promoters are responsive to MDV ICP27 and MDVICP4. Furthermore, MDV ICP27 *in vitro* translation products did not specifically retard any DNA fragments derived from the pp38 and pp14 promoter region as determined by mobility-shift assay (Data not shown). This observation suggests that MDV ICP27-mediated transactivation may not directly involve DNA-protein interactions. However, it

is equally possible that modification present on ICP27 (e.g. phosphorylation) are required for direct DNA binding. Ongoing studies with mutant pp14 and pp38 promoters constructs will further define which cis-acting elements, if any, within these promoters, are critical for MDV ICP27- or MDV ICP4- mediated transactivation (Abujoub and Coussens, 1996).

There are considerable differences in ICP27 homologs with respect to modulating TK gene promoters. For example, HSV-1 ICP27 by itself has no or little effect on HSV-1 TK promoter activity, but represses HSV-1 ICP4- or ICP0-mediated transactivation of the HSV-1 TK promoter (McCarthy *et al.*, 1989; Rice and Knipe , 1990). In addition, HSV-1 ICP27 mediated transrepression of the HSV-1TK promoter occurs via a post-transcriptional regulatory mechanism which depends on the 3' RNA processing signals (Sandri-Goldin *et al.*, 1992). In contrast, VZV ORF4 can independently transactivate both homologous TK promoter as well as HSV-1 TK promoter (Inchauspe and Oatrove, 1989). Like HSV-1 ICP27, MDV ICP27 also possesses both positive and negative regulatory activities. It not only positively modulates certain MDV gene promoters, but also strongly represses the MDV TK gene promoter. Interestingly, the 3'RNA processing signals dose not affect MDV ICP27-mediated promoter transactivation. In contrast, MDV ICP27 failed to transrepress the TK promoter, when the SV40 small T splicing site and the early gene poly(A) were substituted with MDV ICP27 poly(A) signals (Figure 3.6A). However, it is not clear if MDV ICP27 is involved in mRNA processing. These results suggest that MDV ICP27-mediated transactivation and transrepression may be via the different transcription machinery. This apparent functional conflict prompted us to further analyze target promoter sequences. The MDV

TK promoter sequence displays some features different from those found in the HSV-1 TK promoter. C/EBP (CCAAT box), Sp1 (two sites), OTF-1 (octamer motif) *cis*-acting elements in HSV-1 TK promoter region are not found in the MDV TK promoter. Whether these promoter differences lead to functional results such as MDV ICP27-mediated transrepression of the TK promoter is, at present, not clear.

Our studies clearly demonstrate that MDV ICP27 can either positively or negatively regulate certain MDV gene promoters. Our further experiments using deletion mutants indicated that the amino acid segment from 207 to 378 is critical for MDV ICP27-mediated repression on MDV TK promoter. However, we failed to map a specific region which involves MDV ICP27-mediated transactivation activity. Lacking transactivating activity of all three deletion mutants of MDV ICP27 suggested that MDV ICP27-mediated positive regulatory is not a single region, but seems a multiple region related activities. Although evidence obtained from these deletion mutants implies that MDV ICP27-mediated both positive and negative regulatory functions possess the genetically separable activities, we still do not know if these two sets of regulatory activities are entirely independent.

Two MDV IE gene promoter constructs (M-ICP4CAT and M-ICP27CAT) and one MDV late gene promoter construct (gBCAT) revealed no measurable response to M-ICP27CMV co-transfection (Figure 3.4 B). However, M-ICP4CAT and M-ICP27CAT display very low basal CAT activities in transient transfection assays (Data not show). As a result, these reporter constructs may be not sensitive in detecting repression activity if MDV ICP27 has negative effects on MDV IE gene expression. In HSV-1 infection, ICP27 is required for efficient late gene expression. However, VZV

ORF4 protein has little or no effect on VZV late gene expression (Sandri-Goldin *et al.*, 1992; Perera *et al.*, 1994). Failure of MDV ICP27 to augment expression from gB promoter/reporter construct imply that MDV ICP27 may play the minimal role for expression of MDV late genes.

Co-infection of susceptible birds with MDV and ALV leads to enhanced incidence of avian leukosis virus-induced lymphoid leukosis (Bacon *et al.*, 1989). *In vitro,* MDV gene products or cellular proteins induced by MDV infection efficiently transactivate Rous Sarcoma virus long terminal repeat (RSV-LTR) promoters (Tieber *et al.*, 1990). MDV-responsive elements within RSV-LTR promoters were localized to a 28-bp segment from -109 to -137 within RSV-LTR promoter. Furthermore, we also demonstrated that MDV ICP4 was, at least, partially responsible for MDV-mediated transactivation of RSV-LTR in vitro (Banders and Coussens, 1994). However, MDV ICP4-mediated transactivation on RSV-LTR was much less efficient than with intact MDV, suggesting that other MDV-encoded products are likely to play a role in MDV-mediated transactivation of RSV-LTR promoter. Interestingly, here we demonstrated that MDV ICP27 was also able to transactivate RSV-LTR promoter. Although MDV ICP27 and ICP4 mediated transactivation of RSVLTR promoter via a consensus region (-137 to +11), molecular mechanism underlying the *trans* regulatory activities of MDV ICP27 or MDV ICP 4 should be addressed in future studies. It is notable that our present studies provide another worthy candidate for studying the interaction between MDV and ALV.

