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IDENTIFICATION OF AN IMMEDIATE-EARLY GENE IN MAREK'S DISEASE VIRUS LONG INTERNAL REPEAT REGION AND CHARACTERIZATION OF ITS GENE PRODUCT, PP14

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IDENTIFICATION OF AN IMMEDIATE-EARLY GENE IN MAREK'S DISEASE VIRUS LONG INTERNAL REPEAT REGION AND CHARACTERIZATION OF ITS GENE PRODUCT, PP14

By

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ABSTRACT

IDENTIFICATION OF AN IMMEDIATE-EARLY GENE IN MAREK'S DISEASE VIRUS LONG INTERNAL REPEAT REGION AND CHARACTERIZATION OF ITS GENE PRODUCT PP14

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Marek's Disease Virus (MDV) is an oncogenic avian herpesvirus whose genomic structure is similar to herpes simplex virus and varicella-zoster virus. Repeat regions of the MDV genome have been intensively investigated due to a potential relationship to MDV oncogenicity and abundant expression of immediate-early transcripts. However, controversy regarding size, number, and direction of transcripts derived from these regions has been present. No gene product encoded by the transcripts was reported. By Northern hybridization analysis, a 1.6 kb immediate-early transcript was localized to the BamHI-I_2 region. The viral genomic DNA of that region was subsequently sequenced without finding a continuous (>100 amino acids) open reading frame (ORF) in either direction. Through cDNA cloning and sequencing, two cDNAs of 1.4 kb (C1) and 1.35 kb (C2) originating from the BamHI I_2 region were identified. Both cDNAs are derived from spliced mRNAs spanning the BamHI-H and I_2 fragments. C1 and C2 use the same splice acceptors and 3' ends, but differ at their 5' ends and utilize different splice donors. With a combination of primer extension and sequence analysis, the upstream promoter-enhancer region of C1 cDNA has been defined as a bidirectional regulatory region shared by the MDV pp38 gene. Sequencing analysis shows two small open reading frames (ORF) within each cDNA (ORF1a and ORF2 in C1, ORF1b and ORF2 in C2). Potential ORFs

of the sequence have no significant homology with any known protein in the Swiss-Protein data base. To detect any protein product encoded by these two cDNAs, DNA fragments encoding ORF1a and ORF1b were cloned into pGEX-3X vectors to produce GST-fusion proteins and induce antisera. In Western blot analysis of MDV infected cell lysates, a 14 kDa polypeptide (p14) was identified by antisera against both ORF1a and ORF1b. This 14 kDa protein is expressed in cells which are lytically infected with MDV, strains GA passage 8 (oncogenic), Md11 passage 14 (oncogenic) and passage 83 (attenuated), as well as in the MDV latently infected and transformed MSB-1 cell line. Furthermore, we demonstrate that p14 is MDV serotype 1 specific and highly phosphorylated (designated as pp14). Further analysis reveals that pp14 is predominantly found in the cytoplasmic fraction of MDV infected cell lysates and can be detected in cytoplasm of MDV infected cells by immunofluorescence.

In memory of my mother.

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LIST OF ABBREVIATIONS

bp	base pairs
BHV	bovine herpes virus
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CEF	chicken embryo fibroblasts
cDNA	complementary DNA
CHX	cycloheximide
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
CsCl	cesium chloride
Da	dalton
DEF	duck embryo fibroblasts
DR	direct repeat
E	early
EBV	Epstein-Barr virus
EBNA-1	EBV nuclear antigen
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein-5'-isothiocyanate
gB	glycoprotein B
gC	glycoprotein C

GST	glutathione-S-transferase
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HP	high passage
HSV	herpes simplex virus
HVT	herpesvirus of turkeys
ICP0	(HSV) infected cell protein No. 0
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
IgG	immunoglobulin G
IR_L	internal repeat long
IR_S	internal repeat short
kbp	kilobase pairs
kDa	kilodalton
IUdR	5'-iodo-2'-deoxyuridine
L	late
LAT	latency -associated transcript
LP	low passage
LTR	long terminal repeat

MD	Marek's disease
MDV	Marek's disease virus
meq	Marek's EcoRI Q fragment encoded gene
MIEP	major IE enhancer-containing promoter-regulatory region
Oct-1	octamer binding factor-1
ORF	open reading frame
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PE	phycoerythrin
SDS	sodium dodecyl sulfate
TK	thymidine kinase
TM	tunicamycin
TR_L	terminal repeat long
TR_S	terminal repeat short
ts	temperature-sensitive
U_L	unique long
U_S	unique short
VP16	(HSV)virion protein No. 16
vvMDV	very virulent marek's disease virus
VZV	varicella-zoster virus

Chapter I

Literature review

1. Introduction

Marek's disease virus (MDV) is an oncogenic avian herpesvirus which causes a highly contagious lymphoproliferative disease in chickens, named Marek's disease (MD) (Churchill and Biggs,1967; Nazerian and Burmaster,1968). MD was first described by Joseph Marek, a Hungarian veterinarian in 1907 (Payne, 1985), but discovery of MDV as the etiologic agent of Marek's disease (MD) did not occur until the late 1960's (Churchill and Biggs,1967; Nazerian and Burmaster,1968). Accordingly, MDV became one of the first herpesviruses directly associated with a neoplastic disease.

Prior to discovery of the etiologic agent and development of effective vaccines, MD constituted a serious economic threat to the worldwide poultry industry. MD has been effectively controlled by vaccination with attenuated MDV or an antigenically related but apathogenic herpesvirus of turkey (HVT) (Churchill et al., 1969; Okazaki,et al.,1970; Witter,1985). However, frequent vaccine breaks, attributed in part to the emergence of more virulent MDV strains, prompted significant interest in developing better methods for prevention and control of MDV.

In addition to economic importance, MDV offers a superb model for herpesvirus oncology. MDV reproducibly induces neoplasms in its natural host and virus isolates representing a wide spectrum of oncogenic potential are available. The development of HVT or attenuated MDV as effective vaccines, which prevent the symptoms of MD, provides the first example of a naturally occurring malignant lymphomatous disease to be effectively controlled by vaccination. However, the molecular mechanism of MDV oncogenicity and immunoprotection by vaccines are still unclear. In recent years, a tremendous amount of information on MDV molecular biology has been published,

greatly enhancing our knowledge of MDV pathogenesis and tumorigenesis.

2. Biology of Marek's disease virus (MDV)

A). MDV virions

MDV has a typical herpesvirus structure, which consists of 162 capsomeres arranged with icosahedral symmetry (Nazerian and Burmaster, 1968; Schat, 1985). The nucleocapsid contains a core of double-stranded viral DNA and a protein shell (capsid) assembled in the nuclei of infected cells. The nucleocapsid is surrounded by an amorphous tegument and can be enveloped or not. Enveloped virions, 150-160 nm in diameter, are principally associated with nuclear membrane where the envelope is derived and budded to form nuclear vesicles in MDV infected cells. Naked virions are 85-100 nm in diameter and usually found in the nuclei of infected cells (Nazerian and Burmester, 1968; Nazerian, 1971; Hamdy et al., 1974). Naked and enveloped virions can sometimes be seen in the cytoplasm and, rarely, in the extracellular space. The only place where large numbers of cytoplasmic enveloped virus particles, 270-400 nm in diameter, can be detected is in the feather follicle epithelium (FFE) of infected chickens (Calnek et al, 1970). MDV derived lymphoblastoid cell lines and tumor cells occasionally contain naked virus particles which are essentially similar to virions observed in infected cell cultures.

B). MDV serotypes

Marek's disease virus is classified as three serotypes, based on agar gel precipitation (AGP) and indirect fluorescent antibody (IFA) assays (von Bulow and Biggs 1975; Schat and Calnek, 1978). Serotype classification has been further confirmed by

restriction enzyme pattern analysis of viral genomes (Ross et al., 1983). Oncogenic strains and their attenuated derivatives form serotype 1, while naturally occurring, nononcogenic strains of MDV are classified as serotype 2. Nononcogenic herpesvirus of turkey (HVT) is classified as serotype 3. Serotype 1 is further subdivided as very virulent (vvMDV), virulent, mild or attenuated based on variable pathogenicity or oncogenicity in chickens. Very virulent strains, such as MD/5 and RB-1B, can cause high incidence of MD lymphomas in all chickens, except vaccinated birds from genetically resistant lines. Virulent strains including GA and JM can cause high incidence of MD in genetically susceptible but not in resistant birds. Mild strains can cause tumors in only a minority of very susceptible chickens (Schat, 1985). Repeat passage of very virulent or virulent serotype-1 MDV in cell culture leads to attenuation resulting in loss of oncogenicity (Churchill et al., 1969; Nazerian 1970; Schat, 1985). Attenuated serotype 1 strains, together with serotype 2 and 3, have been employed to produce monovalent, bivalent or trivalent vaccines against MD-induced tumors (Witter,1985).

C). MDV isolation and cultivation

MDV is a highly cell associated virus with the exception of feather follicle epithelium where cell-free infectious virions may be readily isolated (Calnek, et al., 1969; Schat, 1985). Cell-associated MDV can be isolated from viable lymphocytes and, in the case of serotype 1 virus, from lymphoma cells. After initial isolation, MDV can be propagated in primary fibroblast cells obtained from various avian embryos, such as chicken and duck embryos (Purchase et al., 1971). In cell culture, discrete foci can be observed 2 to 7 days post-infection with the characteristics of refractile rounded cells and

syncytia formation. Due to strict cell-association *in vitro*, little or no infectious cell free MDV can be recovered from medium (Calnek et al.,1970; Churchill and Biggs, 1967; Schat, 1985).

3. Pathology of MDV

MDV is a highly contagious agent which spreads horizontally by direct or indirect contact with infected chickens and via an airborne route (Payne, 1985; Sevoian et al., 1963). Once MDV enters a chicken, there are three types of virus-cell interactions: 1) productive infection, 2) non-productive latent infection, and 3) non-productive neoplastic infection or transforming (Payne, 1985; Schat, 1985; Calnek and Witter, 1991). Productive infection is cytolytic, characterized by replication of viral DNA and synthesis of numerous viral antigens. Productive infection can be further divided into fully productive and productive-restrictive, or semi-productive infection. Fully productive infection with MDV only occurs in feather follicle epithelium (FFE) and characterized by production of large numbers of enveloped, fully infectious virions, accompanied by cell death. Semi-productive infection occurs in B lymphocytes, some epithelial cells and in most cultured cells. This type of infection, in which infectivity remains cell-associated, results in production of abundant naked nuclear virions, production of viral antigens and leads to cytolysis (Calnek and Witter, 1991).

As a consequence of the production of viral antigens, T lymphocytes are activated and non-productive latent infection (latency) is established coincidently with development of host immune responses (Payne,1985; Calnek, 1985). Latent infection is detected primarily in lymphocytes and is characterized by persistence of the viral genome in cells without expression of most viral antigens or virions. Latent infection can persist for the

lifetime of the animal and virus can be rescued by co-cultivation with permissive cells or inoculation into chickens (Payne, 1985).

Non-productive neoplastic infection (transformation) describes neoplastic lymphoma cells, transplantable lymphomas and lymphoma-derived cell lines (Payne, 1985). In this type of infection, viral genomes persist in cells with limited antigen expression, including Marek's associated tumor surface antigen (MATSA: activated T-cell marker) (McColl et al, 1987). Transformed cells, in general, are activated T-helper cells which are $CD4^+$ $CD8^-$ (Schat et al, 1991). MDV transformed cell lines are used to investigate mechanisms of latent infection and oncogenesis of MDV, a more extensive literature review on the properties of these cell lines will be presented in the *MDV latency* section.

Based on experiments with oncogenic MDV infection of antibody-free, genetically susceptible chickens, MDV infection can be generally divided into four stages: 1) early cytolytic infection, 2) latent infection, 3) permanent immunosuppression and 4) transformation. These stages are sequential but often overlapped (Calnek, 1985).

Primary infection by MDV usually occurs via the respiratory tract, where MDV is picked up by phagocytes and then disseminated from lung to lymphoid cells (Payne, 1985; Calnek and Witter, 1991). Cytolytic infection of MDV initially affect lymphoid tissues, primarily B lymphocytes and a few T lymphocytes. Tissue changes which accompany initial infection are infiltration of macrophages and granulocytes and reticular cell hyperplasia, followed by degeneration of spleen, bursa, and thymus. From 5 to 7 days post infection, the cytolytic infection of primary B-lymphocytes changes to latent infection of predominantly T-lymphocytes coincidence with temporary recovery from

immunosuppression. Latently infected T-lymphocytes are dispersed to various organs and tissues via the circulatory system (Payne, 1985). Another common place involved in MDV infection is peripheral nerve system, characterized by two main pathological processes: 1) neoplastic lymphoproliferation and 2) segmental, cell-mediated demyelination. At 2 to 3 weeks post infection, transformation of T-lymphocytes occurs, accompanied by permanent immunosuppression. Massive lymphomas can be observed in visceral organs (including kidney, gonad organ, heart, lung, liver etc.), skin, muscle, and neural tissues are also involved (Payne, 1985). Association of atherosclerosis with MDV infection in the coronary, aortic, celiac, gastric and mesenteric arteries can be found as a late change with mildly pathogenic MDV (Payne, 1985).

Clinical signs of MD usually appear at 3-4 weeks post-infection. Two pathological forms of Marek's disease, classical and acute, have been defined. Classical MD predominantly affects peripheral nerves and is associated with asymmetricly progressive paralysis of one or more extremities. A very common characteristic attitude in MD is recumbency of chickens with one leg stretched forward and the other backward. Uneven gait, lameness, torticollis, droopy wings, or closed eyelids are also characteristics depending upon the locations of involved peripheral nerves (Purchase, 1985; Calnek and Witter,1991). While only a minority of cases in classical MD develop lymphomas, acute MD is more virulent and characterized by multiple lymphomatous of various visceral organs and tissues (Payne, 1985). The final consequence of MDV pathogenesis depends upon virus strain and dose, host genotype, age, sex, and immune status (Payne, 1988).

4. Molecular biology of MDV

A). MDV genome structure.

The genome of MDV is a linear, double-stranded DNA and contains nicks or gaps, a property shared with other herpesviruses. The molecular weight of MDV DNA is 110-120 x 10^6 daltons, as calculated by sedimentation analysis and contour length measurements of electron microscopy. Total size of MDV DNA is approximately 180 kbp for serotype 1 and 167 kbp for HVT (Lee et al., 1971; Hirai et al., 1979; 1988; Cebrian et al., 1982; Fukuchi et al., 1984; Wilson and Coussens, 1991). The density of MDV DNA in CsCl gradients was determined to be 1.705 g/cm, indicating a 46-47% composition of guanine and cytosine (Lee et al., 1971; Hirai et al., 1979; 1985). This density is similar to that of chicken cell DNA and complicates the purification of viral DNA by gradient centrifugation.

MDV was initially classified as a gamma-herpesvirus based on its biological properties, especially its lymphotropism which is similar to Epstein-Barr Virus (EBV) (Roizman et al., 1981). MDV genome structure, however, was found to be remarkably similar to that of human alpha-herpesvirus (e.g., herpes simplex virus and varicella-zoster virus) with a characteristic of Herpesviridae group E genome, as determined by electron microscopy and restriction enzyme mapping (Roizman and Sears, 1991; Cebrian et al., 1982; Fukuchi et al., 1984). The basic structure of MDV DNA consists of unique long and unique short regions (U_L, U_S) flanked by internal inverted repeats (IR_L, IR_S) and terminal repeats (TR_L, TR_S) (Cebrian et al., 1982; Fukuchi et al., 1984). In addition to extensive inverted repeats, several sets of direct repeats (DR1 to DR5) have been found in MDV DNA. These DR sequences consisting of more than 100 bp are mostly located

in internal or terminal repeat regions (Hirai, 1988). Among them, a 132-bp direct repeat (DR1) is amplified from 1-3 copies to over 30 copies coincident with the attenuation of MDV oncogenicity (Bradley et al., 1989, b; Chen and Velicer, 1991). This region will be further discussed in *MDV tumorigenicity* section. By using Southern blot hybridization and DNA nucleotide sequencing, Kishi et al. (1991) have reported ***a***-like sequence in serotype 1 MDV inverted repeat region with a similar structure to the ***a*** sequence of HSV-1. HSV-1 ***a*** sequences are located at the junction between the L and S components and at the termini of the HSV-1 genome. These ***a*** sequences are related to intramolecular recombination and cleavage/packaging of viral concatamers during rolling-cycle replication (Jacob et al., 1979; Roizman, 1979; Vlazny et al., 1982). Although the structure of the putative ***a***-like region of serotype-1 MDV is similar to the HSV ***a*** sequence, there is no nucleotide sequence similarity to the HSV ***a*** sequence. Similarly, an ***a***-like sequence has been identified in MDV serotypes 2 and 3 (Reilly and Silva, 1993; Camp et al., 1993). These data further support the similarity of MDV genome with that of alpha-herpesviruses.