ACKNOWLEDGMENTS

We thank Dr. A. A. Abujoub for kindly providing pp14CAT and pp38CAT constructs, Mr. Ronald Southwick and Ms. Carolyn Cook for excellent technical assistance. This work was supported, in part through the Michigan Agricultural Experiment Station, the Center for Animal Production Enhancement, and USDA NRI Competitive Grant #

Figure 3.1 (A) Transcript maps of MDV ICP27 and MDV ICP4. The location and orientation of both gene transcripts are indicated by arrows. (B) Structures of MDV ICP27 and ICP4 transient expression constructs. A human cytomegalovirus immediate-early promoter (CMV, open bars) was fused with each gene coding region (cross-hatched bars) followed by their own 3'RNA region (ploy A, unbroken lines)

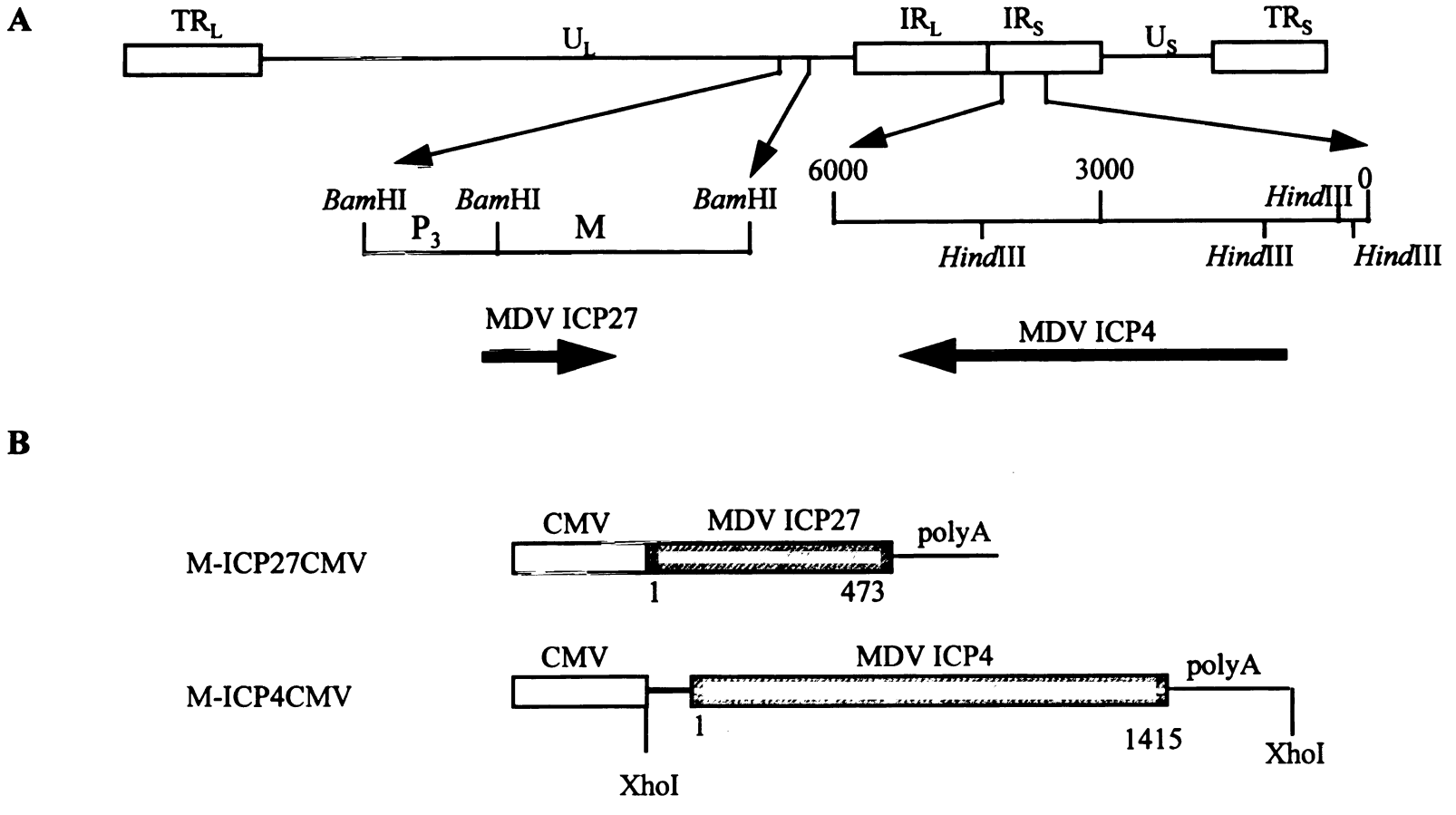

A
TRL
UL
IRL
IRS
US
TRS
BamHI
BamHI
BamHI
P3
M
6000
3000
0
HindIII
HindIII
HindIII
HindIII
MDV ICP27
MDV ICP4
B
M-ICP27CMV
CMV
MDV ICP27
polyA
1
473
M-ICP4CMV
CMV
MDV ICP4
polyA
1
1415
XhoI
XhoI

Figure 3.2 Subcellular localization of MDV ICP27 by indirect immunofluorescence staining. Virus or Mock-infected CEF cells were incubated with GST-ICP27 polyclonal antiserum at a dilution of 1:40 and were stained with Goat anti-rabbit IgG conjugated with FITC at a dilution of 1: 20. The cells were visualized with Laser Scanning Confocal Microscope using 488 nm argon laser line and 520/560 barrier filter. (A) CEF cells infected with MDV-1 strain Md11. 50X. (B) CEF cells infected with a fowlpox virus recombinant expressing MDV ICP27 (rFPV/ICP27). 50X. (C) Mock-infected CEF cells. 20X. (D) CEF cells infected with fowlpox virus wild type. 50X.