B). MDV physical map and gene arrangement

Physical maps of the three MDV serotypes have been constructed using different restriction endonucleases (RE) (Fukuchi et al., 1985; Igarashi et al., 1987; One et al., 1992). Unexpectedly, viruses of serotype 1, 2 and 3 each have a unique restriction enzyme pattern, in spite of their antigenic similaity (Ross, et al., 1983; Hirai et al., 1979). Conflicting results on DNA sequence similarity between the three serotypes varied from 5% to 80% (Ross et al., 1983; Hirai et al., 1986; 1984; Gibbs et al., 1984). The

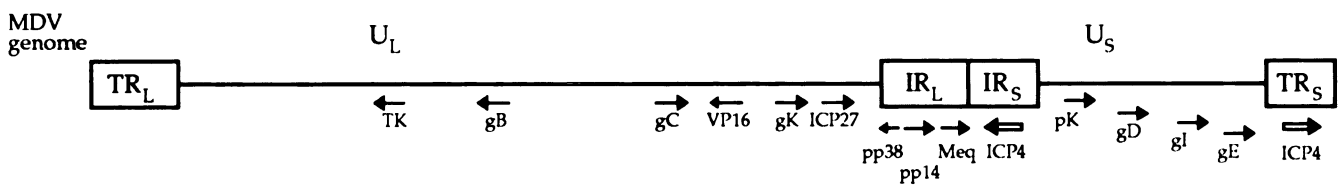
development of RE maps and availability of genomic clones of MDV DNA established a foundation for molecular studies on MDV.

Although nucleotide sequence analysis of MDV has only recently begun, mapping data indicates that genes encoded in the unique long and unique short regions are collinear with varicella zoster virus and herpes simplex virus genes (Buckmaster et al., 1988; Ross et al., 1991; Brunovskis and Velicer, 1992). Two MDV glycoproteins, A and B antigen, have been characterized as HSV gC and gB homologs, respectively (Coussens and Velicer, 1988; Isfort et al.,1987; Ross et al., 1989; Chen and Velicer, 1992). Based on random sequencing analysis, thirty-five MDV genes were identified by comparison to varicella-zoster virus (VZV) (Buckmaster et al., 1988). In addition, extensive colinearity of alphaherpesvirus homologous genes in the MDV U_S region has been recognized (Ross et al., 1991; Brunovskis and Velicer, 1992). Recently, homologs of HSV ICP4, ICP27, VP16 and gK have been identified and sequenced from serotype 1 MDV (Anderson et al., 1992; Yanagida et al., 1993, Ren et al., 1994). Together, these results have provided a basis for MDV reclassification as an oncogenic alphaherpesvirus (Roizman, et al., 1992).

In contrast to the unique region genes, genes in repeat regions are more specific to individual viruses. A 38 kDa phosphoprotein (pp38) and a *fos/jun* oncogene homolog (*meq*), both expressed in MDV transformed cell lines, were localized to the IR_L of the MDV genome within BamHI-H and BamHI-I_2 fragments, respectively (Chen et al., 1992; Cui et al.,1991; Jones et al., 1992). Recently, a 14 kDa MDV specific protein encoded by a cDNA spanning BanHI-H and I_2 regions has been identified (Hong and Coussens, 1994) and is expressed in transformed cell lines. The relationship between genes encoded in repeat regions and MDV tumorigenicity has attracted intensive investigation in these

regions. Genes within repeat regions will further discussed in the *MDV tumorigenicity* section. A brief illustration of MDV gene transcription is summarized in Figure 1.

Figure 1. Location of Genes on MDV Genome



C). Temporal gene expression of herpesviruses

As with other herpesviruses, MDV gene expression is coordinately regulated and sequentially ordered in a cascade fashion (Nazerian and Lee, 1976; Maray et al.,1988; Schat et al., 1989; Wagner, 1991). Generally, three major kinetic classes of genes are expressed: 1) immediate-early (IE, or alpha, α), 2) early (E or beta, β), and 3) late (L or gamma, γ). Immediate- early (IE) genes are expressed immediately upon infection and do not require de novo viral protein synthesis. Their gene products are required for subsequent activation of early and late virus genes, as well as autoregulation of IE genes. Metabolic inhibitors, such as cycloheximide (CHX) will cause accumulation of IE gene transcripts. Early (E) genes are the next class expressed and their synthesis requires the activity of at least one IE protein. Early gene expression is enhanced, rather than reduced, in the present of drugs that block viral DNA synthesis, such as phosphonoacetic acid (PAA). The expression of late genes requires both viral protein synthesis and viral DNA replication (Wagner, 1991). While early genes primarily encode proteins which are required for viral DNA replication, late genes encode structural proteins required for virion assembly and VP16, an IE gene transactivator (Roizman and Sears, 1991).

Comparing with other herpesviruses, studies on MDV gene expression and regulation are still far behind, partly due to the cell associated property of MDV. Information from other herpesviruses, such as herpes simplex virus (HSV), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), are very valuable and greatly facilitate our studies on MDV. Thus, in this next section, the review will start from HSV, the most extensively studied herpesvirus, and focus on immediate-early gene products and their biological properties.

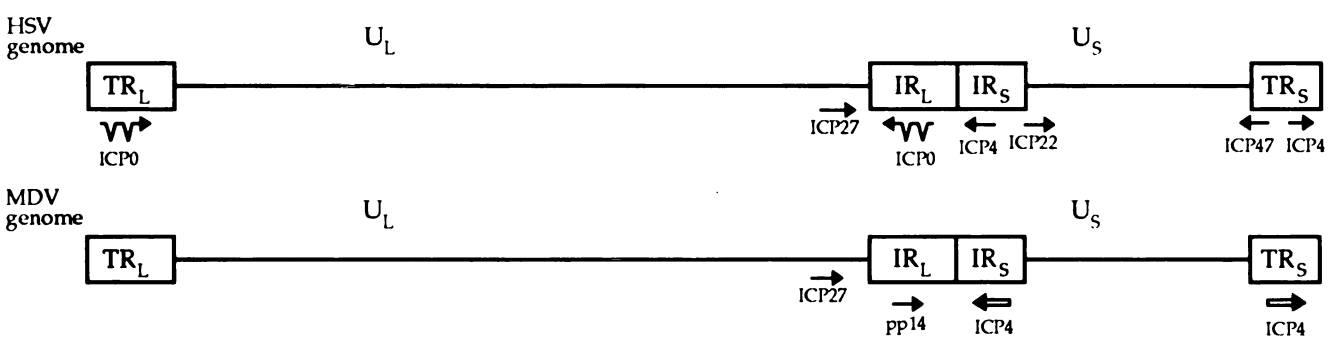
i). Herpes simples virus (alpha-herpesvirus) IE gene expression

HSV is one of the most intensively investigated viruses. It has a double-stranded DNA genome of about 150 kb and encodes more than 70 genes (McGeoch et al., 1988; Wagner, 1991). HSV gene expression is regulated primarily at the transcriptional and post-transcriptional levels during the productive replication cycle (Weinheimer and McKnight, 1987; Godowski and Knipe, 1986; Roizman and Sears, 1991). Although immediate-early genes are the first group of genes expressed, IE genes are induced by a viral structural protein (late gene product), VP16 (or α-trans-inducing factor, α-TIF, ICP25, UL48, Vmw65). VP16 is a component of the virion tegument which is transported into the nucleus different from that of viral DNA. VP16 specifically stimulates viral IE gene expression through direct interaction with the cellular Oct-1 transcription factor system. Oct-/VP16 recognizes a specific cis-acting element (TAATGARAT) in IE gene promoters (Roizman and Sears, 1991).

HSV DNA contains five immediate-early genes, IE-1 (α1), IE-2 (α27), IE-3 (α4), IE-4 (α22), and IE-5 (α47), corresponding to gene products ICP0 (Vmw110), ICP27 (Vmw63), ICP4 (Vmw175), ICP22 (Vmw68), and ICP47 (Vmw12), respectively (Everett et al., 1991; Roizman and Sears, 1990). All HSV IE genes map to near the termini of the L and S components (Figure 2). IE-1 and IE-3 are located within inverted repeats of the L and S components, respectively, and therefore are diploid in each genome. In the circular arrangement of viral DNA, IE genes form two clusters of IE-1, 3, 4, and IE-5, 3 and 1. Each cluster contains an origin of DNA replication (Ori_S) between IE-3 and IE-4 or IE-3 and IE-5. In spite of the clustering, each IE gene has its own promoter-regulatory region, as well as transcription initiation and termination sites (Roizman and

Sears, 1991). IE genes are transcribed by the host RNA polymerase II. Synthesis of IE proteins can be detected 30 minutes post infection and reaches a peak at 2-4 hr post-infection. All HSV IE proteins, with the exception of ICP47, have been shown to have regulatory functions important for transcription of early and late HSV genes (Roizman and Sears, 1991).

Figure 2. Comparison of the Locations of IE Genes on the HSV and MDV Genomes



ICP0. ICP0 is the gene product of IE-1 which is the only spliced IE gene in HSV. The IE-1 gene is located in the repeated sequences which flank the unique long region of HSV genomes. Nucleotide sequence analysis, coupled with S1 nuclease mapping and cDNA analysis revealed that IE-0 is 3,587 bp long and composed of three coding exons separated by two introns (Perry, et al., 1986; Everett, et al., 1991; Zhu et al., 1991). Based on the sequence of the coding region, ICP0 is predicated to be composed of 775 amino acids with a molecular weight of 78,452. In SDS-polyacrylamide gels, however, the apparent molecular size of ICP0 is about 110 kDa (O'Hare and Hayward, 1985; Perry et al., 1986). ICP0 is a highly phosphorylated protein and predominantly located in the nuclei of infected cells (Ackermann, et al., 1984; Perry, et al., 1986; Wilcox et al., 1980). As deduced from the predicted amino acid sequence, ICP0 contains a highly acidic N-terminus, adjacent to two zinc-finger motifs, which are in DNA binding domains of several transcription factors. Two proline-rich regions are in the middle of ICP0 (Perry, et al., 1986; Everett, 1988; Freemont et al., 1991). Proline-rich regions have been shown to act as transcription activation domains in CTF/NF-1, OTF-2, and AP-2. Despite identification of these structural characteristics, the mechanism by which ICP0 regulates gene expression is still unclear.

In vitro transient assays demonstrate that ICP0 is a potent and promiscuous trans-activator of gene expression. ICP0 is able to activate promoters from HSV genes of all three kinetic classes (IE, E, L), as well as heterologous promoters, including cellular genes, the SV40 early promoter and the human immunodeficiency virus (HIV) long terminal repeat (LTR) (Everett et al., 1991). In spite of action on a wide range of promoters, ICP0 does not require specific cis-acting sequences. However, specific

sequences in HIV LTR promoters that are required for response to ICP0 are reported (Mosca, et al., 1987). ICP0 transactivates promoters by itself, or in synergy with ICP4, a major regulatory protein encoded by IE-3. Synergistic activation is much greater than that found with either of protein alone (Everett, 1984; Gelman and Silverstein, 1986). Based on serial in-frame insertion or deletion mutations throughout the ICP0 polypeptide, it has been found the carboxyl terminal and cysteine-rich zinc-finger domains (regions 3 and 5) are most crucial for both synergistic activation with ICP4 and normal intranuclear distribution of ICP0. Whereas region 1 in the second exon is required for ICP0 activity in the absence of ICP4 (Everett et al., 1990) Therefore, ICP0 may act through two different mechanisms depending upon the presence of ICP4 (Roizman and Sears, 1991; Everett et al., 1991).

In vivo studies of HSV mutants with lesions affecting both copies of ICP0 show that ICP0 plays an important, but not essential role during lytic infection in cell culture. ICP0 mutants replicate in a multiplicity-dependent fashion, i. e., they grow poorly following low-multiplicity infection but well following high-multiplicity infection (Sacks and Schaffer, 1987; Stow and Stow, 1986). In low multiplicity infection, ICP0 deletion mutants exhibit substantial impairment in viral polypeptide synthesis, delay expression of early and late gene transcripts, are defective in replication and have reduced initiation of plaque formation (Chen and Silverstein, 1992; Everett et al., 1991). Observations on relative importance of ICP0 regions are mostly agreeable between mutant virus *in vivo* experiments and *in vitro* transfection assays. The mechanism by which ICP0 activates transcription, however, remains obscure. Several possible mechanisms have been suggested, including binding to DNA indirectly through cellular or other viral proteins;

acting at post-transcriptional level; and making DNA more accessible to the transcriptional machinery (Everett et al., 1991; Cai and Schaffer, 1992). Alternatively, ICP0 could activate a cellular transcription factor, or suppress negative regulatory factors (Preston et al., 1988).

ICP0 has also been suggested to play a role in efficient establishment and reactivation of latency, in which viral gene expression is limited to transcription of the latency-associated transcript (LAT) and no infectious virus can be detected (Leob, et al., 1989). Viruses with mutations in both copies of ICP0 do not reactivate as efficiently as wild type virus. In an *in vitro* latency system, the product of the IE-1 gene, cloned in an adenovirus expression vector is sufficient to reactivate latent virus (Zhu et al., 1988). When mutant IE-1 genes were examined, the transactivation domains of ICP0 were shown to be required for reactivation (Zhu et al., 1990). Difficulties associated with explanation of the effects of ICP0 mutations are due to overlap of the ICP0 and LAT genes, which are transcribed in an opposite direction and have been also implicated in reactivation (Feldman, 1991). Overlap of these two genes results in ICP0 mutants also containing mutations in LAT transcripts and raises the question of how to separate effects of mutations in ICP0 from effects of mutations in LAT transcripts. Cai et al. (1993) reported a mouse ocular model to study establishment and reactivation of latency by a series of ICP0 nonsense, insertion and deletion mutant viruses. Among these mutations, all nonsense mutants can induce synthesis of near-wild type levels of the 2 kb LAT which means LAT transcription is not disrupted. These mutants exhibite less efficient replication and reduced reactivation. In addition, when a single copy of the ICP0 gene is inserted into an ICP0$^-$ LAT$^-$ double mutant virus genome, replication and reactivation of the mutant is

restored to one-half the wild-type level. These results demonstrate the role of ICP0 is distinct from that of the LATs in establishment and reactivation of latency.

The role of ICP0 in establishment of latency is to increase efficiency of virus replication at the site of primary infection and in ganglionic neurons, the site of latent infection (Leib et al., 1989). In reactivation from latency, ICP0 can boost viral gene expression in neurons at the onset of reactivation. Therefore, an alternative pathway independent to VP16-Oct-1 interaction may exist for activating IE gene expression, particularly in the early stage of reactivation from latency where VP16 is absent (Elshiekh et al., 1991). This idea is supported by an experiment in which Vero cells were transfected with infectious mutant and wild-type viral DNA (no virion protein VP16 is present) (Cai and Schaffer, 1992). The reduction of ICP4 expression in mutant viral DNA transfection can be reversed by cotransfection of an ICP0 expression plasmid, suggesting ICP0 may play a back-up role to VP16 in activating IE gene expression. However, the mechanism by which ICP0 genes are regulated to exert a role in reactivation of latency is unclear. Multiple copies of the octamer consensus binding site in the ICP0 promoter region have been reported. A 15-bp critical promoter region of ICP0 is mutually bound by two cellular proteins and is required for efficient constitutive expression of ICP0 (O'Rourke and O'Hare, 1993; Elshiekh et al., 1991).

ICP4. ICP4, encoded by IE-3 (IE175, or α4), is the major trans-activator of HSV genes. IE-3 encodes 1,298 amino acids with a predicted protein molecular weight of about 133 kDa (Roizman and Sears, 1991). ICP4 polypeptide localizes to the nuclei of infected cells and is phosphorylated such that at least three modified species (4a, 4b, 4c) are observed on denaturing polyacrylamide gels with molecular weights of 160 kDA, 163

kDa, and 170 kDa, respectively. One of the phosphorylated species (170 kDa) is stable throughout the course of infection, whereas phosphate cycles on and off at least two other species (Wilcox et al., 1980). Through studies involving viral genetics, transient assays, and biochemical analysis, it has been established that ICP4 is essential for productive viral infection and is a complex multifunctional protein (Dixon and Schaffer, 1980; Roizman and Sears, 1991). Viruses carrying temperature-sensitive (ts) deletion, and nonsense mutations which impair expression or activity of ICP4 fail to synthesize both early and late gene products and exhibit an overproduction of ICP4 and other IE proteins (Dixon and Schaffer, 1980; Roizman and Sears, 1991; Shepard et al., 1989). Consistent with *in vivo* experiments, results of transient expression assays utilizing a cloned ICP4 gene and reporter genes containing promoters of IE, early and late viral genes demonstrate that ICP4 transactivates early and late viral gene promoters while repressing IE gene promoters (DeLuca et al., 1984; 1985; Everett, 1984; O'Hare and Hayward, 1985, a; b). From these observations, it was concluded that ICP4 has at least two major regulatory activities: (i) induction-enhancement of early and late gene expression, and (ii) repression of ICP4 and possibly other immediate-early genes. As described above, ICP4 has a synergistic effect with ICP0. Recently, Zhu et al. (1994) reported that ICP4 can enhance nuclear localization of ICP0. These results suggest cooperative regulation by these IE genes.

Analysis of primary amino acid sequences of ICP4 in HSV and comparison with analogous proteins in other herpesviruses (VZV, PRV etc.) indicate existence of five highly conserved intramolecular domains (McGeoch et al., 1986). Using deletion mutations, partial polypeptides of ICP4 conferring different functions have been reported

(DeLuca and Schaffer, 1988; Shepard et al., 1989). The N-terminal 774 amino acids of ICP4 are proficient for DNA binding, autoregulation, and transactivation of some viral genes based on genetic approaches. The ability of ICP4 to form specific protein-DNA complexes is correlated with both transactivation and autoregulation activities (DeLuca and Schaffer, 1988; Shepard et al., 1989).