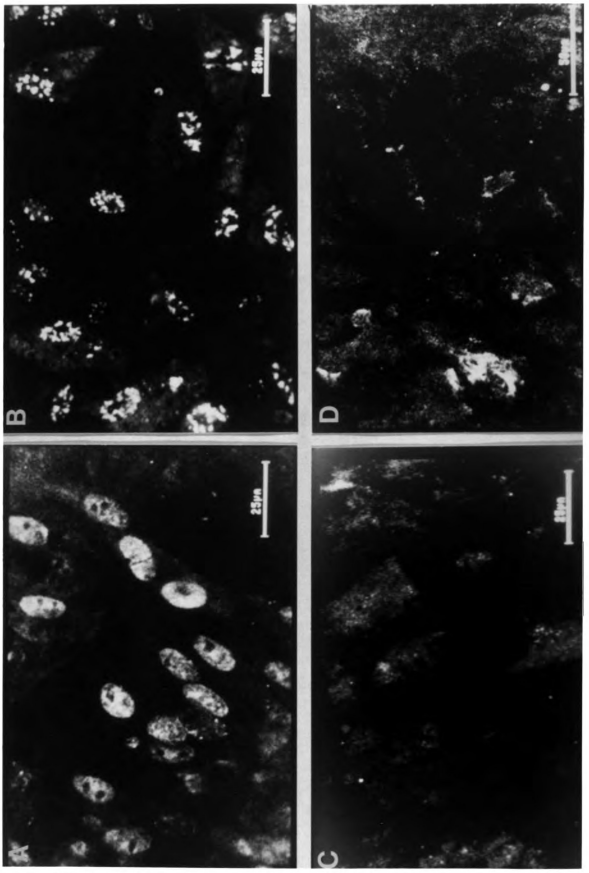
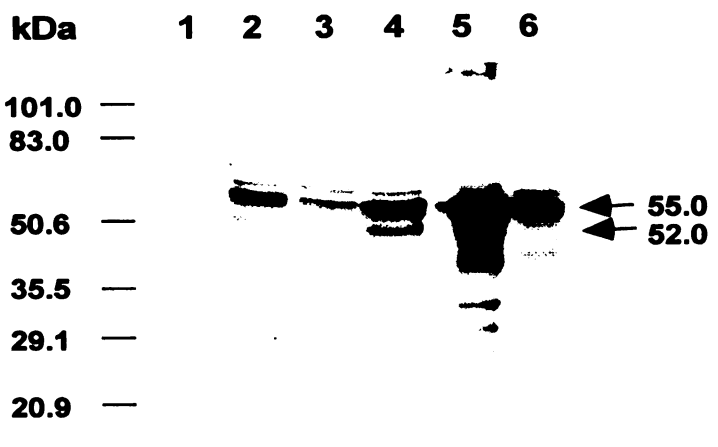

A
B
C
D
20μm
20μm
20μm
20μm

Figure 3.3 Characterization of MDV ICP27 polypeptides. (A) Western blot analysis of MDV ICP27. CEF cells infected with: Lane 1, Mock; Lane 2, MDV serotype 1 strain GA; Lane 3 and 4, MDV serotype 1 strain Md11 low and high passages respectively; Lane 5, rFPV/ICP27; Lane 6, MDV ICP27 *in vitro* translation product. Specific polypeptides were indicated by arrow. Molecular weight standards were indicated on the left. (B) Immunoprecipitation analysis of labeled MDV ICP27. CEF cells were infected with rFPV/ICP27 (lane2), FPV wild type (lane 3), and mock (lane 4). CEF cells were labeled in [α^{32}P] orthophosphate for 6 hours. Cell lysates were precipitated with GST-ICP27 polyclonal antiserum as described in Materials and Methods. Lane1, MDV ICP27 *in vitro* translation polypeptide was labeled with ^{35}S methionine and used as positive control. (C) MDV ICP27 polypeptide dephosphorylation analysis. Protein samples are identical to that in (B), except the precipitates were treated with phosphatase (CIP).

A



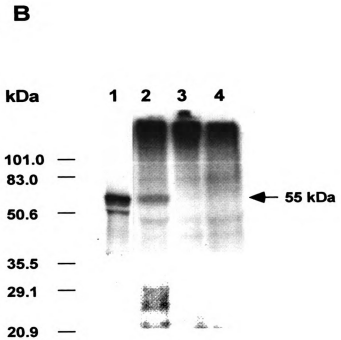

B
kDa
1
2
3
4
101.0
83.0
50.6
35.5
29.1
20.9
55 kDa
C
kDa
1
2
3
4
101.0
83.0
50.6
35.5
29.1
20.9

Figure 3.4 (A) Schematic structures of reporter constructs where different target promoters (unbroken lines) were fused with CAT gene (open box) followed with a SV40 small T 3' intron and an early poly A signal. Restriction endonuclease sites used in the construction of reporter constructs are indicated with their positions of relative to transcriptional start site (arrow) or initiation ATG codon. (B) Effects of MDV ICP27 on different reporter constructs. For each $2X10^6$ CEF cell mixture, 2 ug of reporter constructs (except pSph) were transfected along or cotransfected with 1.5 ug of M-ICP27CMV using electroporation. In pSph transfection, the same amount of effector was used as described above but only 0.2 ug of reporter (pSph) was used. The basal CAT activity of reporter promoter in absence of effector was arbitrarily set as 1.0. Fold changes of cotransfection of reporter construct with M-ICP27CMV was calculated relatively to the basal CAT activity. The standard division of mean was indicated.

A

B

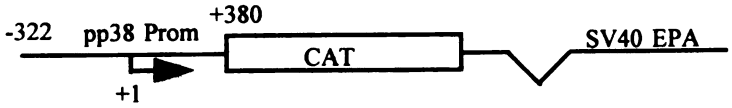
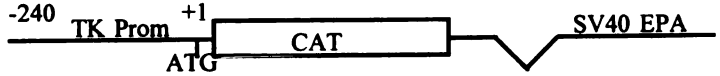
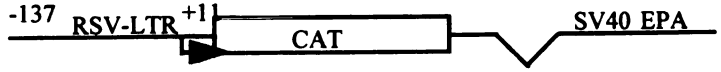
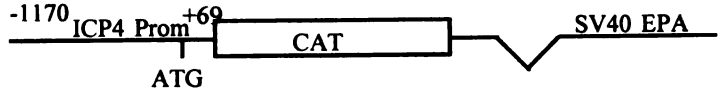
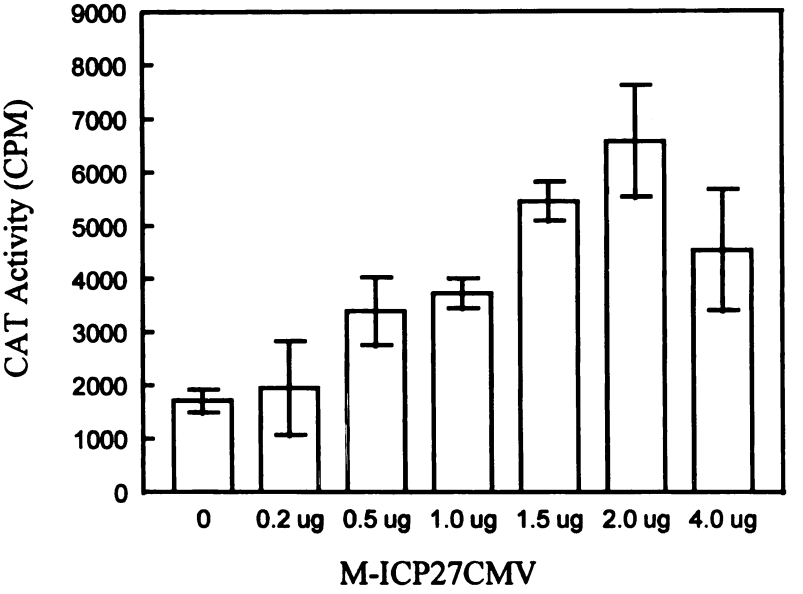
Reporter Constructs		Effect of M-ICP27CMV (Fold-Induction)
pp14CAT		3.0 ± 0.6
pp38CAT		12.5 ± 4.5
TKCAT		0.08 ± 0.02
pSph		2.0 ± 0.2
M-ICP4CAT		Negative effect
M-ICP27CAT		Negative effect
gBCAT		Negative effect

Figure 3.5 Dose-response feature of MDV ICP27-mediated transactivation or transrepression activities. 2 ug of pp14CAT (A) and TKCAT (B) were co-transfected with increasing amount of M-ICP27CMV. Amount of M-ICP27CMV plasmid used for each individual experiment was indicated. CAT activities were exhibited as CPM. The standard division of mean was shown as bars.

A


CAT Activity (CPM)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
0.2 ug
0.5 ug
1.0 ug
1.5 ug
2.0 ug
4.0 ug
M-ICP27CMV

B

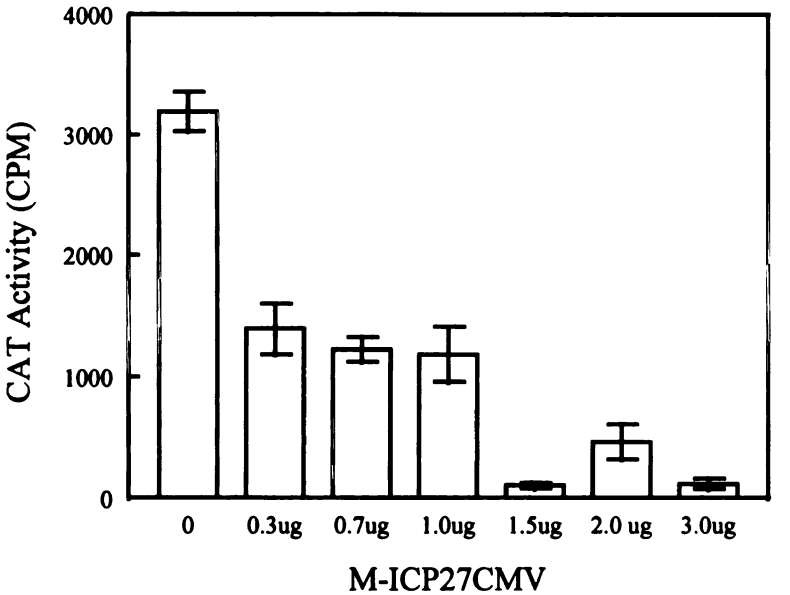

CAT Activity (CPM)
4000
3000
2000
1000
0
0
0.3ug
0.7ug
1.0ug
1.5ug
2.0 ug
3.0ug
M-ICP27CMV

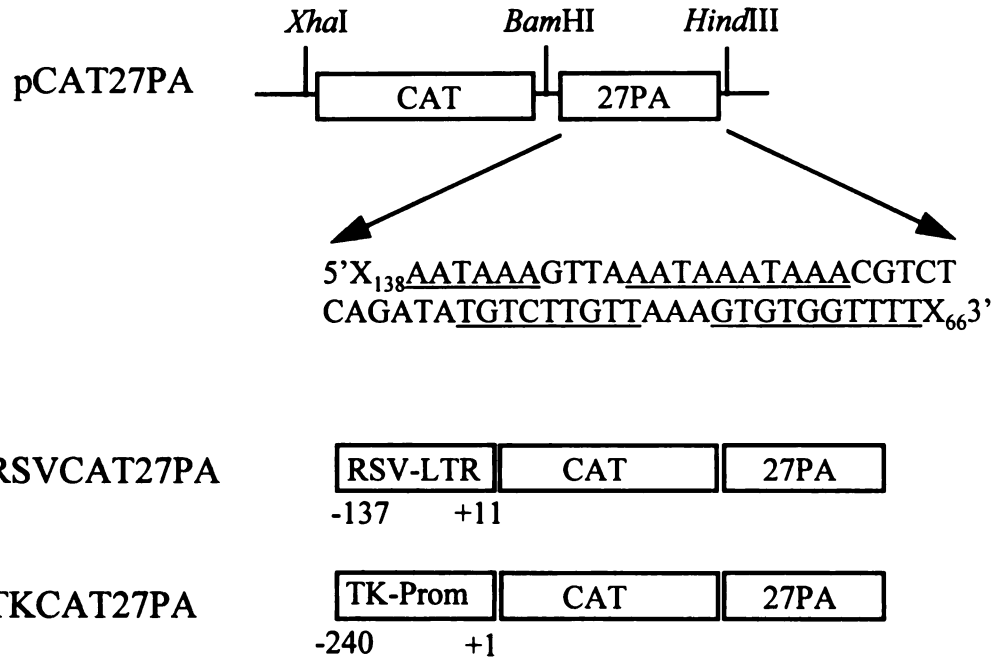
Table 3.1 Effects of MDV ICP27 and ICP4 on Target Promoters

Reporter Constructs [a]	Effector Plasmids (Fold-induction)[b]		
	M-ICP27CMV	M-ICP4CMV	M-ICP27CMV & M-ICP4CMV
pp14CAT	3.0 ± 0.6	1.9± 0.4	3.6 ± 0.7
pp38CAT	12.5 ± 4.5	12.0 ± 3.0	13.7 ± 5.7
pSph	2.0 ± 0.2	1.5 ± 0.3	2.1 ± 0.3

a. 2 ug of pp14CAT, 2 ug of pp38CAT, and 0.2 ug of pSph were used in $2x10^6$ cell mixtures.
b. 1.5 ug of effector plasmid was used for co-transfection.