Although it has been well defined that ICP4 is a DNA-binding protein, controversies on whether it binds to specific DNA sequences exist. ICP4 was first shown to interact with the sequence ATCGTCNNNNYCGRC (where R=purine, Y=pyrimidine, and N=any base). One such ICP4-binding site overlaps the start site of ICP4 transcription, leading to the suggestion that ICP4 binding to the start site of transcription suppresses expression by preventing RNA polymerase from associating with the mRNA cap site (Faber and Wilcox, 1986; Muller, 1987). However, other immediate-early genes lacking similar ICP4 binding sites within their promoter do not support this hypothesis. It has been reported that ICP4 can utilize a common DNA binding domain to interact directly with many different DNA sequences found in or near HSV gene promoters with different affinity, depending on target sequences (Shepard et al., 1989; Imbalzano and DeLuca., 1992; Michael et al., 1988).

ICP4 can stimulate transcription of minimal promoters in which the only recognizable cis-acting element is a TATA homology. Therefore, it was suggested that ICP4 may operate though the transcriptional machinery acting at TATA boxes (Imbalzano and DeLuca, 1992; DiDonato and Muller, 1989). Recently, Smith et al. (1993) reported that ICP4 forms a tripartite complex with TFIIB and either the TATA-binding protein (TBP) or TFIID by using gel retardation and footprinting assays. Formation of the

complex was not simply a result of tripartite occupancy of DNA, but a consequence of protein-protein interactions. In the presence of all three proteins, the affinity of ICP4 and TBP for their respective binding sites was substantially increased. The ability of ICP4 to bind to DNA is necessary but not sufficient for the formation of the tripartite complex, and the ability of ICP4 to form this complex correlates with its ability to activate transcription (Simth et al., 1993).

ICP27. ICP27 is encoded by the IE-2 gene, which is located in the unique long region of HSV-1 genomes with an unspliced transcript of 1.8 kb (Sandri-Goldin, 1991). The coding sequence of 1,536 bp encodes a protein of 512 amino acids. Like other IE gene products, ICP27 accumulates in the nucleus and is highly phosphorylated, resulting in the observed molecular weight 63 kDa larger than the predicted weight of 55 kDa (Sandri-Goldin, 1991). ICP27 contains both stable phosphate groups and phosphate groups which cycle on and off during infection (Rice et al., 1993). The analysis of viral mutants containing temperature-sensitive mutations and deletion mutations demonstrate that ICP27 is an essential regulatory protein in viral infection (McCarthy et al., 1985; Sacks et al., 1985).

Transient-expression (chloramphenicol acetyltransferase CAT assay) experiments provide evidence of the regulatory activities of ICP27 (Block and Jordan, 1988; Everett 1986). Several studies suggest that ICP27 exerts both positive and negative effects on ICP0- and ICP4-induced gene expression (Rice and Knipe, 1990; Sekulovich et al., 1988; Su and Knipe, 1989). In the presence of ICP4 and ICP0, ICP27 represses IE and E gene expression, whereas L gene expression is enhanced. However, in the absence of ICP4 and ICP0, ICP27 has little or no trans-regulatory effect on target genes. Cis-elements in

reporter genes which mediate positive and negative regulation have not been definitively identified, but recent studies have indicated that mRNA processing signals are critical to regulation of a reporter gene by ICP27 (Chapman et al., 1992; Sandri-Goldin and Mendoza, 1992). Specifically, 3' sequences involved in pre-mRNA cleavage and polyadenylation mediate positive regulation by ICP27, while intron sequences appear necessary for transrepression. These studies suggest that ICP27 can regulate gene expression at a post-transcriptional level in transfected cells, possibly by affecting mRNA processing (Rice et al., 1993). ICP27 is also partly responsible for post-translational modification of ICP4. ICP4 migrates faster in wild-type virus infected cells and in cells cotransfected with ICP4- and ICP27-expressing plasmids than in cells infected with ICP27 null mutants or cells transfected with an ICP4-expression plasmid only (McMahan and Schaffer, 1990; Rice and Knipe, 1988; Su and Knipe, 1989). In contrast to ICP4, ICP27 inhibits nuclear localization of ICP0. The inhibitory effect depends on ICP27 expression levels (Zhu et al., 1994).

Assessment of the predicted amino acid sequence of ICP27 reveals that the protein can be divided into two halves with a hydrophilic amino terminal half and hydrophobic carboxyl terminal half (Sandri-Goldin, 1991). Mutational studies show the C-terminal half is required for both activation and repression functions (Hardwicke et al., 1989; McMahan and Schaffer, 1990). Although this region is not similar in nature to those of other trans-activator proteins, it does contain a consensus sequence resembling metal binding domains or "zinc-finger" motifs. The N-terminal half contains a high proportion of acidic amino acid residues (25 of the first 64 residues) and nine serine residues which are potential phosphate acceptors (Sandri-Goldin, 1991). Recently, Rice et al. (1993) have reported that

the acidic N-terminal half of ICP27 is required for efficient transrepression in transfected cells and is required for an essential lytic function. It appears ICP27 is a multifunctional protein which contains multiple functional domains to mediate distinct activities.

ICP22/47. ICP22 and 47 are encoded by HSV IE gene 4 and 5 (US1 and US12), respectively, and are present in one copy per genome (Roizman and Sears, 1991). The predicted translated and apparent molecular weights of ICP22 are 46.521 and 72.0 kDa, respectively (Roizman and Sears, 1991). Although ICP22 is regulated as an IE gene (Post and Roizman, 1981), the specific function(s) of ICP22 remains to be elucidated. Sears et al. (1985) demonstrated that ICP22 affects expression of some late genes. ICP22 deletion mutants can be propagated in some cell lines but not others, suggesting that permissive cells may complement the ICP22 protein by utilizing a host cell factor. ICP47 is the only IE protein without an obvious regulatory function on viral gene expression. Deletion mutants of ICP47 grow as well as wild-type virus in cell culture (Mavromara-Nazos et al., 1986). Based on characteristics of wide host range of HSV, it is conceivable, by analogy with the studies on the ICP22 mutant, that the function of ICP47 is to complement certain functions missing in some cells infected by HSV in its natural host. However, no direct evidence for this hypothesis has been reported. Therefore, the function of ICP47 remains to be determined.

(ii) Human cytomegalovirus virus (beta-herpesvirus) IE gene.

Cytomegaloviruses are species-specific viruses that have the largest genome (230-240 kb) in the herpesvirus family (Stinski, 1991). In HCMV, three distinct segments of genomes are expressed at IE times, including major IE genes, UL36-38, and US3. The

major IE region is the most extensively studied, consisting of two transcription units, IE region 1 (IE1, UL123) and IE region 2 (IE2, UL122) (Stenberg et al., 1984; 1985). These two genes are driven by a single promoter regulatory region, referred to as the major IE enhancer-containing promoter-regulatory region (MIEP), located upstream of IE1 (Stinski, 1991; Boshart et al., 1985; Thomsen et al., 1984). This regulatory region is unusually strong and contains multiple sets of highly conserved repetitive elements. The repeat elements are in two regions designated region I and II. Region I contains a potential serum response element (SRE) and a consensus binding sequence for NF1/CBP which overlaps a 13-bp repeat element. Region II contains five GC-boxes for SP1 binding, an AP1 site, and four different repeat elements of 16-, 18-. 19-, and 21-bp which have consensus binding sites for CRE, ATF, and NF-κB. It is likely that one repeat element interacts with more than one nuclear factor or presence of one repeat element influences binding to one or more other repeat elements. MIEP containing many cis-acting sites for eukaryotic cell transcription factors makes it highly efficient during virus infection. However, MIEP itself is also positively and negatively regulated by virus-specific proteins which will be discussed later.

By alternative splicing, IE1 and IE2 yield a family of mRNAs which encode a series of unique, but related proteins (Stinski, 1991; Stenberg et al, 1989). The IE1 transcription unit consists of the first four exons with a translation initiation codon in exon 2. IE1 codes for a 1.95-kb mRNA that translates into a 72-kDa protein (IE72). IE2 includes exon 1 through 3 and exon 5. Transcripts derived from this region encode several mRNAs of 2.25-, 1.70-, and 1.40-kb, resulting in of translation proteins 86- (IE86), 55- (IE55), and 40-kDa, respectively (Stenberg et al., 1989; 1985; 1990). All of the major IE

proteins share a common N-terminus of 85 amino acids derived from exons 2 and 3, with the exception of the 40 kDa protein that originates solely from exon 5 (Stenberg et al., 1985). Most of the proteins derived from these two IE regions are phosphorylated with apparent molecular weights larger than that calculated from primary sequence data. The functional characteristics of different HCMV IE proteins differ dramatically, although they share many domains.

While 86-kDa IE2 protein can activate most promoters of HCMV assayed to date, IE1 only augments transactivation activity of IE2 on viral promoters (Klucher et al., 1993; Malone et al., 1990). Both IE1 and IE2 are capable of transactivating heterologous promoters, such as HIV LTR or the heat shock protein 70 (HSP70) promoters. In addition to its function as a transactivator, the 86-kDa IE2 protein negatively autoregulates its own expression. This 86-kDa IE2 is a sequence specific DNA-binding protein that interacts directly with a DNA sequence, termed the *cis* repression signal (CRS), that is located between the TATA box and the transcriptional start site of the MIEP of HCMV (Arlt et al., 1994; Cherrington et al., 1991; Lang et al., 1993; Liu et al., 1991). In contrast, the 72-kDa IE1 and the 55-kDa IE2 proteins can transactivate MIEP (Barachini et al., 1992; Cherrington et al., 1989).

Analysis of amino acid sequences of HCMV IE proteins reveals some characteristics similar to those of other transcription factors, including three amphipathic helices at the N-terminus of all IE proteins; single zinc finger motifs in 86-kDa IE2 and 72-kDa IE1; a leucine zipper in the 72-kDa IE1 protein; and a leucine-rich region in the 86-kDa IE2 protein. However, mechanisms whereby HCMV IE proteins execute their functions remain largely undefined. In principle, two main mechanisms of action are

possible: 1) A direct interaction with DNA could occur, thereby tagging an activation domain close to the basal transcription machinery; 2) Alternatively, protein-protein interaction could play a role (Stinski, 1991).

(iii) Epstein-Barr virus (gamma-herpesvirus) IE genes.

Studies on EBV IE genes are different from other herpesviruses due to lack of appropriate *in vitro* culture systems. However, three immediate-early genes, BMLF1 (Mta), BRLF1 (Rta), and BZLF1 (Zta) are identified after induction of the EBV lytic cycle in lymphocytes (Hayward and Hardwick, 1991). The BZLF1 protein is the key immediate-early trans-activator of early lytic gene expression and its expression is sufficient to disrupt viral latency. BZLF1 is located just downstream of BRLF1 gene, therefore, BZLF1 gene expression can be derived from two promoters (Hayward and Hardwick, 1991; Kieff and Liebowitz, 1991). The more 3' BZLF promoter drives transcription of a 1.0-kb mRNA which encodes only the BZLF1 gene product, whereas an upstream BRLF1 promoter drives transcription of a 2.8-kb bicistronic mRNA which encodes both BZLF1 and BRLF1 gene products (Hayward and Hardwick, 1991). BZLF1 gene yields a spliced transcript which contains three exons. It encodes a 34- to 38-kilodalton nuclear protein which resembles a *c-fos* leucine zipper protein, containing a basic DNA-binding domain adjacent to a coiled-coil dimerization domain (Hayward and Hardwick, 1991; Farrell et al., 1989; Kouzarides et al., 1991). The BZLF1 protein not only binds to an AP-1 site, like *c-fos*, but also binds to additional non-AP-1 sites referred to as Z response elements (ZREs) (Chang et al., 1990; Urier et al., 1989). Thus, BZLF1 can activate a number of early EBV promoters by a direct binding mechanism (Kenney

et al., 1989; Roony et al., 1989). BZLF1 also binds directly to the EBV lytic origin of replication (Ori-lyt) and is required for lytic replication (Fixman et al., 1992; Schepers et al., 1993). Some cellular proteins, such as NF-κB and p53 can physically and functionally interact with BZLF1, resulting in inhibition of BZLF1 and consequently an inhibition of the switch from latent to productive infection (Gutsch et al., 1994; Zhang et al., 1994).

BRLF1 protein is also a transcription activator and sequence-specific DNA-binding protein (Chevallier-Greco, et al., 1986; Hardwick et al., 1988; Kenney et al., 1989; Gruffat et al., 1990; Manet et al., 1989). Three EBV promoters (the BMLF1 IE promoter, the BHRF1 early promoter, and the DR early promoter) have been reported to be transactivated by BRLF1 protein (Chevallier-Greco, et al., 1986; Hardwick et al., 1988; kenney et al., 1989). Interestingly, all three promoters have upstream enhancer elements, and the BRLF1 response region in each promoter is mapped to these enhancer elements. Consequently, BRLF1 gene has been referred to as an enhancer factor (Manet et al., 1989; Urier et al., 1989). However, it was reported that BRLF1 can transactivate a HIV type-1 long terminal repeat (LTR) with a deleted enhancer element (Quinlivan et al., 1990). BRLF1 also positively autoregulates its own promoter through a nonbinding mechanism (Zalani et al., 1992). Thus, the BRLF1 gene product may activate promoters by more than one mechanism, involving certain enhancer elements or enhancer independent; direct binding or indirectly through modulation of cellular transcription factors .

Both BZLF1 and BRLF1 transactivate independently, but they also act synergistically (Chevallier-Greco, et al., 1986; Cox et al., 1990; Giot et al., 1991; Quinlivan et al., 1993). In these cooperative functions, both Z and R binding sites are required, suggesting BZLF1 and BRLF1 both bind to DNA directly (Quinlivan et al.,

1993). In addition, BZLF1 functions as either an enhancer or repressor of R-induced transactivation, depending upon the presence or absence of functional Z binding sites (Quinlivan et al., 1993).

The third EBV IE transactivator called BMLF1, is encoded by the BLF1-BSLF2 ORF. It contains several mRNAs generated by alternative splicing and driven by two promoters of promoter M (PM) and more upstream promoter M1 (PM1) (Buisson et al., 1989). The BMLF1 gene product analyzed on denaturing polyacrylamide gene migrates as a polypeptide family (molecular weights, 45, 000 to 70,000), with the major product being a phosphorylated 60-kilodalton nuclear protein (Hayward and Hardwick, 1991). In transient assays, BMLF1 stimulates chloramphenicol acetyltransferase (CAT) expression controlled by several different EBV promoters. However, this BMLF1-induced increase of CAT activity is not accompanied by a significant increase in steady-state-level CAT mRNA, suggesting a post-transcriptional mechanism (Buisson et al., 1989; Kenney et al., 1988; 1989). In addition, when the CAT reporter gene is changed to growth hormone, transactivation by BMLF1 is lost (Kenney et al., 1989). Reporter-gene dependence of BMLF1 activation suggests that BMLF1 is not directly involved in promoter activation, but instead may function to increase level of some unknown protein(s) required for EBV infection (Kenney et al., 1989). In addition, the PM promoter of BMLF1 is activated by both BZLF1 and BRLF1 IE gene products (Buisson et al., 1989).

(iv) MDV IE genes

Studies on MDV IE genes have been hampered due to the cell-associated nature of MDV *in vitro*. Characterization of RNA transcripts isolated from MDV-infected cells

treated with a metabolic inhibitor, such as cycloheximide (CHX) indicate that transcripts from IE genes are mainly clustered in repeat regions similar to locations of other herpesvirus IE genes (Maray et al., 1988; Schat et al., 1989). IE Transcripts, ranging from 0.6 to 4.4 kb, derived from BamHI fragments-A, D, H, I_2, I_3, L, and M regions are reported by different groups (Maray et al., 1988; Schat et al., 1989). However, all these reports were based only on Northern hybridization analysis, without exact gene and gene product identification.

Recently, two HSV IE homologs were identified with similar localization in MDV (Figure 2) (Anderson et al., 1992; Ren et al., 1994). The MDV ICP4 gene is located in BamHI-A fragment within the inverted repeat region. The gene is 4,245 nucleotide long with an AUG translation start site located at position 1,264. The predicted protein structure of MDV ICP4 is similar to its counterparts in VZV and HSV. MDV ICP4 contains five regions in which regions 2 and 4 are more conserved than others, and a serine-rich tract located towards the amino terminus (McGeoch et al., 1986; Anderson et al., 1992). However, the MDV serine run differs from those of other alpha-herpesvirus ICP4 homologs in that it is flanked on both sides by regions enriched in prolines and basic amino acids, whereas the HSV, VZV, and PRV serine runs are preceded by regions enriched in prolines and basic amino acids but are followed by strongly acidic regions. Also, a number of potential transcriptional regulatory sites are identified within or adjacent to the MDV ICP4 sequence, including ICP4 binding site, Oct-1 site, and TAATn3A sequence similar to VP16 recognizing sequence of TAATGARAT (Anderson et al., 1992). However, neither a protein product nor a regulatory function is known.

The HSV ICP27 homolog of MDV is mapped to the EcoRI-B fragment of MDV

DNA (Ren et al, 1994). The MDV ICP27 gene is 1,419 nucleotide long and encodes 473 amino acids with a predicated molecular weight of 54.5 kDa. Comparison of the predicted amino acid sequence of MDV ICP27 with that of HSV ICP27 and VZV ORF4 (HSV ICP27 homologue) shows a strikingly similarity (37.3% identity between MDV ICP27 and HSV-1 ICP27; 32.7% identity between MDV ICP27 and VZV ORF4) within the C-terminal region, which is the functional domain for HSV ICP27 and VZV ORF4. In addition, a conserved zinc finger motif is in the C-terminus of MDV ICP27. The zinc finger motif in HSV ICP27 is involved in DNA, RNA and protein-protein interaction (Sandri-Goldin, 1991; Smith et al., 1991). Using antibody raised against TrpE-ICP27 fusion protein, a 55 kDa gene product was identified by western blot analysis (Ren et al., 1994). Information regarding functional assays of ICP27 of MDV is not yet available.

Recently, a 1.6 kb immediate-early transcript is localized to the MDV BamH I_2 region by Northern hybridization analysis of RNA isolated from MDV infected cells treated with CHX (Hong and Coussens, 1994). By cDNA cloning and sequencing, two cDNAs of 1.4 kb (C1) and 1.35 kb (C2) are identified as spliced transcripts spanning MDV BamHI-H and I_2 fragments. C1 and C2 use the same splice acceptors and 3' ends, but they differ at their 5' ends and utilize different splice donors. Despite abundant transcription detected in Northern blot analysis, sequencing analysis shows only two small open reading frames (ORFs) within each cDNA. C1 cDNA contains ORF 1a (83 amino acids) and ORF 2 (107 amino acids), while C2 cDNA contains ORF 1b (76 amino acids) and the same ORF 2 in C1. All potential ORFs within C1 and C2 were searched against the Swiss data base without finding any significant homology, suggesting that any protein encoded by these ORFs would be MDV specific. A 14 kDa polypeptide (p14) is detected

by Western blot analysis using antisera raised against ORF1a- and ORF1b-GST fusion proteins (Figure 2). This 14 kDa protein is expressed in cells which are lytically infected with MDV oncogenic strains (GA, md11), and their attenuated derivatives, as well as in the latently MDV-infected, transformed MSB-1 cell line. Like most IE gene products in other herpesviruses, p14 is a phosphoprotein and therefore, designated as pp14. However, both Western blot analysis of subcellular fractions and immunofluorescence detection reveal that p14 is predominately a cytoplasmic protein (Hong et al., 1994). Whether pp14 has a regulatory function on other MDV gene expression is still unknown at this point.

In contrast to most other herpesvirus, MDV IE gene transcripts and gene products are identified in latently infected and transformed lymphoblastoid cell lines (Maray et al., 1988; Schat et al., 1989; Hong and Coussens, 1994). Therefore, it is tempting to speculate that MDV IE gene products are involved in induction and/or maintenance of the latency or transformed state. If true, this would make MDV latency similar to EBV.

(V). Early and late genes.

Early genes of herpesviruses are defined as genes which start transcription and protein synthesis at approximately 3 hr post-infection, peak by about 5-7 hr post-infection, and decline thereafter. Early proteins are involved in nucleotide precursor metabolism and viral DNA synthesis. Most early gene products, therefore, are metabolic enzymes and proteins for virus replication (Roizman and Sears, 1991). In HSV, the most well characterized early proteins include viral ribonucleotide reductase, major DNA-binding protein, thymidine kinase (TK), and DNA polymerase. Some MDV early genes homologs of HSV have been identified, such as TK, DNA polymerase, DNA-binding protein etc.

(Buckmaster et al., 1988). Also, pp38, a MDV specific gene is expressed in infected cells treated with PAA, suggesting it may be an early protein (Chen and Velicer, 1992). Appearance of early proteins signals onset of viral DNA synthesis, and subsequently induction of late gene expression.

Based on dependence for viral DNA replication, late genes can be subdivided into γ1 and γ2. Transcription of γ1 genes occurs prior to initiation of viral DNA synthesis and thus does not depend stringently on viral DNA replication. As viral DNA synthesis begins, high level expression of γ1 genes occurs and γ2 gene expression begins. Both γ1 and γ2 protein synthesis continues throughout the remaining replication cycle (Wagner, 1991; Roizman and Sears, 1991). Most late gene products are structural proteins for virion capsid, tegument, and envelope. Eight glycoproteins, including gB, gC, gD, gE, gG gH, gI and gJ are well characterized in HSV (Roizman and Sears, 1991). The functions of HSV glycoproteins are defined and involve virus attachment, penetration and cell fusion. In MDV, six HSV glycoprotein homolog genes (gB, gC, gD, gE, gI and gK) are identified and sequenced (Buckmaster et al., 1988; Chen and Velicer, 1992; Coussens and Velicer, 1988; Isofort et al., 1987; Ross et al., 1989; 1991; Brunovskis and Velicer, 1992, Ren et al., 1994). Among them, gB and gC (also designated as A and B antigen, respectively), have been extensively studied at antigen level. MDV gB is located in BamHI-K_3 and I_3 regions and is processed into a family of proteins known as gp100, gp60 and gp49 (Chen and Velicer, 1991; Ross et al., 1989). Antibodies against MDV gB can neutralize MDV in cell culture (Ikuta et al., 1984). Recombinant fowl pox virus (FPV) expressing an MDV gB homolog *in vivo* shows complete protection against Marek's disease (Nazerian et al, 1992).

MDV gC gene is located in BamHI-B fragment (Isfort et al., 1986; Coussens and Velicer, 1988). It encodes a N-linked glycoprotein of 57-65 kilodalton with a precursor of 44 kDa (Isofort et al., 1986). MDV gC is a secreted protein and its expression is significantly reduced in attenuated MDV strains (Bulow and Biggs 1975; Ikuta et al., 1983; Isfort et al., 1986). However, the mechanism leading to reduced gC expression and its relationship with MDV oncogenicity or attenuation is still unclear. Based on comparison of the protein level, gene structure, steady-state RNA and transcription rates of MDV gC between oncogenic and attenuated MDV isolates, Wilson et al. (1994) suggest that reduced expression of MDV gC is directly related to a reduction in transcription rate of MDV gC genes in attenuated strains. Lack of DNA sequence alternations in gC gene coding regions and promoters in attenuated strains, Wilson et al. further suggest that reduced transcription of gC is due to alternation of viral or cellular proteins which regulate gC promoter activity in attenuated MDV (Wilson et al., 1994).

5. MDV latency

An important biological property of all three subfamilies of herpesviridae is their ability to establish latent infections in their natural hosts. Alpha-herpesvirus latency occurs primarily in nervous tissue, including sensory and autonomic nerve ganglia and the central nervous system. Beta-herpesviruses can establish latent infection in secretory glands, lymphoreticular cells, kidney and other tissues. Gamma-herpesvirus latency is confined to lymphoid tissues (Roizman, 1991). Although MDV genomic structure is similar to that of alpha-herpesviruses, latent infection of MDV occurs predominantly in lymphocytes, similar to gamma-herpesviruses (Payne, 1985). As described previously, MDV infection

switches from lytic infection of primary B-lymphocytes to latent infection of predominantly T-lymphocytes at about one week post-infection, coincident with a temporary immune recovery (Payne, 1985). Most latently infected T cells are activated, $CD4^+CD8^-$ T helper cells (Schat et al., 1991).

Studies on MDV latency are mostly conducted on MDV transformed lymphoblastoid cell lines which are considered as latently infected. Virus can be rescued from most of these tumor cell lines by co-cultivation with permissive cells, such as CEF, DEF, or by inoculation of tumor cells into chickens (Schat, 1985). However, the ability to rescue virus can be lost after prolonged in vitro passage of MDV tumor cell lines. Based on the capability of virus to be recovered and viral antigen expression, MDV transformed cell lines are distinguished as producer cell lines and non-producer cell lines. Producer cell lines are those cell lines from which virus can be rescued after *in vitro* co-cultivation or following innoculation into susceptible chickens. Producer lines can be further classified as expression or non-expression lines based on proportion of cells which spontaneously express viral antigens. Expression lines, such as MDCC-CU36, MSB-1, contains a high proportion of antigen expression cells which can be detected by immunofluorescence (IF) tests using conventional anti-MDV sera. While non-expression cell lines, such as MDCC-CU41, MKT-1, contain none or only a few cells expressing viral antigen. Treatment of non-expression cell lines with 5-iodo-2-deoxyuridine (IUdR) can induce viral antigen expression detectable by anti-MDV sera. In non-producer cell lines, such as MDCC-HP1 or MDCC-RP1, viral antigens are not detectable and virus can not be rescued by co-cultivation. All MDV lymphoblastoid cell lines are free of infectious virion particles, but contain multiple copies of the viral genome, typically between 5 to

15 copies per cell (Akiyama and Kato, 1974; Nazerian, 1977; Schat et al., 1989; Silver et al., 1979). The status of MDV genomes in transformed cell lines has been reported as closed circular DNA by several investigators using ethidium bromide-CsCl equilibrium centrifugation to separate viral and cellular DNA (Lee et al., 1971; Tanaka et al., 1980; Hirai et al., 1979). Recently, Delecluse and Hammerschmidt (1993) observed MDV DNA integration into the host cell chromosomes in all six MDV transformed cell lines by Gardellar gel electrophoresis and in situ hybridization techniques. MDV integration sites are preferentially located at telomeres of large- and mid-size chromosomes or on mini-chromosomes. Only a minor population of MDV genomes were detected as linear or covalently circular DNA. Based on these observations with MDV and a similar phenomena of EBV DNA integration in a number of Burkitt's lymphoma cell lines, the authors proposed that herpesvirus DNA integration is common and may provide clues for understanding virus tumorigenicity (Delecluse and Hammerschmidt, 1993).

Similar to other herpesvirus latency, MDV transformed cell lines only have limited viral gene expression compared with many viral transcripts in lytically infected cells (Silver et al., 1979; Sugaya et al., 1990). Since MDV latency and oncogenicity are closely related, genes expressed in transformed cell lines may be important to initiate or maintain latency and/or transformation by MDV. However, contradictory results are reported by several groups. Maray et al. (1988) reported 29 transcripts detected in Northern hybridization dispersed over almost the entire MDV genome in MSB-1 cells. Schat et al. (1989) compared transcription of MDV genomes in lytically infected cells and different types of lymphoblastoid cell lines. Between 4 and 7 transcripts are detected in MDCC-HP1 (non-producer cell line) and MDCC-CU41 (non-expression cell line), respectively.

These RNAs are transcribed from IE genes located mainly in repeat regions flanking U_L and U_S and in U_S. Additional transcripts are identified when IUdR is added to culture medium. In MDCC-CU36, an expression cell line with a high percentage of antigen-positive cells, most of the transcripts in lytically infected cells are detected (Schat et al., 1989). Sugaya et al. (1990) reported 32 viral transcripts in a non-producer cell line, MKT-1. These transcripts are clustered in the short and long repeat regions, similar to the observation of Schat et al. (1989). The most abundant transcripts were encoded in BamHI-I_2 region. Together, these data suggest that repeat regions of MDV encode genes which may play important roles in MDV latency and transformation.

Consistent with transcripts identified in Northern blot analysis, three genes and their gene products have recently been identified in MDV transformed cell lines. All of them are located in repeat regions. Phosphoprotein 38 (pp38) was first identified as one of three viral proteins (41, 38, and 24 kDA) detected by a monoclonal antibody against a λgt11 fusion protein containing MDV BamHI-H fragment sequence (Silva and Lee, 1984). Based on Northern blot analysis and DNA sequencing, the gene encoding pp38 is mapped to the BamHI-H fragment region and spans the junction of MDV long unique (U_L) and long internal repeat (IR_L) regions. The pp38 gene is translated from unspliced mRNA in a leftward direction. The gene product, phosphoprotein 38, is 290 amino-acids in length and is phosphorylated primarily at serine residues (Nakajima et al., 1987). The protein is relatively rich in acidic residues with glutamic and aspartic acid composing 15% of the predicted amino acid sequence (Nakajima et al., 1987; Cui et al., 1991; Chen et al., 1992). Pp38 is only expressed in serotype-1 (oncogenic and attenuated) MDV infected cells, but not in nononcogenic serotype-2 and serotype-3 infected cells. It also

has been shown that pp38 is abundantly expressed in the latently infected MSB-1 lymphoblastoid cell line without IUdR induction (Chen et al., 1992).

Another MDV phosphoprotein, pp14, was recently identified and is also located in the long internal repeat (IR_L) region. Encoded by spliced RNA(s) which span the BamHI-H and I_2 fragments, pp14 is transcribed in a righward direction, opposite to that of pp38 (Hong and Coussens, 1994). On the basis of transcriptional mapping and alignment with published BamHI-H viral genomic DNA sequence (Bradley et al., 1989 b), the upstream promoter regulatory region of pp14 is defined to be a bidirectional promoter-enhancer region shared by pp38 (Hong and Coussens, 1994). Similar to pp38, pp14 is expressed in MDV transformed cell line, MSB-1, and is MDV serotype-1 specific antigen. While pp38 is insensitive to phosphonoacetic acid inhibition, suggesting that pp38 is an early viral protein, pp14 is derived from an immediate-early gene. Western blot analysis of subcellular fractions and indirect immunofluorescence reveal that both proteins are cytoplasmic (Cui et al., 1991; Hong and Coussens, 1994). The functions of pp38 and pp14 are yet to be determined.

A third gene, designated as *meq*, is highly expressed in MDV infected lymphoblastoid cell lines and is located in BamHI-I_2 and EcoRI-Q regions (Jones et al, 1992). The *meq* gene encodes a protein of 362 amino acids, which has a leucine zipper repeat and an upstream domain rich in basic amino acids, both characteristics of the *fos/jun* family of transcriptional activators. The C-terminal region of *meq* contains a proline-rich domain (26% proline) and is rich in acidic amino acids, which are features of another class of transcription factors, such as AP-2, C/EBP, OCT-2. Using antiserum raised against a synthetic peptide corresponding to the leucine zipper region of *meq*, a 40

kDa protein is detected in the MDV transformed RP4 cell line. Although EcoR-Q derived transcription can be detected in MDV (strain JM) acutely infected DEF by Northern blot analysis, the 40 kDa protein is not present in cell lysates of DEF lytically infected with MDV (Jones et al., 1992).

Based on DNA-protein binding and DNA-protein immunoprecipitation, Wen et al. (1988) reported a 28 kDa nuclear protein, MDNA, which is expressed in MDV transformed cell lines (MKT-1 and MSB-1), but not in lytically infected cells. MDNA is bound to two loci in the BamHI-A fragment of the MDV genome. By DNase footprint, Wen et al. (1988) demonstrated that MDNA protect regions containing AT-rich and palindromic sequences. Therefore, the authors suggest that MDNA may function analogous to EBNA-1 of EBV in immortalized cells. However, there has been no functional analysis to confirm this hypothesis.

6. MDV tumorigenicity

The discovery of DNA tumor viruses was several decades later than that of RNA tumor viruses. Unlike RNA tumor viruses which all belong to a single group of retrovirus, DNA tumor viruses are distributed among six major families. Among them, polyomavirues, adenoviruses, and papillomaviruses are best understood, perhaps due to their small genomic size and ease of manipulation. Herpesviruses are associated with tumor induction, such as a link between EBV and Burkitt's lymphoma, or nasopharyngeal carcinoma in humans, MDV with Marek's disease in chickens. However, it was considered that herpesvirus in tumors might be a passenger rather than a causal agent. The successful recovery of infectious MDV virions from feather follicle epithelium of Marek's

disease infected chickens and reproduction of lymphomas in specific-pathogen-free chickens provided the first solid evidence of herpesvirus as a oncogenic agents in their natural host (Churchill and Biggs, 1967; Nazirian and Burmester, 1968). Further proof came with discovery of live virus vaccines, made from an antigenically related but apathogenic herpesvirus of turkey (HVT), which can protect chickens against MD tumors (Churchill et al., 1969). Although MDV is the first tumor virus effectively controlled by vaccination, the mechanism of tumorigenicity and vaccine protection is poorly understood.

After a brief cytolytic infection and short latency, MDV induces lymphoma within three weeks, suggesting MDV is an acutely oncogenic herpesvirus. Studies of MDV oncogenicity have focused on several aspects, including comparison of gene expression in oncogenic MDV with their attenuated derivatives or with nononcogenic serotype-2 and -3; identification of genomic structure changes during attenuation; characterization of gene(s) specifically expressed in MDV transformed tumor cell lines. Among them, repeat regions of the MDV genome have been intensively investigated because of speculation that transcripts within this region may be important in MDV-induced tumorigenicity.

Several investigators have shown that serial *in vitro* passage of oncogenic MDV results in loss of MDV tumorigenicity. Attenuation is strongly correlated with an expansion in two particular regions (BamHI-H and -D), present in MDV TR_L and IR_L regions, respectively (Fukuchi et al., 1985; Silva and Witter, 1985). It was later discovered that expansion was due to amplification of a specific 132-bp direct repeat sequence located within BamHI-H and -D regions (Maotani et al., 1986). Tumor induction studies in chickens shows that cloned virus populations which exhibit an amplification in this region have decreased tumorigenic capability. By contrast, viruses which do not

contain amplified BamHI-H or -D regions efficiently induce tumors in chickens (Fukuchi et al., 1985). However, in all cases, changes in other regions of the genome are present. These results led to the hypothesis that the existence of one or more genes within the expanded regions is responsible for initiation or maintenance the tumorigenic state of MDV.

Bradley et al. (1989) reported that a 1.8-kb gene family is transcribed rightwardly from the expanded region in MDV BamHI-H fragments. Nucleotide sequencing and transcriptional mapping data suggest the presence of a group of spliced transcripts (1.4 to 1.8 kb) which are composed of two exons. The first exon has two species with the same 5 'end and different 3' ends near or within the expanded region. The second exon contains five species with identical 3' ends and various 5' ends mapped outside of the expanded region. Therefore, this gene family shares a 5' initiation site and a 3' termination site, but contains different introns. Furthermore, the 1.8-kb RNA family is only expressed in CEF infected by oncogenic strains of MDV and in cell lines established from MDV-induced tumors, but not in CEF infected with an attenuated strain of MDV (Bradley et al., 1989). Later, Bradley et al. (1989) demonstrated that disappearance of the 1.8 kb transcript is due to truncation of the 1.8-kb transcript into a 0.4-kb transcript. These data suggest that the 1.8 kb gene family is associated with MDV tumorigenicity. This hypothesis is supported by antisense inhibition of proliferation of MDV-induced lymphoblastoid cell lines using an oligonucleotide complementary to the putative splice donor sequence of the 1.8 kb gene family (Kawamura et al., 1991).