Figure 3.6 Effects of 3'RNA processing signals on MDV ICP27-mediated transactivation activities. (A) Schematic structures of pCAT27PA, RSVCAT27PA, and TKCAT27PA. (B) Effects of M-ICP27CMV and M-ICP4CMV on reporter constructs which contain different 3'RNA processing signals were represented with fold-induction. In pSph and RSVCAT27PA transfection, 0.2 ug of reporter plasmid and 1.5 ug of effector plasmid were used. In TKCAT and TKCAT27PA transfection, 2.0 ug of reporter and 1.5 ug effector were used. ND, not determined.

A

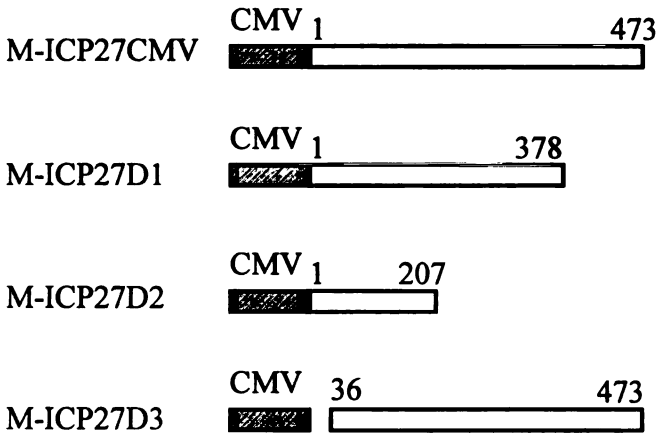


B

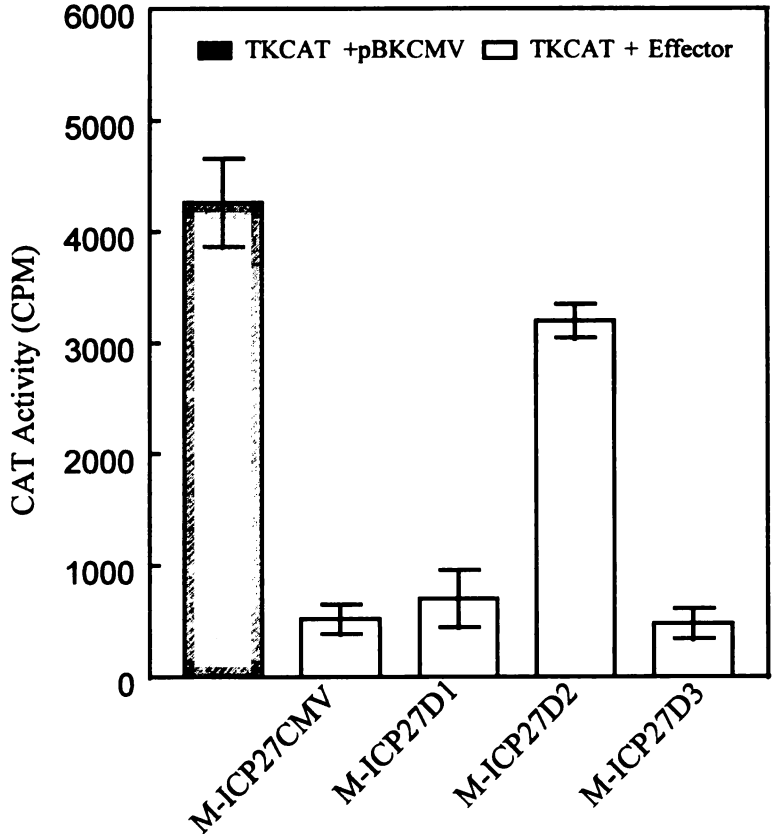
	M-ICP27CMV	M-ICP4 CMV	M-ICP17CMV& M-ICP4CMV
pSph	2.0 ± 0.2	1.5 ± 0.3	2.1 ± 0.3
RSVCAT27PA	1.9 ± 0.3	1.7 ± 0.4	2.7 ± 0.4
TKCAT	0.08 ± 0.02	ND	ND
TKCAT27PA	1.0 ± 0.3	ND	ND

Figure 3.7 Functional dessection of MDV ICP27 polypeptides. (A) Schematic structures of wild type and three mutated MDV ICP27 constructs. Representation of polypeptides are represented as open boxes and the amino acid residues are indicated. Promoter (CMV) is identical to that of wild type construct. (B) 2.0 ug of TKCAT was used as reporter, while 1.5 ug of wild type M-ICP27CMV or mutated effector was used for cotransfection. The CAT activity of each combination was shown with CPM. Standard division of mean was shown as bars. (C) Western blot analysis of alternative polypeptides of MDV ICP27 expressed from the deletion mutant constructs. $2x10^6$ CEF cells were transfected with 3.0 ug of wild type M-ICP27CMV or deletion mutant constructs. Cells were harvested after 48 h transfection. Lane 1, pBK/CMV vector control; lane 2, M-ICP27CMV; lane 3, M-ICP27D1; lane 4, M-ICP27D2; lane 5, M-ICP27D3; lane 6, CEF cells infected with wild type MDV strain Md11. Specific polypeptides were indicated with arrows.

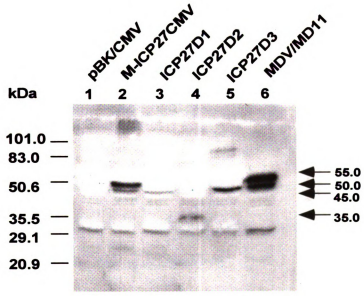
A


M-ICP27CMV
CMV 1
473
M-ICP27D1
CMV 1
378
M-ICP27D2
CMV 1
207
M-ICP27D3
CMV
36
473

B


TKCAT +pBKCMV
TKCAT + Effector
CAT Activity (CPM)
6000
5000
4000
3000
2000
1000
0
M-ICP27CMV
M-ICP27D1
M-ICP27D2
M-ICP27D3

C


pBK/CMV
M-ICP27CMV
ICP27D1
ICP27D2
ICP27D3
MDV/MD11
kDa
1
2
3
4
5
6
101.0
83.0
50.6
35.5
29.1
20.9
55.0
50.0
45.0
35.0

Chapter IV

DISCUSSION

1. Summary and Conclusion

Herpesvirus gene expression is temporally regulated in a cascade fashion and has been classified as immediate-early, early, and late genes. Most IE gene proteins are transcriptional regulators which are required for both early and late gene expression. IE genes have been extensively investigated in the α-herpesvirus, HSV-1. HSV-1 ICP27 is one of two essential IE genes for expression of viral genes and for viral replication. HSV-1 ICP27 plays multifunctional roles during HSV-1 lytic infection and is the only IE gene which is conserved in all three herpesvirus subfamilies. Molecular biology studies have indicated that MDV genomic structure and gene homology are extremely similar to that of HSV-1. Therefore, we speculated that HSV-1 ICP27 homolog should be conserved in MDV. The overall aims of this project have been to identify the ICP27 homolog in MDV and to further investigate its regulatory functions.