Chen and Velicer (1991) reported four groups of transcripts derived from BamHI-H and D region based on cDNA analysis and S1 nuclease protection assay. These

transcripts can be either initiated or terminated within or near the expanded region of the 132-bp direct repeat at multiple sites and are transcribed in both rightward and leftward directions. Therefore, these RNAs contain a partial copy or one or more full copies of the 132-bp repeat at either their 5' or 3' end. Each 132-bp repeat contains one TATA box and two polyadenylation consensus sequences in each direction. The authors hypothesized that the 132-bp repeat may serve as a bidirectional promoter region to generate a diversity of transcripts (Chen and Velicer, 1991). Since these four groups are transcribed in opposite directions and complementary RNAs function as antisense RNA, a function in regulation of gene expression is possible (Chen and Velicer, 1991). In agreement with Bradley's paper, Chen and Velicer also speculate that the transcripts derived from the repeat regions are associated with the tumorigenic potential of MDV.

Iwata et al. (1992) also reported a cDNA library derived from MDV strain Md5-infected cells. Twelve clones, containing 1.1- to 1.8-kb insertions, localized to the BamHI-H fragment. All these cDNAs are derived from unspliced mRNAs transcribed rightwardly. Iwata et al., therefore, concluded that the majority of mRNA from the BamHI-H region is composed of unspliced transcripts.

Another cDNA library was constructed from MDV strain RBIB-infected cells by the group that identified the 1.8 gene family (Peng et al., 1992). Three classes of cDNA (four cDNAs) from the long inverted repeat region are identified, and all are transcribed rightwardly. Class I is represented by two cDNAs, one is a nonspliced 1.69-transcript which is completely located in BamHI-H fragment and contains two copies of the 132-bp repeat sequence; while another one is a spliced 1.5-kb transcript which share the same initiation site with the nonspliced 1.69-kb cDNA, but spanning BamHI-H and I_2 region.

Class II represents the 1.9-kb spliced transcript which shares the same splice acceptors and 3' ends as the 1.5-kb class I cDNA, but differs at the 5' end and at splice donors. Class III cDNAs are represented by the 2.2-kb cDNA which is derived from a nonspliced mRNA spanning the BamHI-H and BamHI-I_2 fragments of MDV DNA. Sequence analysis demonstrats two potential open reading frames in each of the cDNAs. According to the authors, two cDNAs of class I, the nonspliced 1.69-kb transcript and the spliced 1.5-kb transcript, belong to the 1.8 -kb BamHI-H gene family. The putative 63-amino-acid protein encoded by the first ORF (ORF-A) in the 1.69-kb cDNA and the putative 75-amino-acid protein encoded by the first ORF (ORF-B) in the 1.5-kb cDNA shows limited homology with the mouse T-cell lymphoma (TLM) oncogene and the *fes/fps* family of kinase-related transforming proteins (Peng et al., 1992). Recently, Peng et al. (1994 a) reported that the 1.69-kb and 1.5-kb cDNA can induce prolonged proliferation and reduced serum dependence of primary CEF cells in transfection assays. In addition, Peng et al. (1994 b) synthesized a polypeptide corresponding to the C-terminus of ORF-A in the 1.69-kb cDNA to raise antibody. A 7-kDa protein is detected in CEF cells infected with MDV oncogenic strain RBIB and MDV-induced tumor cell line, MSB-1.

As described above, a cDNA library constructed by Hong and Coussens (1994) also identified two spliced cDNAs (C1 and C2) which span BamHI-H and -I_2 fragments. These two cDNAs are derived from IE transcripts. One cDNA (C2) has the same splice donor and acceptor as the 1.9-kb cDNA reported by Peng et al. (1992). Therefore, there are at least three cDNAs, which utilize different splice donors but share a common splice acceptor site, in the BamHI-H and I_2 fragment of MDV. Each cDNA contains only two small ORFs. Protein similarity searches of putative C1 and C2 encoded polypeptide

against the Swiss-protein data base reveal only limited homology with a zinc-finger protein and *myc* proto-oncogene. However, these similarities are not in conservative or functional regions and, therefore, are not considered as significant. Using antisera raised against fusion protein of two putative ORFs (ORF1a of C1, ORF1b of C2), a 14 kDa phosphoprotein is detected in MDV (oncogenic and attenuated) lytically infected cells, and MDV transformed cell lines (Hong and Coussens, 1994). Since the protein is encoded by an ORF which spans a 2.3-kb intron, including the 132 bp repeat, the detection of this 14 kDa protein in attenuated strains suggests that removing the 132-bp repeat by splicing does not affect protein expression. However, subtle changes in amino acid content coincident with attenuation and expansion of the 132-bp repeat region cannot be ruled out.

Interestingly, three proteins (pp14, pp38, and *meq*) expressed in MDV-transformed cell lines are all located in the repeat region of MDV. These data support the posible association of this region with MDV tumorigenic potential. Among them, only *meq* is a partial homolog of *jun/fos* oncogene. Both pp14 and pp38 are MDV unique, serotype-1 specific antigens. Therefore, whether MDV encodes a specific oncogene, or pp38 and pp14 play indirect roles in tumorigenicity, such as stimulation of other cellular factors, will be important issues for understanding MDV oncogenicity. Furthermore, these proteins are detected in MDV transformed cell lines, whether they are expressed in MDV tumors has not yet been tested. The mechanism by which latent infection changes to neoplastic proliferation needs to be investigated further.

To understand MDV pathogenesis and tumorigenicity, we are interested in studying gene expression and regulation, especially genes in repeat regions of the MDV genome. The subject of this dissertation is focused on: 1) identification and localization

of an IE gene in the IR_L region, 2) identification of the gene product encoded by this IE gene and characterization of the properties of the protein.

Charter II

Identification of an Immediate-early Gene in the Marek's disease Virus Long Internal Repeat Region Which encodes a unique 14 kDa polypeptide

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ABSTRACT

Marek's Disease Virus (MDV) is an oncogenic avian herpesvirus whose genomic structure is similar to herpes simplex virus and varicella-zoster virus. Repeat regions of the MDV genome have been intensively investigated due to a potential relationship to MDV oncogenicity and abundant expression of immediate-early transcripts. In this report, a 1.6 kb immediate-early transcript was localized to the BamHI-I_2 region by Northern hybridization analysis. With cDNA cloning and sequencing, two cDNAs of 1.4 kb (C1) and 1.35 kb (C2) were identified. Both cDNAs are derived from spliced mRNAs spanning the BamHI-H and I_2 fragments. C1 and C2 use the same splice acceptors and 3' ends, but differ at their 5' ends and utilize different splice donors. The upstream promoter-enhancer region of C1 cDNA has been defined as a bidirectional regulatory region shared by the MDV pp38 gene. Sequencing analysis shows two small open reading frames (ORF) within each cDNA (ORF1a and ORF2 in C1, ORF1b and ORF2 in C2). Potential ORFs of the sequence have no significant homology with any known protein in the Swiss-Protein data base. DNA fragments encoding ORF1a and ORF1b were cloned into pGEX-3X vectors to produce GST-fusion proteins and induce antisera. In Western blot analysis of MDV infected cell lysates, a 14 kDa polypeptide was identified by antisera against both ORF1a and ORF1b. This 14 kDa protein is expressed in cells which are lytically infected with MDV strains GA, Md11 passage 16 (oncogenic) and Md11 passage 83 (attenuated), as well as in the MDV latently infected and transformed MSB-1 cell line.

INTRODUCTION

Marek's disease virus (MDV) is a highly cell-associated avian herpesvirus which induces T-cell lymphomas and peripheral nerve demyelination (Marek's disease) in chickens (Calnek, 1985; Churchill and Biggs, 1967). Although MDV has been effectively controlled by vaccination with an antigenically related but apathogenic herpesvirus of turkeys (HVT), the mechanism of MDV tumorigenicity is still unknown. Recent efforts in this regard have been focused on identification of genes which may be responsible for, or related to malignant transformation by MDV.

MDV was classified as a gamma-herpesvirus, based on its biological characterization (Roizman and Sears, 1991). However, the overall genome structure of MDV is more similar to human alpha-herpesviruses (e.g., varicella-zoster virus and herpes simplex virus), consisting of unique long (U_L) and unique short (U_S) regions, each bounded by a set of inverted repeats (TR_L, IR_L, IR_S and TR_S) (Cebrian et al., 1982; Fukuchi et al.,1984). As with other herpesviruses, MDV gene expression is coordinately regulated and sequentially ordered in a cascade fashion (Maray et al., 1988; Nazerian and Lee, 1976; Schat et al., 1989). Three major kinetic classes of genes are expressed as immediate early (IE), early (E), and late (L) genes. IE genes are expressed immediately upon infection and do not require de novo viral protein synthesis. Characterization of RNA transcripts isolated from MDV infected cells treated with cycloheximide (CHX) indicates that transcripts from IE genes are clustered in repeat regions similar to the locations of other herpesvirus IE genes (Roizman and Sears., 1991; Schat et al., 1989). In addition, MDV IE transcripts can be detected not only in lytically infected cells but also in transformed cell lines in which MDV infection is considered latent. To understand

MDV gene expression and regulation during MDV tumor induction, we are interested in examining IE transcripts in MDV repeat regions and investigation of related IE gene products.

Repeat regions of the MDV genome have been intensively investigated due to speculation that transcripts within this region may be important in MDV induced tumorigenicity. Several investigators have demonstrated that serial *in vitro* passage of virulent MDV in primary chicken embryo fibroblast cells results in loss of MDV tumorigenicity. This attenuation was found to correlate with amplification of a specific 132 bp repeat sequence found within the MDV TR_L and IR_L (BamHI-H and D, respectively) (Bradley et al. 1989, a; b; Chen and Velicer, 1991; Fukuchi et al., 1985; Maotani et al., 1986; Silva and Witter, 1985). Bradley et al. reported that a 1.8 kb gene family is transcribed rightwardly from the expanded region of the BamHI-H fragment. According to Bradley et al. these transcripts are expressed only in oncogenic MDV, but absent or truncated in attenuated MDV. Antisense inhibition of proliferation of a MDV-derived lymphoblastoid cell line using an oligonucleotide complementary to the putative splice donor sequence of the 1.8 kb gene family supports the hypothesis of an association between this gene family and the tumorigenic potential of MDV (Kawamura et al., 1991). Recently, Peng et al. (1992) further developed a cDNA library from this BamHI-H gene family and identified four cDNA clones. While two cDNAs of 1.9 and 2.2 kb, were reported as nonspliced transcripts, two other cDNAs, 1.5 and 1.9 kb were recognized as single spliced transcripts spanning the BamHI-H and BamHI-I_2 fragments of MDV DNA. Protein products associated with these cDNAs or other transcripts from the BamHI-H gene family have not been identified. Other transcripts that are initiated or terminated

within or near the 132 bp repeat region have also been described (Chen and Velicer, 1991). Recently, a 38 kDa phosphoprotein expressed both in MDV lytically infected cells and transformed lymphoblastoid tumor cell lines was localized to the BamHI-H region and is transcribed in a leftward direction (Chen at al., 1992; Cui et al., 1991). A basic-leucine zipper gene, designated *meq*, (identified as a homolog of *fos/jun* oncogene family), has been mapped to the rightward region of BamHI-I_2 fragment, within the MDV IR_L. By using antiserum against a synthetic peptide deduced from the *meq* DNA sequence, a 40 kDa protein was detected in MDV transformed lymphoblastoid cell lines but not in cells lytically infected with MDV strain GA (Jones et al., 1992).

In this study, we report analysis of an IE gene localized within the MDV IR_L. By cDNA cloning and sequencing, we have determined that distinct transcripts are derived from this same gene by altered splicing patterns. In all cases examined, the 132 bp repeat region is not present in mature mRNA species. Computer sequence analysis revealed only small open reading frames within our cDNA transcripts, consistent with previous report (Peng et al., 1992). Using antisera raised against fusion proteins, we demonstrate that a 14 kDa protein encoded by two small ORFs in these transcripts is expressed in cells lytically infected with both oncogenic and attenuated MDV, as well as in cells latently infected and transformed by MDV. Our results have important implications regarding the role of proteins encoded within the MDV IR_L in MDV-induced tumorigenicity.

MATERIALS AND METHODS

Cell culture and virus.

Duck embryo fibroblast (DEF) cells and chicken embryo fibroblast (CEF) cells were prepared, maintained, and infected with MDV as described previously by Glaubiger et al. (1983). Cell-associated MDV strain GA passage 7, Md11 passage 16 , designated as a low passage (LP) and passage 83, designated as a high passage (HP) used in this study were obtained from the Avian Disease and Oncology Laboratory (ADOL), U.S. Department of Agriculture, East Lansing, MI., and have been described (Wilson et al., 1994). To obtain immediate early RNA transcripts, 100 ug/ml cycloheximide (CHX) was added at the time of infection. RNA was extracted 16 to 24 hours post infection/CHX treatment. Early RNA was obtained by treating cells with 100 ug/ml phosphonoacetic acid (PAA) at the time of infection. Fresh medium containing 100 ug/ml PAA was added to infected and control cultures after 24 hours. RNA was extracted after an additional 24 hours incubation in PAA. The MSB-1 cell line (a producer, expression cell line) (Akiyama and Kato, 1974) was used as a representative of MDV-induced lymphoblastoid cell line and was cultured in Leibovitz L15-McCoy 5A medium supplemented with 10% fetal calf serum at 41°C, in a humidified atmosphere containing 5% CO_2 .

RNA isolation and Northern blot analysis.

Total cellular RNA was isolated from uninfected and MDV-infected DEF cells using the guanidinium-phenol:chloroform method (Chomczynski and Sacchi, 1987). Polyadenylated [poly(A)$^+$] mRNAs were purified from total RNA using the polyATract mRNA kit (Promega, Madison, WI) according to the manufacturer's specifications.

For Northern blot analysis, 0.5 ug poly(A)$^+$ mRNA was loaded per well, electrophoresed through 1.2% formaldehyde/agarose gels and transferred to Hybond-N nylon membranes (Amersham corp., Arlington Heights, IL) essentially as described (Sambrook et al., 1989). α-^{32}P-labeled probes were generated using a random primed labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) as recommended by the manufacturer. Hybridization was performed in high stringency conditions (50% formamide, 3X SSC, 5% dextran sulfate, 50 mM phosphate buffer, 5X denhardt's, 0.1% SDS and 100 ug/ml salmon sperm DNA, at 42° C for 12-16 hs). Transcription sizes were determined by comparison with a 0.24 to 9.5 kb RNA ladder (Bethesda Research Labs, Bethesda, MD) run on the same gel. RNA blots were stripped of probe DNA and rehybridized with a chicken β-actin gene probe (Cleveland et al., 1980). Intensity of RNA bands on autoradiographies were analyzed on a FB910 Densitometer using Zeineh 1-D Videophoresis II software (FisherBiotech).

cDNA library construction, screening and Southern blot analysis.

To enrich for cDNAs derived from IE genes, poly(A)$^+$ mRNAs isolated from MDV infected DEF cells, treated with cycloheximide, were utilized. Construction of a cDNA library was performed as described by Hu et al. (Hu et al., 1992). Briefly, plasmid pBluscript II KS+ was digested with the restriction enzyme EcoRV to generate blunt ends. Oligo (dT) tails were added to the blunt ends with T4 terminal transferase. After removal of the oligo(dT) tail at one end by digesting with SmaI, poly(A)$^+$ mRNA was annealed to the oligo (dT) tail of the remaining end and cDNA was synthesized directly along the vector-primer. Following digestion of RNA and second strand cDNA synthesis, blunt

ends were generated by treating with T4 polymerase and religated to generate recombinant cDNA clones. cDNA clones were transformed into *E.coli* DH10B cells (Bethesda Research Labs, Bethesda, MD) and the library was screened by in situ hybridization as described (Sambrook., et al., 1989). Positive colonies were isolated and expanded for analysis of plasmid DNA by Southern hybridization.

Primer extension.

Primer extension studies were performed essentially as described previously (Ausubel et al., 1991). Three oligonucleotide primers were designed based on cDNA sequences described in the text. Oligonucleotide primer 1 is: 5'-AGGAAATATATCGGG-GTACGGCCGT-3'; oligonucleotide primer 2a is: 5'-ATGGAAAGTGGGTCCGCAGTC-AATG-3'; and primer 2b is 5'-GTCAATGCATCCGGGGTCGTTCCCA-3'. The primers were 5'- end labeled with [γ-^{32}p] ATP and annealed to 30 ug total RNA isolated from uninfected and MDV-infected DEF cells for 90 minutes at 65°C. After cooling at room temperature for 30 minutes, reverse transcription was carried out at 42°C for one hour. Reactions were terminated by phenol-chloroform extraction and ethanol precipitation. Precipitated nucleic acids were resuspended and analyzed on 9% polyacrylamide/7 M urea sequencing gels.

Viral genomic DNA and cDNA clone sequencing.

Viral genomic DNA sequencing was initially performed on a 2.3 kb BamHI-XbaI subfragment of the BamHI-I_2 fragment from an MDV strain GA BamHI library (kindly provided by M.Nonoyama). This subfragment was subcloned into pUC18 and used to

generate overlapping deletions using ExoIII and S1 nucleases. At the same time, Sau3A and TaqI libraries of the 2.3 kb BamHI-XbaI fragment were constructed in M13 vectors. Nucleotide sequences of both strands were obtained by the dideoxy-chain termination method (Sanger et al., 1977) using the Sequenase enzyme (United States Biochemical Corp., Cleveland, OH). Both forward and reverse 17-mer universal primers were used to sequence the ends of subcloned DNA. For acquiring the junction sequence of BamHI-I_2 and BamHI-H, a HindIII-XbaI subfragment was isolated from the EcoRI-F fragment of a MDV strain GA EcoRI library (kindly provided by R. F. Silva, ADOL, East lansing, MI). This subfragment was cloned into pUC18 and sequenced in both directions as described above. For cDNA clone sequencing, the dideoxy-chain termination method was conducted as described for viral genomic DNA clones. T7 forward and KS reverse primers were used for the end sequences of each cDNA.

Computer analysis of DNA sequence.

Nucleotide sequences were analyzed using the Genepro program (Riverside Scientific Enterprises, Seattle,WA) and MacVector 3.5 (International Biotechnologies, Inc., New Haven, CT). Amino acid sequences of the putative polypeptide encoded by open reading frames (ORF) were searched against the protein sequence data deposited within Swiss-Protein data bases using the Genetics Computer Group program FASTA from the University of Wisconsin (Devereux and Smithies, 1984).

GenBank accession number.

The nucleotide sequences of the C1 and C2 cDNA reported in this paper have

been given GenBank accession numbers L26394 and L26395, respectively.

Expression of GST fusion proteins and antibody production.

The vector system used to express C1 and C2 ORFs in *E.coli* is plasmid pGEX-3X (Pharmacia, Alameda, CA) which contains a glutathione S-transferase (GST) gene under the control of an isopropylthiogalactopyranoside (IPTG)-inducible *tac* promoter. DNA fragments containing ORF1a of C1 cDNA and ORF1b of C2 cDNA were ligated into the 3' end of the GST ORF as in-frame insertions. The respective GST fusion proteins were purified using glutathione-sepharose 4B (pharmacia, Alameda, CA) as recommended by the manufacturer. Purified fusion proteins were applied to a 0.1% sodium dodecyl sulfate (SDS)-10% polyacrylamide gel. Positions of the fusion proteins were identified by Coomassie brilliant blue staining. Sizes of fusion proteins were estimated by comparison with protein MW standards run on the same gel.

New Zealand White rabbits were initially immunized with 400-500 ug of purified fusion proteins in Titer-Max adjuvant (CytRx Co., Norcross, GA). The rabbits were boosted with the same amount of protein in Titer-Max every four weeks and bled ten days after final boost. The immune sera were preabsorbed with GST carrier protein to remove cross-reacting antibody. Antibody titers were determined by Enzyme-Linked Immunosorbent Assay (ELISA) as described (Sjogren and Jeansson, 1990).

Western immunoblot analysis.

Cultured cells were lysed with triple-detergent lysis buffer (Sambrook et al., 1989) and separated on 12.5% or 15% polyacrylamide gels containing 0.1% SDS. Proteins were

electrophoretically transferred to nitrocellulose filters. Immune detections were performed using an Amersham ECL™ western blot kit according to the manufacturer's specifications. The filters were blocked with 5% nonfat milk and probed with anti-ORF1a and ORF1b sera at 1:100 dilution. Donkey anti-rabbit immunoglobulin conjugated with horseradish peroxidase was used as second antibody. Proteins were detected by luminescence reagent and exposed to X-ray film. Protein sizes were estimated with reference to prestained protein MW standards (Bio-RAD, Richmond, CA) run on each gel.

RESULTS

Detection of an immediate early transcript within BamHI-I_2 region.

Previous studies have shown that MDV IE genes are mainly located in repeat regions, but there have been conflicting reports on the sizes and numbers of transcripts from these regions (Bradley et al., 1989, a; b; Chen and Velicer, 1991; Maray et al., 1988; Peng et al., 1992; Schat et al., 1989). To avoid problems associated with unprocessed precursor transcripts and introns, poly(A)$^+$ mRNAs isolated from uninfected, MDV-infected DEF cells, and infected DEF cells treated with CHX or PAA were purified for Northern blot analysis. Initial studies revealed that the BamHI-I_2 fragment hybridized to a 1.6 kb transcript. This 1.6 kb BamHI-I_2 transcript was abundantly expressed in cycloheximide treated cells (enhanced 3.5 fold over untreated MDV infected cells normalized relative to a chicken actin probe), and is identified in PAA treated cells after longer exposure (data not shown), suggesting it is expressed with immediate-early kinetics (Figure 1A). To verify location of this 1.6 kb IE transcript, various subfragments of the 5.1 kb BamHI-I_2 fragment were utilized as probes in Northern hybridization. These studies confirmed localization of the 1.6 kb transcript to the leftward BamHI-XbaI region of BamHI-I_2, juxtaposed to the right region of the BamHI-H fragment (Figure 1B). Thus, this 1.6 kb transcript may be related or identical to the 1.8 kb transcripts which hybridize to the BamHI-H fragment (Bradley et al., 1989 b), and is also consistent with the 1.7 kb transcript of BamHI-I_2 identified by Peng et al. (1992). Smaller RNA species (1.0 kb, 0.5 kb and 0.4 kb) expressed with immediate-early kinetics were also observed in Northern hybridizations along with the 1.6 kb transcript (Figure 1A, and 1B). As described later

in this report, smaller RNA species are most likely derived from undegraded intron RNAs.

Viral genomic DNA sequence of the BamHI-XbaI region from a MDV BamHI-I_2 fragment.

Data from Northern blot hybridization indicated that an immediate-early transcript was encoded within the leftward BamHI-XbaI region of the BamHI-I_2 fragment. This region of MDV DNA was subcloned and completely sequenced as described in Materials and Methods. Despite abundant transcription in infected cells, no large open reading frame (ORF) was found in either direction of this region, though several smaller (<100 amino acids) ORFs were identified (data not shown). To confirm the accuracy of sequence data and acquire the junction sequence between the BamHI-H and BamHI-I_2 fragments, a HindIII-XbaI subclone derived from the EcoRI-F fragment was also sequenced. As before, no continuous large open reading frames were observed (data not shown). Two possibilities were considered to explain a lack of ORFs within an abundantly transcribed region: (i) transcripts from the BamHI-I_2 region may function similar to latency associated transcripts (LATs) in HSV-1 infected neurons (Rock et al., 1987; Spivack and Fraser, 1987), or (ii) RNAs encoded within this region are extensively spliced. Data from Peng et al. (1992), Bradley et al. (1989 a; b), and Chen and Velicer (1991), suggested the later case was more likely. However, a protein product from this region had not been identified by any of these groups.

Isolation of cDNA and cDNA sequence analysis.

In an attempt to distinguish between the possibilities described above, a cDNA library was constructed using poly(A)$^+$ mRNA from DEF cells infected with MDV strain GA and treated with cycloheximide. In construction of this library, we considered both the reported sequence of BamHI-H and our own sequence of the MDV BamHI-I_2 fragment. Both sequences contain many stretches of high AT content. Using oligo dT to prime cDNA synthesis in this area could lead to multiple starts within transcripts. For this reason, we chose a modified Okayama-Berg procedure (Hu et al., 1992) as described in Materials and Methods. This procedure is less likely to support priming within transcripts. The resulting cDNA library was screened with a 5.1 kb BamHI-I_2 fragment by in situ hybridization. Three cDNA clones (C1 through C3) were isolated. Insert sizes for these clones were estimated to be 1.4 kb for C1, 1.35 kb for C2, and 0.7 kb for C3 by Southern blot analysis. The three clones were sequenced on both strands, and compared to MDV genomic sequence data. C1 and C2 were identified as derived from different spliced RNAs and C3 was a partial cDNA clone. C1 and C2 transcripts both extend from BamHI-H into BamHI-I_2 in a rightward direction (Figure 2). C1 and C2 start from different 5' initiators, have different splice donor sites within the BamHI-H region, but share the same splice acceptor site and 3' end within the BamHI-I_2 region (Figure 2).

The complete nucleotide sequences of C1 and C2 are presented in Figure 3. The C1 cDNA clone has an insertion of 1295 base pairs before the poly(A) tail. The 5' 38 bp of C1 are identical to nucleotides 701 to 738 of a previously published BamHI-H sequence (Bradley et al., 1989, b). Beginning with nucleotide 39, C1 sequence was aligned with the BamHI-I_2 genomic sequence from nucleotide 224 to 1489. Sequence alignment revealed the presence of a 2.3 kb intron, which encompass the 132 bp repeat

region. A potential TATA sequence was found 42 bp upstream of the 5' end of C1 and a putative polyadenylation signal sequence (ATTAAA) is located 17 bp upstream of the 3' end of our C1 cDNA clone. This spliced transcript utilized a splice donor, GAAGGA, beginning at nucleotide 739 of BamHI-H sequence and a splice acceptor, ATCGTTGCAG, at nucleotides 214 to 223 of the BamHI-XbaI subfragment of BamHI-I_2.

The C2 cDNA contains a 1275 bp insert. The beginning 18 base pairs were identical to nucleotide 2071 to 2088 of the BamHI-H sequence (Bradley et al., 1989, b). The remaining sequence of C2 was aligned to the BamHI-I_2 fragment, which is identical to C1 cDNA. C2 cDNA initiates 512 bp downstream of the 132 bp repeat region and ends at the same 3' site as C1 cDNA. The C2 transcript thus contains a 1.0 kb intron. This intron employs a splice donor, GTATGC, located at nucleotides 2088 to 2093 of the BamHI-H fragment, and the same splice acceptor used by the C1 transcript. C2 cDNA therefore appears to be analogous to cDNA 3 identified by Peng et al. (1992).

Despite significant attempts, we were unable to identify any cDNA clones which initiated within the 132 bp region of MDV BamHI-H.

Transcriptional mapping of C1 and C2 cDNA.

In order to determine the precise 5' ends of our cDNA clones, primer extension experiments were conducted. Three oligonucleotide primers were designed, based on our cDNA sequence. The position and direction of these primers are schematically depicted in Figure 2. The first primer (P1), 5' AGGAAATATATCGGGGTACGGCCGT 3', was complementary to C1 cDNA sequence positions 14-38, which is just upstream of the

intron. The 42 bp extension observed in reactions with P1 and RNA isolated from MDV GA infected cells (data not shown) indicated the C1 transcript starts 13 base pairs downstream of a putative TATA sequence within BamHI-H. This location is consistent with the start site of the 1.8 kb gene family described by Bradley et al. (1989, b). An ATG translation start site was found 37 bp down stream of the putative TATA sequence. However, the optimal context surrounding AUG (A at -3 position, G at +4 position) was not found (Kozak, 1991). The TATA sequence, upstream of the C1 start site, belongs to a putative promoter-enhancer region containing a variety of potential transcription regulatory elements (Bradley et al., 1989, b; Cui et al., 1991). This promoter-enhancer region has been cloned into a chloramphenicol acetyl transferase (CAT) reporter plasmid in both orientations in our laboratory. Transient expression assays indicate that this region is, in fact, a bidirectional promoter activated by infection with MDV (Abujoub and Coussens, unpublished observations).

The second primer (P2a) 5' ATGGAAAGTGGGTCCGCAGTCAATG 3' is complementary to C2 cDNA sequence position 29 to 53, which is 10 base pairs downstream of the splice acceptor. The third primer (P2b) 5' GTCAATGCATCCGGGGTCGTTCCCA 3' is complementary to C2 cDNA nucleotides 9 to 33 and spans the exon/intron junction. Primer extension bands of 135 bp, 99 bp, 77 bp and 54 bp in length were observed using P2a and P2b primers hybridized to RNAs isolated from MDV GA infected cells (data not shown). The 77 bp band matched the C1 initiation site. It is difficult to determine which extension product represents the actual initiation site of C2 RNA, as we can not exclude the presence of other spliced transcripts using the same splice acceptor as C1 and C2 or presence of unspliced transcript

precursors.

To confirm the origin from which our cDNA clones were derived, Northern hybridization was conducted using the C1 cDNA insert as a probe. A 1.6 kb transcript was detected in poly(A)$^+$ mRNA isolated from MDV strain GA infected cells and enhanced in mRNA from infected cells treated with cycloheximide (Figure 4, lanes 2 and 3). No hybridization to uninfected cell mRNA was observed (Figure 4, lane 1). This result indicates that the cDNA was derived from the 1.6 kb immediate early RNA previously detected by a BamHI-I_2 fragment probe. Consistent with sequencing and primer extension data, the C1 cDNA is 1.3 kb in length, without a poly(A) tail. Interestingly, minor transcripts of 1.0 kb, 0.5 kb and 0.4 kb observed in Northern hybridizations probed with the BamHI-XbaI subfragment of BamHI-I_2 were not detected when using the C1 cDNA insert as a probe (Figure 4). This result suggests that these smaller RNA species are most likely derived from undegraded intron RNAs. In support of this observation, unspliced RNA precursors (3.3 kb and 2.6 kb) as well as the 2.3 kb intron are readily detected in Northern blots of total RNA from MDV infected cells (data not shown).

Analysis of potential open reading frames in MDV BamHI-I_2 related cDNAs.

Nucleotide sequences of C1 and C2 cDNA were analyzed using the Genepro, Macvector, and GCG programs. Translation of the cDNAs in all six reading frames identified two small potential open reading frames in both C1 and C2. In C1 cDNA, ORF1a could encode 83 amino acid residues, resulting in a putative protein of 9,307 Da. ORF1a has two potential casein kinase consensus phosphorylation sequences (amino acid 5 and 66), four potential histone kinase consensus phosphorylation sequences (amino acid

9, 42, 49 and 63), and three N-glycosylation sites (amino acid 3, 34 and 64). ORF2 could encode a 107 amino acid protein of 12,332 Da. ORF2 has four sites of histone kinase consensus phosphorylation sequence (position 7, 43, 44 and 71) and one potential site of N-glycosylation (position 63). C2 cDNA could encode two ORFs of 76 (ORF1b) amino acids and 107(ORF2) amino acids , respectively. The predicted size of an ORF1b encoded protein is 8,381 Da with one potential casein kinase phosphorylation consensus sequence (position 59), three potential histone kinase phosphorylation sequences (position 35, 42 and 56) and two potential N-glycosylation sites (position 27 and 57). ORF2 of C2 cDNA is the same as ORF2 in C1(Figure 5) and corresponds to ORF-F reported by Peng et al (1992). All potential ORFs were compared with protein sequence data deposited within the Swiss-Protein data base without finding highly significant homology. However, limited homologies were found with mouse zinc finger protein ZFP-27(mkr4) and *myc* proto-oncogene protein. ORF1a has a 29% identity to mouse zinc finger protein ZFP-27 (mkr4) (21 of 78 amino acids overlapping) and ORF1b has a 28% identity to ZFP-27 (14 of 50 amino acids overlapping) (Chowdhury et al., 1988). The N-terminus of ORF2 has a 30% homology with the *myc* proto-oncogene exon 3 (19 of 66 amino acids overlapping) (van Beneden et al., 1993) (data not shown). A lack of continuous open reading frames within our cDNA sequences is consistent with results of Peng et al. (1992) and raises questions regarding the function of these abundantly expressed spliced transcripts.

Identification of a 14 kDa Protein of ORF1a and ORF1b gene products.

Previous analyses of transcripts encoded by the BamHI-H and I_2 regions of MDV DNA have failed to determine if any protein product is produced by these extensively

spliced RNAs. Given the potential importance of this region in viral oncogenicity, identification of a protein product associated with BamHI-H and I_2 transcripts would be of considerable interest. To generate adequate amounts of protein for antibody production, DNA fragments which contain ORF1a (83 amino acids) of C1 and ORF1b (76 amino acids) of C2 were cloned into the 3' end of the glutathione S-transferase (GST) gene of pGEX-3X vectors (in frame insertions) and induced by IPTG to express fusion proteins in *E.coli* cells. Two fusion proteins GST-ORF1a and GST-ORF1b were generated and purified by glutathione-affinity chromatography. Antibodies against these proteins were raised in rabbits as described in Materials and Methods. The immune antisera were preabsorbed with purified vector protein (GST) to remove cross-reacting antibodies. After the second boost, antisera titer reached a 1:1000 dilution as determined by ELISA test (data not shown).

Western blot analysis was conducted to identify putative protein products of the C1 and C2 clones. Both ORF1a and ORF1b antisera detected a 14 kDa protein in CEF infected with MDV strain GA , but not in control CEF cells (Figure 6 A, B, lane 1 and 2). As described previously, ORF1a spans a 2.3 kb intron which includes the 132 bp repeat region amplified in the genome of attenuated MDV. To determine whether ORF1a and ORF1b gene products, identified in this study, incur any structural changes when the 132 bp region expands in attenuated MDV, we employed Md11p16 as a representative of low passage (oncogenic) virus, and Md11p83 as a high passage (attenuated) virus. Both fusion protein antisera detected a 14 kDa protein in lysates of cells infected with low and high passage Md11(Figure 6 A, B, Lane 3 and 4). Since these gene products originate from the repeat region, which is speculated to be associated with MDV tumorigenicity,

we further explored the gene products using the MDV lymphoblastoid cell line MSB-1. A 14 kDa protein was readily detected by both ORF1a and ORF1b antisera in the MSB-1 tumor cell line without IUdR induction (Figure 6 A, B , lane 5). These results suggest that ORF1a and ORF1b indeed encode polypeptides in MDV infected cells. Furthermore, both oncogenic and attenuated strains of MDV produce similar amounts of this polypeptide.