The MDV *Eco*RI-B fragment was chosen to initiate a search for the ICP27 gene, and two open reading frames (ORFs), ORF53 and ORF54, were identified within this region. By predicted amino acid sequence analysis, we determined that ORF53 encodes 354 amino acids and is homologous to the HSV-1 glycoprotein K, whereas ORF54 encodes 473 amino acids and displays significant homology to the HSV-1 immediate-early protein, ICP27.

The predicted amino acid sequence of MDV ICP27 was compared to HSV-1 ICP27 as well as VZV ORF4. The amino acid sequence identity and similarity between MDV ICP27 and HSV-1 ICP27 are 25.8 and 42.5%, respectively and between

MDVICP27 and VZV ORF4 are 29.5 and 49.4 %, respectively. The primary structure of MDV ICP27 can be divided into a highly hydrophilic N-terminal half and a relatively hydrophobic C-terminal half. Importantly, MDV ICP27 shares a very high structural domain conservation to HSV-1 ICP27. First, a cystein-rich region and a potential zinc-finger motif which are highly conserved within ICP27 protein family are also found in the C-terminus of MDV ICP27. It has been demonstrated that this region is critical for HSV-1 ICP27-mediated positive and negative regulatory activities. MDV ICP27 also contains a very basic domain from amino acids 151 to 200. A similar basic region, including two arginine-rich domains, was reported in the N-terminal half of HSV-1 ICP27 (Sandri-Goldin, 1991). A high acidic and serine-rich domain is also conserved in both MDV ICP27 and HSV-1 ICP27. Based on amino acid sequence and structural similarities, we speculated that MDV ICP27 may have regulatory functions in MDV gene regulation.

To detect MDV ICP27 gene transcript and determine the gene classification, cycloheximide was used to enhance IE gene transcription and assess if MDV ICP27 is expressed in immediate-early time. Using a *Bam*HI-*Kpn*I DNA fragment probe (which maps within the ICP27 gene ORF), 1.6 and 2.9 kb transcripts were detected in MDV infected cells treated with cycloheximide. However, only the larger transcript (2.9kb) was detected using the probe *Cla*I-*Cla*I (which maps within the gK ORF). This suggested that MDV gK and MDV ICP27 transcripts overlap, sharing a common 3'RNA region, most likely due to read-through transcription of the MDV gK gene, since no poly(A) signal sequence was found between the two ORFs.

The bacteria fusion proteins trpE-ICP27 and GST-ICP27, which correspond to the C- and N-terminal half of MDV ICP27, respectively, were expressed and polyclonal rabbit antisera were produced. Using immunoprecipitation assays, the two antisera specifically reacted to individual MDV ICP27 *in vitro* translation products. Two specific polypeptides (55 & 52 kDa) were detected in the MDV 1 infected cells using GST-ICP27 antiserum. In contrast, only a 55 kDa polypeptide was detected in MDV serotype 1 infected cells using trpE-ICP27 antiserum. Failure to detect the smaller polypeptide with trpE-ICP27 antiserum may be due to the relative poor titer of this antibody, since the fragment used for production of this polypeptide was derived from a region of predicted poor antigenicity in the C-terminal region of MDV ICP27. The most likely explanation for the presence of two distinct ICP27 species is post-translational modification. We also demonstrated that MDV ICP27 is a phosphoprotein by immunoprecipitation and protein dephosphorylation analysis.

Like most herpesvirus IE proteins, MDV ICP27 is predominantly located in the cell nucleus. The predominantly nuclear localization of MDV ICP27 in MDV serotype 1 infected cells and in CEF cells infected with rFPV/ICP27 suggested that MDV ICP27 may contain signal peptides which direct the protein to the cell nucleus during MDV infection. We noted that a strong nuclear localization signal (NLS) and a nucleolar localization signal (NuLS) have been identified and mapped within the N-terminal half of HSV-1 ICP27 (Mears *et al.*, 1995). The NLS of HSV-1 ICP27 is similar to the bipartite NLSs found in *Xenopus laevis* nucleoplasmin (Robbins *et al.*, 1991). The NuLS in HSV-1 ICP27 includes a strong NLS, and a RGG box that is a putative mRNA binding

motif. (Mears *et al.*, 1995). Although neither the bipartite elements nor RGG box has been observed in MDV ICP27, MDV ICP27 contains a very basic amino acid region, including 24% of Arg and 16% & His and Lys from amino acid residues 151 to 200. Highly basic amino acid conservation in this region of MDV ICP27 implies that may play a role for its predominant nuclear localization property. However, more defined mutagenesis studies should be required to verify this possibility.

Transient expression assays were used to determine if MDV ICP27 is a transcriptional regulator. The MDV ICP27 gene was placed under control of the CMV promoter and expressed in CEF cells by transient DNA transfection. Initially, seven gene promoters were used as the target promoters, including six homologous promoters derived from all three MDV kinetic classes, and one heterologous promoter from the RSV-LTR U3 region. We reported that MDV ICP27 can significantly transactivate the MDV pp14 and pp38 gene promoters in our transient transfection experiments. MDV pp14 and pp38 genes are located within MDV genomic repeat regions (IR_L and TR_L). MDV pp14 and pp38 genes share a common control region which has been defined as a bi-directional promoter (Cui *et al.*, 1991; Chen and Velicer, 1992; Hong and Coussens 1994). There are several *cis*-acting elements including two TATA boxes, two Sp1 sites, two CAAT sits and one Octamer motif within this control region (Cui, *et al* 1991). However, the MDV ICP27 *in vitro* translation product did not specifically retard any small DNA fragments derived from the pp38 and pp14 promoter region determined by mobility-shift assays. This suggested that MDV ICP27-mediated transactivation did not directly involve a DNA-protein interaction. We have not determined which cis-acting

site is critical for MDV ICP27-mediated transactivation at this time, but this should be addressed in future studies.