DISCUSSION

Reports on size, number, and direction of transcripts derived from MDV repeat segments (IR_L and TR_L), especially around the 132 bp expansion region, have been well documented (Bradley et al., 1989, a; b; Chen and Velicer, 1991; Maray et al., 1988; Schat et al., 1989). Many reports offer confusing and often conflicting data regarding initiation, splicing, and termination. Transcripts derived from the BamHI-H fragment may comprise a 1.8 kb gene family, including three transcripts of 1.8, 3.0, and 3.8 kb as reported by Bradley et al. (1989, a; b). Four groups of transcripts are postulated to initiate or terminate within or near the 132 bp repeats (Chen and Velicer, 1991). Three transcripts of 4.1, 3.0 and 1.9 kb in BamHI-H and six transcripts from 1.8 to 5.9 kb in BamHI-I_2 were reported by Schat et al. (1989). Two transcripts (5 and 2.5 kb) in BamHI-H and similar sized transcripts in BamHI-I_2 were reported by Maray et al. (1988). Among these transcripts, the 1.9 kb in BamHI-H and 1.8 kb in BamHI-I_2 identified by Schat et al. (1989), as well as the 5 kb and 2.5 kb transcripts in BamHI-H reported by Maray et al. (1988) were cataloged as immediately-early gene transcripts.

Here we report that a 1.6 kb major transcript which hybridized to BamHI-I_2, is highly expressed in MDV strain GA infected and CHX treated DEF cells. Accumulation of this 1.6 kb transcript in CHX treated cells suggests it is expressed with immediate-early gene kinetics. The decrease of this transcript in PAA treated cells is expected, because inhibition of viral replication by PAA can limit new viral infection and subsequently reduce accumulation of viral transcripts in infected cell cultures. Though CHX would also prevent virus spread, IE gene transcription is not limited by feedback inhibition as it would be in PAA treated or untreated cells. Complete nucleotide sequence analysis of

the 2.3 kb BamHI-XbaI fragment of MDV BamHI- I_2 and its positioning relative to previously published MDV BamHI-H sequences (Bradley et al.,1989, b) revealed no large open reading frames in over 5 kb of continual viral genome. Several smaller ORFs (< 100 amino acids) which could comprise exons of a spliced gene were identified within the BamHI-I_2 sequence. In support of this possibility, spliced IE mRNAs have been identified in other herpesviruses. ICP0 of human simplex virus (HSV), IE1 and IE2 of cytomegalovirus (CMV), BZLF1 and BRLF1 of Epstein-Barr virus (EBV), and IE RNA 1 and IE RNA 2 of Bovine herpesvirus 4 (BHV-4) (Lau et al., 1992; Leib et al., 1989; Stinski et al., 1991; van Santen, 1993) all result from spliced transcripts. In CMV, exons 1, 2 and 3 of IE1 can be ligated onto the IE2 by alternate splicing to produce different mRNAs (Stinski et al., 1991). A variant form of splicing which omits the middle exon of BZLF1 to produce a shortened protein is also described in EBV (Lau et al., 1992). It was therefore considered likely that the 1.6 kb transcript identified in our Northern blots was spliced.

Given the confusing and conflicting reports regarding transcription in the IR_L region of MDV, we analyzed the available sequence data for regions which may interfere with cDNA synthesis and S1 nuclease mapping. The sequence of BamHI-H from an internal EcoRI site to the right end BamHI site has been reported (Bradley et al., 1989, b). Within this sequence, multiple stretches of high AT content DNA exist. For example, nucleotides 1701 to 1900 contain two tracts , one with 19 A residues and another with 13 T residues. These sections could dramatically affect S1 mapping and act as sites for oligo dT initiation during cDNA synthesis. In our poly(A)$^+$ mRNA Northern blots, smaller species of RNA (1.0, 0.5 and 0.4 kb) hybridize to the BamHI-XbaI fragment but are not

observed when a cDNA insert is used as probe. These results suggest that the small RNAs may be derived from undegraded or partially degraded intron RNA which can contaminate poly(A)$^+$mRNA, perhaps due to specific AU rich sequences. In Northern blots of total infected-cell RNA, additional bands of 3.3 and 2.6 kb are visible when the RNA is probed with an intact I_2 fragment. Interestingly, these larger species correspond to the predicted sizes of unspliced RNAs from which our 1.6 kb transcript is likely derived. Given these facts, we chose to utilize an oligo dT tailed plasmid for initiation of cDNA synthesis. In this system, the poly (dT) primer extends from a double-stranded plasmid end. Thus, annealing to internal poly(A) tracts is thermodynamically less favored than annealing to terminal poly(A) tracts (e. g. internal annealing could only occur if the RNA bent to accommodate the complimentary plasmid strand). Poly(A)$^+$ mRNA was also used to reduce the contribution of unspliced RNA precursors and spliced introns. In the analysis of over 3000 clones from this library, we detected no transcripts which initiated within the 132 bp repeat region.

As this research was in process, Peng et al. (1992) reported a cDNA library constructed from MDV strain RBIB infected cells. Three classes of cDNA were detected from the long inverted repeat region and all were transcribed rightwardly. Among them, two cDNAs are derived from spliced mRNAs. The class II 1.9 kb cDNA (no.3 cDNA) has the same splice donor and acceptor sites as our C2 cDNA, but is 663 bp longer at the 5' end and about 30 bp shorter at the 3' end. The class I 1.5 kb cDNA (no. 4 cDNA) shares the same splice acceptor as the class II 1.9 kb cDNA, as well as our C1 and C2 cDNA, but uses a different splice donor site which is 250 bp downstream of our C1 cDNA splice donor. Thus, there are at least three cDNAs, which utilize different splice

donors but share a common splice acceptor site, present in the BamHI-H and I_2 fragments of MDV. Two unspliced mRNAs (1.69 kb cDNA 1 and 2.2 kb cDNA 6) from the BamHI H and I_2 regions were also reported by Peng et al. Previously, Iwata et al. (1992) reported construction of a cDNA library derived from MDV strain Md5 infected cells. Twelve clones localized to the BamHI-H fragment, contained 1.1 to 1.8 kb insertions, and all were derived from unspliced mRNAs transcribed in a rightward direction. Iwata et al., therefore, concluded that the majority of mRNA from the BamHI-H region is composed of unspliced transcripts. Chen and Velicer (1991) also reported four groups of unspliced transcripts which initiated or terminated in the BamHI-H 132 repeat region in a bidirectional manner. On the basis of results presented in this report and extensive analysis of cDNA clones, we believe that many of these cDNA result from priming within transcripts and within relatively stable introns containing the 132 bp repeats.

The 5' end of C1 has been defined by a combination of primer extension and sequence analysis. The 5' upstream promoter-enhancer has been characterized as a bidirectional regulatory region, shared with the pp38 gene. The structural arrangement of this bidirectional regulatory region shared by C1 and pp38 is similar to that of the HSV ICP4 and ICP22/47 genes (Preston et al., 1988; Wong and Schaffer, 1991). Whether C1 and pp38 gene expression is coordinately regulated, mutually exclusive, or their gene products are functionally synergistic will be an important issue in extending our knowledge of MDV gene regulation.

Protein similarity searches of putative C1 and C2 encoded polypeptides against the Swiss-protein data base revealed only limited homology with zinc-finger proteins and the *myc* proto-oncogene. However, these similarities were not in conservative or

functional regions. The regions of similarity between ORF1a, ORF1b, and the mouse zinc-finger protein ZFP-27 is not within the zinc-finger motif region (Chowdhury et al., 1988). Similarly, ORF2 has a limited homology with exon 3 of the *myc* proto-oncogene, a region outside the conservative "*myc*-box" (van Beneden et al., 1986). Therefore, we do not believe these similarities have significance regarding potential gene functions. We did not find similarity between ORF 1a or 1b and the mouse TLM oncogene as reported by Peng (1992), because the region in question was not present in our cDNA sequence due to altered splicing. We also did not find any similarity between ORF-F, (corresponding to our ORF2), and the *fes/fps* family of kinase-related transforming proteins as reported by Peng et al. (1992). It is therefore likely that any protein product encoded by these cDNAs are MDV specific.

Using predicted amino acid sequences deduced from our cDNA nucleotide sequence, fusion proteins and specific antibodies have been prepared. A 14 kDa protein was detected in Western blots of MDV infected cell lysates by antisera against both ORF1a and ORF1b. It is possible that these two ORFs encode proteins similar in size which differ slightly at their amino-terminal end. ORF1a is predicted to encode 83 amino acids. An additional 2 amino acids would be added if 5' sequence determined by primer extension is included. ORF1b could encode only 76 amino acids and is 18 amino acids shorter at the amino-terminal end than ORF-D described by Peng et al. (1992). If we assume the 5' end of ORF-D is the same 5' end for our ORF1b, ORF1b could encode 93 amino acids. Thus, the calculated sizes of polypeptides encoded by ORF1a and 1b would be proximately 9.6 kDa and 10.3 kDa, respectively. The predicted sizes of polypeptides encoded by ORF1a and 1b, therefore, are smaller than the polypeptides identified by

ORF1a and 1b fusion protein antisera in SDS-PAGE gels. According to computer data analysis, ORF1a and 1b both could be heavily phosphorylated (six sites for ORF1a and four sites for ORF1b) and glycosylated (three sites for ORF1a and two sites for ORF1b). Thus, post-translation modification, such as phosphorylation or glycosylation, may be an important factor contributing to the discrepancy between predicted and apparent protein sizes. Alternatively, ORF1a and 1b may be exons encoding only part of a larger polypeptide. We believe this latter possibility is remote, since our primer extension data indicate the C1 and C2 transcripts end very near the 5' ends contained in our cDNA clones and we did not detect any larger polypeptide in Western blots.

Our data suggests that the 132 bp repeat region is removed by splicing and does not affect the protein encoded by transcripts C1 and C2 from this region of MDV DNA. These results also suggest that expansion of the 132 repeat may not be as critical in MDV attenuation as was previously thought. However, our data cannot rule out the possibility of subtle changes in amino acid content coincident with attenuation and expansion of the 132 bp repeat region.

Expression of the 14 kDa C1/C2 gene products in an MDV induced lymphoma cell line indicates these proteins may play at least some role in maintenance of the transformed state or latent infection. Detection of the C1/C2 14 kDa proteins in MDV induced lymphoblastoid cells represents the third such MDV antigen identified in latently infected transformed cells after the identification of pp38 (Chen et al., 1992; Cui et al., 1991) and *meq* (Jones et al., 1992). All of the proteins identified in MSB-1 cells are encoded within viral repeat regions. Whether these proteins have coordinated functions in MDV gene regulation and cell transformation remains to be investigated. The 14 kDa

proteins identified in this study are derived from an IE gene. Investigation of whether these proteins can activate or inhibit expression from other MDV genes and whether they execute different roles mediated by altered splicing patterns, will provide important clues regarding the nature of MDV induced tumorigenicity and pathogenicity.

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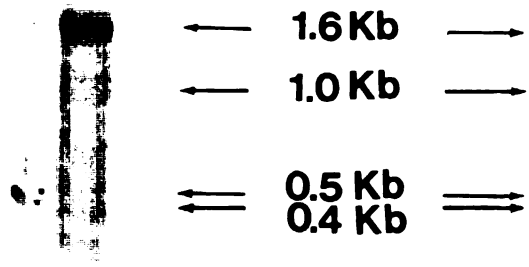
Figure 1. Northern blot hybridization to identify immediate-early gene transcripts in MDV BamHI-I_2. **A**). MDV strain GA, passage 7, was propagated in duck embryo fibroblasts (DEF). To obtain immediate-early and early RNA transcripts, cycloheximide (CHX) or phosphonoacetic acid (PAA) was added at the time of infection and was present during the entire culture process. Poly(A)$^+$ mRNAs were isolated and electrophoresed in 1.2% formaldehyde/agarose gels, transferred to Hybond-nylon membranes and hybridized with the 5.1 kb MDV BamHI-I_2 fragment. Lane 1 is uninfected DEF, lane 2 is DEF infected with MDV GAp7, lane 3 is DEF infected with GAp7 and treated with CHX, lane 4 is DEF infected with GAp7 and treated with PAA. Sizes of various transcripts were determined by comparison to a 0.24 to 9.5 kb RNA ladder. **B**). The same membrane used in panel A was stripped and reprobed with the 2.3 kb BamHI-XbaI subfragment of MDV BamHI-I_2. **C**). Schematic representation of MDV genomic structure with its BamHI sites (Fukuchi et al., 1984). The enlarged portion is a restriction map of the BamHI-I_2 fragment with the heavy line representing the 2.3 kb BamHI-XbaI subfragment probe.

A.

DEF
MDV GAP7
MDV GAP7/CHX
MDV GAP7/PAA

B.

DEF
MDV GAP7
MDV GAP7/CHX
MDV GAP7/PAA



1 2 3 4

Actin

1 2 3 4

C.

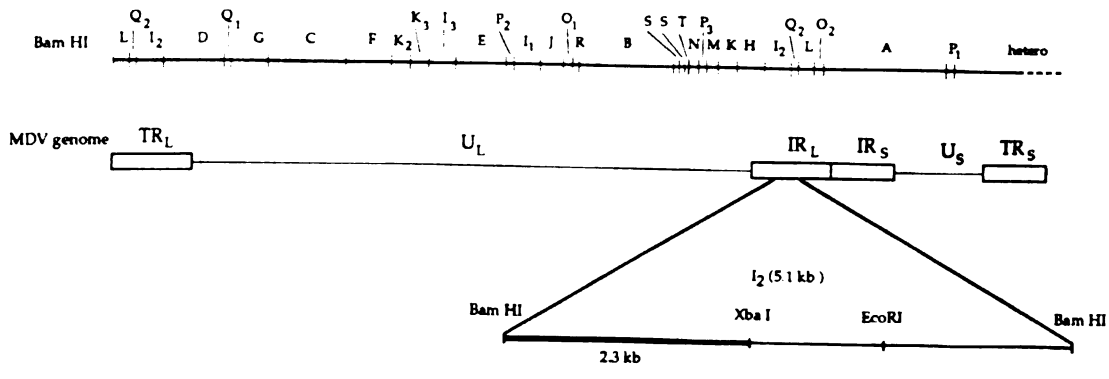


Figure 2. Schematic representation of the location, structure and primer extension analysis of C1 and C2 cDNAs. Partial restriction map of the MDV BamHI-H and I_2 fragments is presented with a putative TATA sequence for the C1 transcript in a horizontal box. The dark line represents the region from which cDNAs C1 and C2 are derived. Grey shaded boxes represent the 132 bp repeat region. Location of C1 and C2 cDNAs is shown with approximate positions of their respective introns. Arrow heads indicate the direction of transcription for C1 and C2. Oligonucleotides used for primer extensions were designed based on cDNA sequence. P1 primer was complementary to C1 cDNA position 14 to 39, just upstream of the intron. Two primers, P 2A and P 2B were designed for the C2 transcript. P 2A is complementary to C2 cDNA sequence position 29 to 53, 10 base pairs downstream of the splice acceptor. P 2B is complementary to nucleotide 11 to 35 of C2 clone and crosses over to the spliced intron. Primers P1, P 2A and P 2B are depicted with small arrows. Dashed lines indicate sequences extended by primer extension analysis.