MDV ICP27 also possesses a negative regulatory activity on target promoters. An MDV early promote, the TK gene promoter was strongly repressed by MDV ICP27. This result suggested that MDV ICP27-mediated transrepression of the MDV TK promoter is functionally distinct from either the HSV-1 ICP27 or VZV ORF4 protein. As described above, the HSV-1 ICP27 showed negative effects on the HSV-1 TK promoter but was dependent on the presence of ICP4 and /or ICP0 (McCarthy *et al.*, 1989; Rice and Knipe , 1990), while the VZV ORF4 protein can independently transactivate both the homologous TK promoter and the HSV-1 TK promoter (Inchauspe and Oatrove, 1989). In addition, the MDV TK promoter sequence also displayed some features different from those of HSV-1 TK promoter. There is a C/EBP (CCAAT box), two Sp1, and an Oct-1 (octamer motif) *cis*-acting elements in the HSV-1 TK promoter region but not in the MDV TK promoter. Whether this promoter difference leads to the functional results of MDV ICP27-mediated transrepression on the TK promoter is, at present, not clear.

Deletion mutant experiments indicated that amino acids from 207 to 378 are critical for MDV ICP27-mediated repression of the MDV TK promoter. The lack of transactivation activity of all three deletion mutants suggested that MDV ICP27-mediated positive regulation is not a single region, but seems to require multiple regions. Western blot analysis indicated that deletion mutants of MDV ICP27 are expressed with the comparable yields and molecular sizes.

Previous observations indicated that MDV infections were able to enhance avian leukosis virus-induced lymphoid leukosis (Bacon *et al.*, 1989). The *in vitro* studies demonstrated that MDV gene products or cellular proteins induced by MDV infection, efficiently transactivate RSV-LTR promoters and a 28 bp segment from -109 to -137 within RSV-LTR is an MDV-responsive element. MDV ICP4 is partially responsible for the activation of the RSV-LTR promoter (Tieber *et al.*, 1990; Banders and Coussens, 1994). Here we demonstrated that MDV ICP27 was also able to transactivate RSV-LTR promoter. Although MDV ICP27- and ICP4-mediated transactivation of RSVLTR promoter via a consensus region (-137 to +11), the molecular mechanism underlying the *trans* regulatory activities of MDV ICP27 or MDV ICP4 should be addressed in future studies. MDV ICP27 may also be involved in the interaction between MDV and ALV.

To determine if 3'RNA processing signals affect MDV ICP27-mediated gene-modulation as described in HSV-1ICP27, we created two alternative reporter constructs, RSVCAT27PA and TKCAT27PA, which were derived from pSph and TKCAT, respectively. Each plasmid differed from its parent only in the 3'RNA processing signals. Transient expression assays indicated that substitution of 3'RNA processing signals did not affect MDV ICP27-mediated transactivation of RSV-LTR promoter. In contrast, the TK promoter showed a negative response to MDV ICP27 when the SV40 small T 3'splicing site and the early poly(A) signal were replaced with the MDV ICP27 gene poly(A) signals in the TK promoter construct. These results taken together suggested that MDV ICP27-mediated transactivation and transrepression activities are involved in the different mechanisms of modulating of gene expression.

We also noted that MDV ICP27 displayed a negative effect on either MDV immediate-early promoters (MDVICP4 and MDV ICP27), or on the MDV late gene gB promoter. Functional roles of MDV ICP27 in MDV IE genes and late gene expression remain to be determined.

The MDV ICP4 was also able to transactivate the MDV pp14, pp38 promoters as well as RSV-LTR promoter. However, addition of MDV ICP27 had no demonstrable effect on promoter activation by MDV ICP4 with any three target promoters. In conclusion, both MDV ICP27 and ICP4 independently transactivate target promoters but exhibit no co-operation.

2. Future Research Directions

Identification and characterization of the MDV ICP27 homolog adds more information about gene expression, especially for studying and understanding the regulation of MDV gene expression. We primarily investigated MDV ICP27 regulatory activities at the transcriptional level , but many questions remain to be answered and should be addressed in future studies.

It would be very important to extend our experiments to *in vivo* studies. Construction of MDV mutants which are functionally defective in MDV ICP27 will greatly contribute to the following issues. First, construction of viral mutants defective in ICP27 will answer if MDV ICP27 is essential for viral growth and for expression of viral genes. Second, the functional role of MDV ICP27 will be precisely elucidated for expression of different viral genes. Since MDV can only be grown in primary cell culture, it was difficult to mutate the essential genes from MDV. Recently, a chicken embryo fibroblast cell line, OU2, was used for MDV infection and MDV OU2 cell lines infected with MDV serotypel Md11 were established (Abujoub and Coussens, 1995). MDV OU2 cell lines are similar to certain lymphoblastoid cell lines, and are capable of transferring MDV infection to primary CEF monolayer cultures. However, MDV OU2 cell lines are also capable of supporting a cytolytic infection of MDV. Thus, MDV OU2 cell lines can be used for creation of either "knock out" or insertion virus mutants which display functionally defective MDV ICP27. It is also necessary to create cell lines which can stable express MDV ICP27 in OU2 cells to provide complementary cells.

We demonstrated that MDV ICP27 can transactivate pp38 and pp14 promoters in transient expression assays. Since it has been reported that both pp38 and pp14 genes are expressed in the MDV transformed lymphoblastoid cell line, MSB-1, it would be interesting if we could introduce the M-ICP27CMV plasmid into MSB-1 and determine if MDV ICP27 displays the same activities.

The defined deletion mutant studies should be considered in the future to identify the nuclear localization signals in the MDV ICP27 polypeptide. These studies should focus on the N-terminal region which has a highly basic domain, similar to that of HSV-1 ICP27.

Our previous studies indicated that MDV ICP27 selectively modulated different target promoters. We also mentioned that MDV ICP27 did not directly bind to target promoter DNAs. Information from HSV-1 and VZV implied that ICP27 may be involved in protein-protein interactions. Thus, *in vitro* experiments should attempt to identify which MDV encoded proteins or MDV induced cellular factors are involved in MDV ICP27-mediated transactivation or transrepression activities. These studies should focus on the pp14, pp38 and TK promoters using deletion or insertion approaches. First, pp14 and pp38 promoters contain several cis-acting element binding sites such as SP1, CAAT, and Oct-1. Using deleted pp14 and pp38 promoters as targets may help to determine which cellular factors are involved in MDV ICP27-mediated transactivation of promoters. The MDV TK gene promoter is distinguished from HSV-1 TK by its lack of SP1, CCAAT box (C/EBP) and Oct-1sequences. Introducing certain *cis*-acting elements into the MDV TK promoter or using the HSV-1 TK promoter as a target for MDV ICP27

will provide clues for the explanation why MDV ICP27 displays functional differences on the TK promoter.

APPENDIX

Supplementary Data

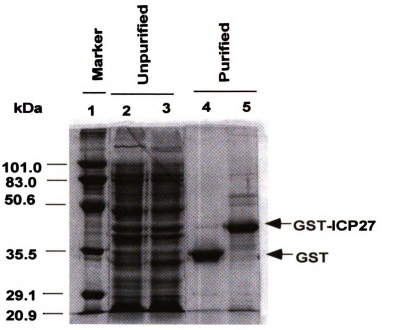
Expression of GST fusion protein in *E. coli*

The vector system used to express MDV ICP27 in *E. coli* is plasmid pGEX2T (Pharmacia, Alameda CA) which contains a glutathione S-transferase (GST) gene under the control of an isopropylthiogalactopyranoside (IPTG)-inducible *tac* promoter. As escribed in Chapter III, a *Bam*HI-*Kpn*I fragment, corresponding to amino acids 23-206 of MDV ICP27, has been cloned into pGEX2T vector and transformed into *E. coli*, DH5α cells. The insertion DNA was confirmed by sequencing. 3 mM IPTG was used for GST induction. Expression and purification of GST-ICP27 have been performed according to the manufacture's specification. New Zealand white rabbits were initially immunized with 20 ug of purified GST-ICP27 fusion proteins in Titer-Max adjuvant (CytRx Co., Norcross, GA). The rabbits were boosted with the same amount proteins in Titer-Max after 4 weeks and bled 10 days following the last injection.

Figure A.1 (A). SDS-PAGE analysis of GST-ICP27 fusion protein. Lane 1, protein standard marker; Lane 2 & 4, GST control; Lane 3 & 5, GST-ICP27 fusion proteins; A 47 kDa fusion protein was purified using glutathione-sepherose 4B (lane 5)

Figure A.1 (B). Cleavage of GST-ICP27 by thrombin. A 20 kDa polypeptide (lane 3) of MDV ICP27 was specifically cleaved from GST-ICP27 fusion protein with thrombin. Lane 1, protein standard marker; Lane 2, GST-ICP27; Lane 3, ICP27 polypeptide cleaved from fusion protein; Lane 4, GST cleaved from fusion protein with thrombin; Lane 5, GST control.

A


Marker
Unpurified
Purified
kDa
1
2
3
4
5
101.0
83.0
50.6
35.5
29.1
20.9
GST-ICP27
GST

B

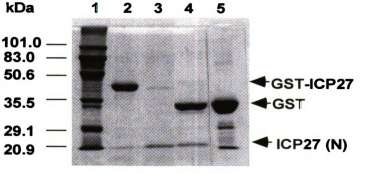
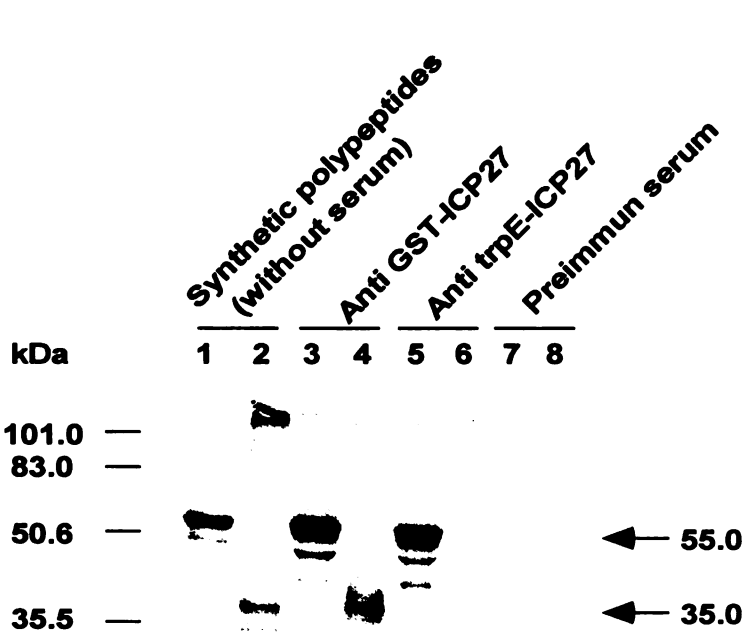

kDa
1
2
3
4
5
101.0
83.0
50.6
35.5
29.1
20.9
GST-ICP27
GST
ICP27 (N)

Figure A.2 Specificity analysis of MDV ICP27 polyclonal antisera

As described in Chapter II, two polypeptides of MDV ICP27 were synthesized using *in vitro* and labeled with ^{35}S methionine. Using TNT/T7 *in vitro* translation system (Promega), a 55kDa polypeptide was translated from plasmid pBlue-ICP27 that contains the entire MDV ICP27 ORF (lane 1), while a 35 kDa polypeptide was translated from pRSET-BK derived from N-terminal half of MDV (lane 2). Two synthetic polypeptides were precipitated with two different polyclonal antiserum. Both synthetic polypeptides were specifically precipitated using GST-ICP27 antiserum (lane 3, and lane 4). In contrast, only 55 kDa polypeptide was precipitated using *trp*E-ICP27 antiserum (lane 5, and lane 6). As expected, both synthetic polypeptides were not precipitated by preimmun serum (lane 7 & 8). These results indicated that two different antisera specifically reacted with polypeptides which were generated from distinct coding region of MDV ICP27.


Synthetic polypeptides (without serum)
Anti GST-ICP27
Anti trpE-ICP27
Preimmun serum
kDa
1 2 3 4 5 6 7 8
101.0
83.0
50.6
35.5
29.1
20.9
55.0
35.0

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