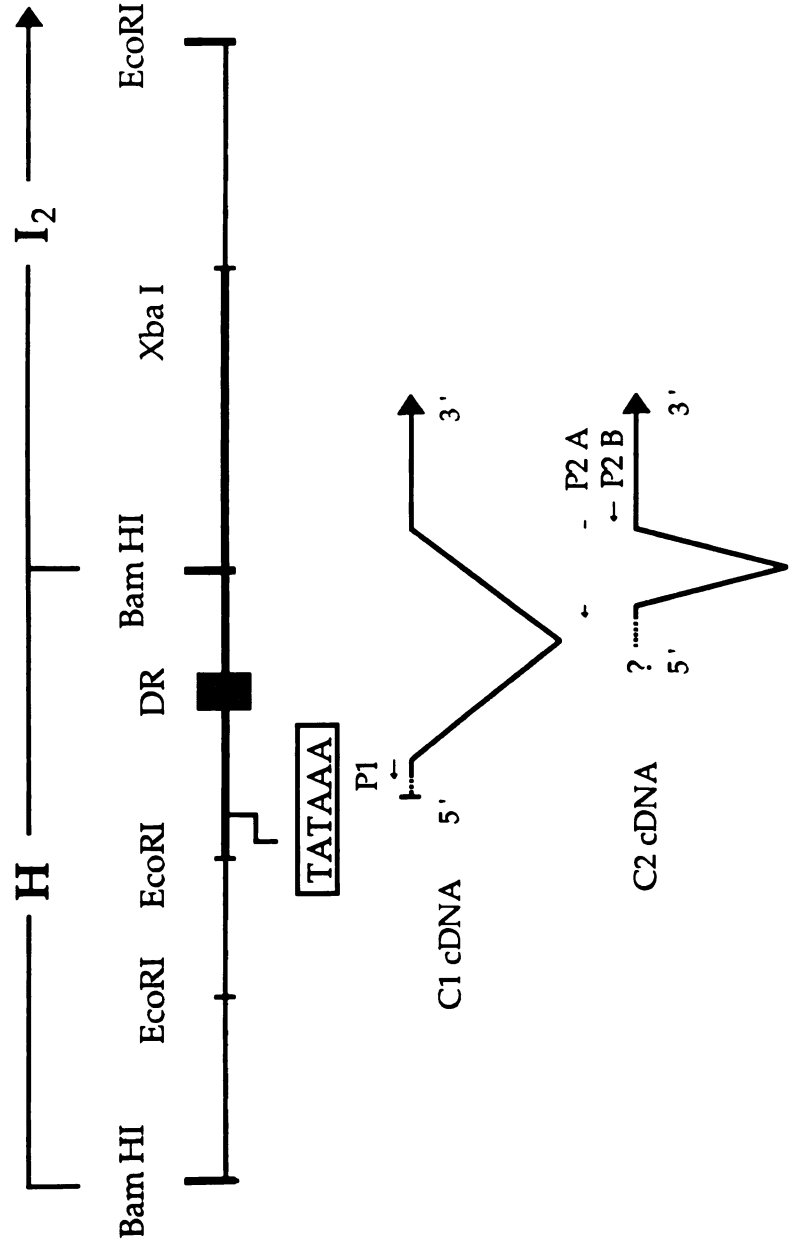
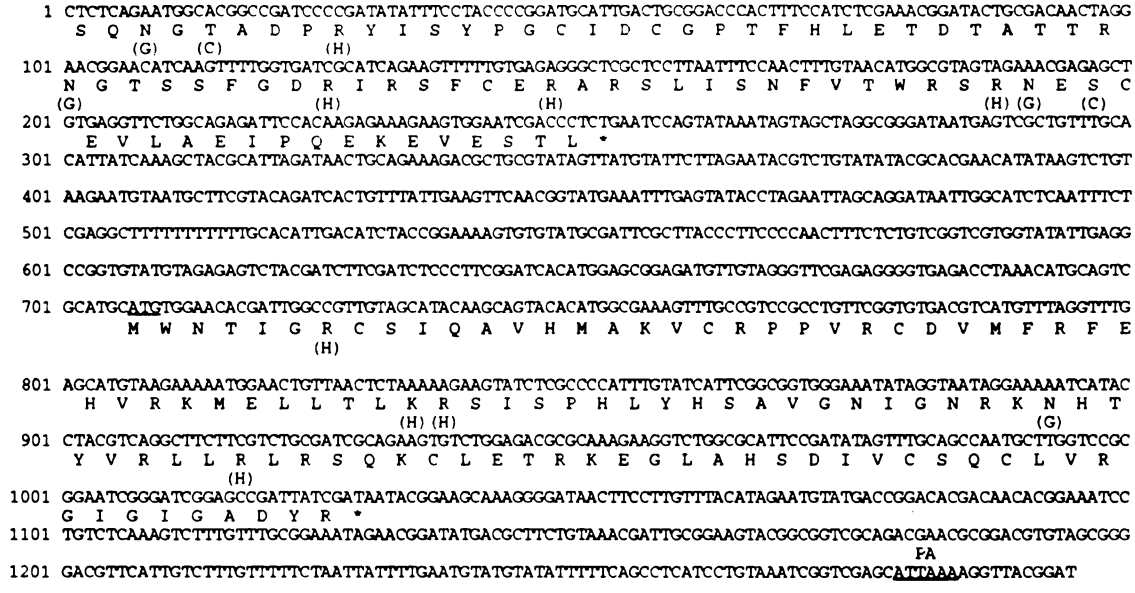

H
I2
Bam HI
EcoRI
EcoRI
TATAAA
DR
Bam HI
Xba I
EcoRI
P1
5'
3'
C1 cDNA
P2 A
P2 B
3'
5'
?
C2 cDNA

Figure 2.

Figure 3. The complete nucleotide sequence of two cDNA clones. The overall lengths of cDNA inserts are: **A**) C1 cDNA, 1295 bp and **B**) C2 cDNA, 1275 bp. The amino acid sequences of potential open reading frames are shown by single-letter code below the appropriate nucleotide sequence. ATG start codons and ATTAAA consensus sequences for polyadenylation (PA) are underlined. N-glycosylation sites (G), casein kinase consensus phosphorylation site (C), and histone kinase consensus phosphorylation sites (H) are also noted.

Figure 3.

A). C1 cDNA 1295 bp



B). C2 cDNA 1275 bp

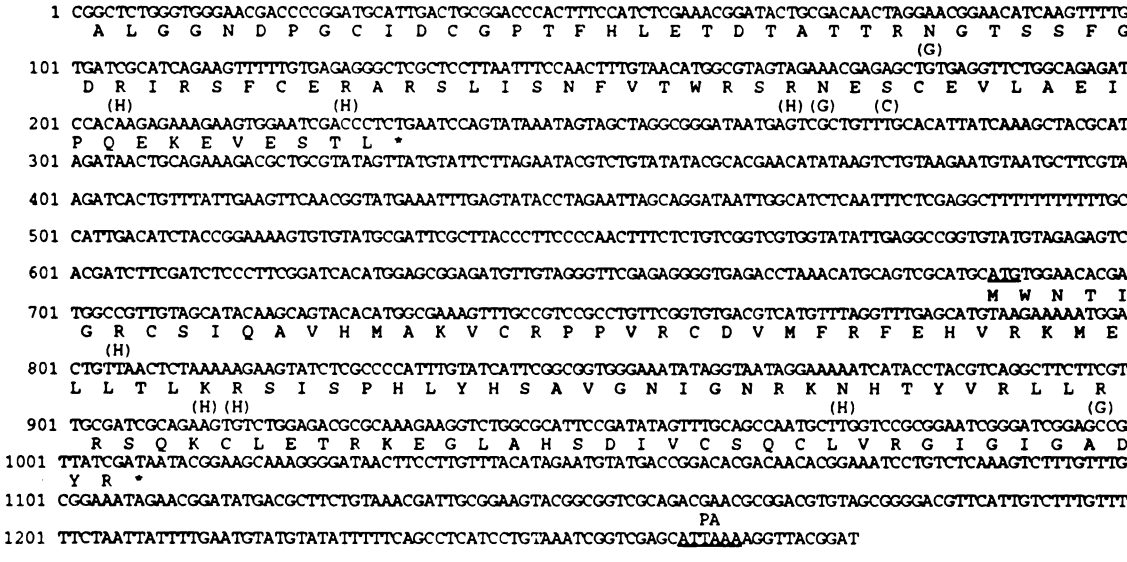


Figure 4. Northern blot hybridization to confirm the origin of cDNA sequence. Poly(A)$^+$ mRNAs from uninfected DEF (lane 1), DEF infected with GAp7 (lane 2), and DEF infected with GAp7 and treated with CHX (lane 3), or PAA (lane 4) were hybridized with the 1.3 kb C1 cDNA insert. Transcript sizes were determined by comparison to a 0.24 to 9.5 kb RNA ladder.

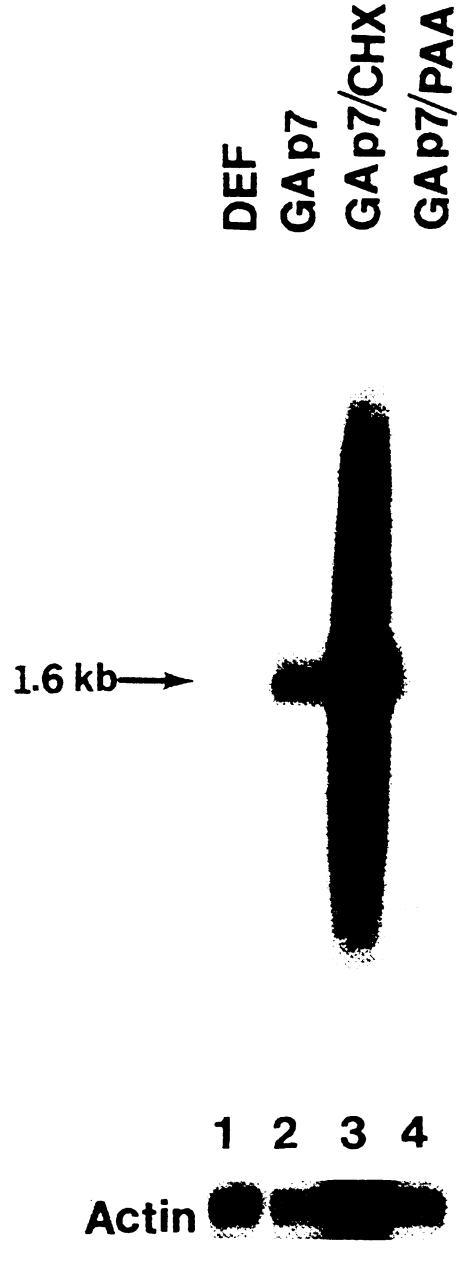
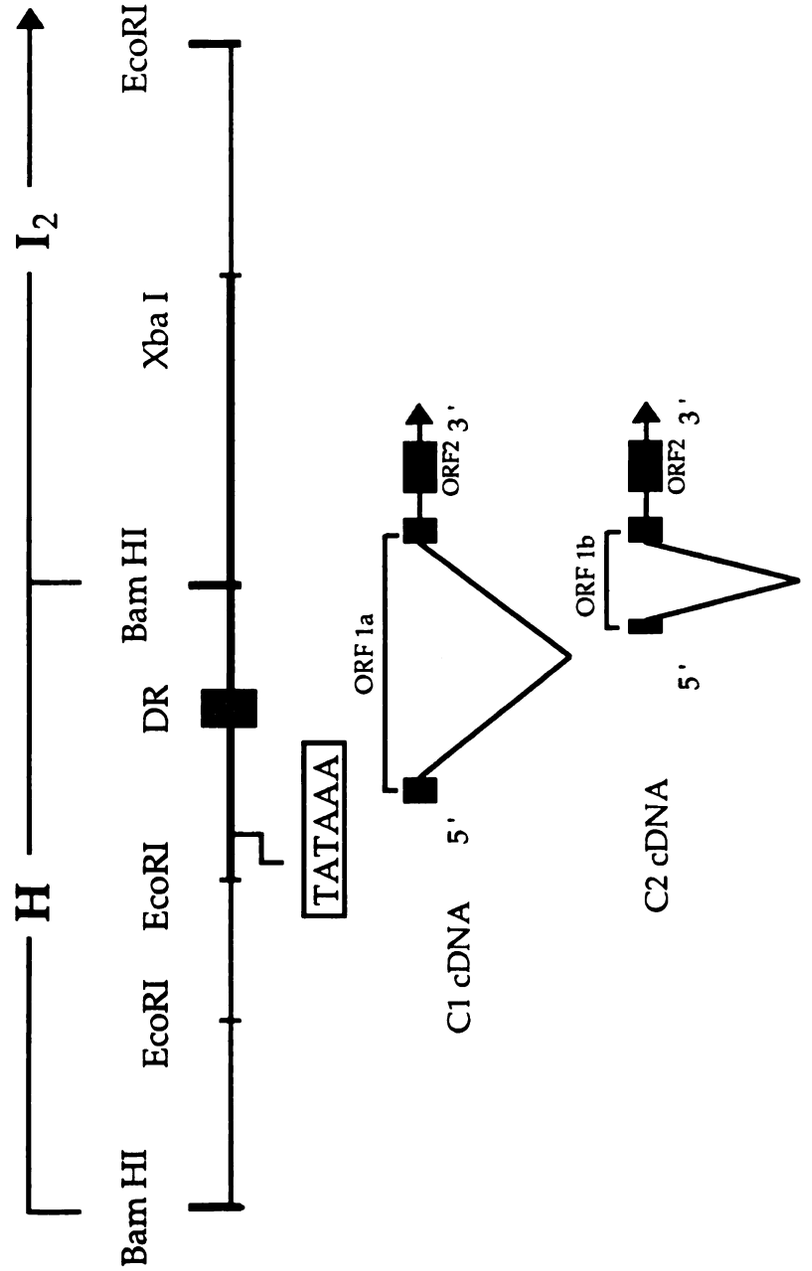


Figure 4.

Figure 5. Schematic representation of the positions of ORFs in C1 and C2 cDNA clones. Nucleotide sequences of C1 and C2 cDNA were analyzed using the Genepro and GCG programs. A partial restriction map of the BamHI-H and I_2 fragments is shown with the putative TATA sequence upstream of C1 clone highlighted. Grey shaded boxes (DR) represent the 132 bp direct repeat region. ORF1a of C1 clone, which is interrupted by a 2.3 kb intron, encodes a putative 83 amino acid polypeptide while ORF1b of C2 (which crosses over a 1.0 kb intron) encodes a putative 76 amino acid polypeptide. ORF2, which is completely located in exon 2, encodes 107 amino acid residues.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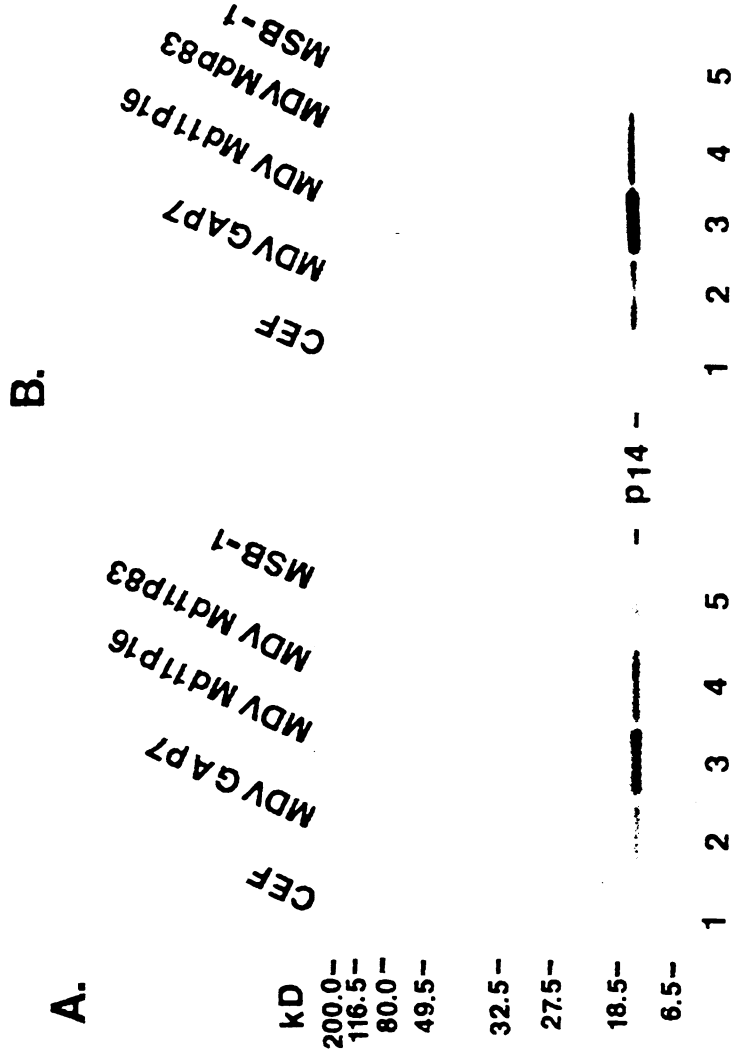
5'
C2 cDNA

Figure 5.

Figure 6. Western blot analysis detection of MDV specific-proteins encoded by ORF1a and ORF1b. A). Cell lysates from uninfected CEF (lane 1), CEF infected with MDV GAp7 (lane 2), Md11 p16 (lane 3), or Md11p83 (lane 4), and MDV-induced lymphoblastoid cell line MSB-1 (lane 5) were resolved in 12.5% SDS-PAGE gels and transferred to nitrocellulose membrane, followed by immunodetection using fusion protein antiserum against ORF1a. **B**). Cell lysates and order are as described for Panel A above and detected by anti-ORF1b serum.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B.
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MDV Md11P16
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MSB-1
1 2 3 4 5

Figure 6.

Chapter III

A 14 kilodalton Immediate-early Phosphoprotein is Specifically Expressed in Cells Infected With Oncogenic Marek's Disease Virus Strains and Their Attenuated Derivatives

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ABSTRACT

Previously, we reported an immediate-early transcript derived from Marek's Disease Virus (MDV) long internal repeat region. With cDNA cloning and sequencing, two cDNAs (C1 and C2) were identified as derived from spliced mRNAs spanning BamHI-H and I_2 fragments of MDV genome. By Western blot analysis, a 14 kDa polypeptide (p14) was detected by antisera raised against fusion proteins containing two small open reading frames (ORFs) from two distinct cDNAs. P14 is expressed in cells lytically infected with both oncogenic and attenuated MDV, as well as in cells latently infected and transformed by MDV (Hong and Coussens, 1994). In this study, we demonstrate that p14 is MDV serotype-1 specific and highly phosphorylated. Given the degree of phosphorylation and lack of homology to known proteins, we propose the name pp14 for the polypeptide encoded by ORF1a and ORF1b. Further analysis reveals that pp14 is predominantly found in cytoplasmic fractions of MDV infected cells and can be detected in cytoplasm of MDV infected cells by immunofluorescence with polyclonal antisera prepared against pp14-Glutathione-S-Transferase (GST) fusion protein.

INTRODUCTION

Marek's disease virus (MDV) is an oncogenic avian herpesvirus which induces lymphoproliferative disease and demyelination of peripheral nerves in chickens (Calnek, 1985; Churchill and Biggs, 1967). MDV-induced disease, Marek's disease (MD), was one of the first neoplastic diseases found to be associated with a herpesvirus. Although MDV has been effectively controlled by vaccination with an antigenically related but apathogenic herpesvirus of turkeys (HVT), the mechanism of MDV-induced tumorigenicity is poorly understood.

The MDV genome is double stranded DNA with a structure similar to that of human alpha-herpesviruses, such as varicella-zoster virus (VZV) and herpes simplex virus (HSV). MDV DNA consists of unique long (U_L) and unique short (U_S) regions, each bounded by a set of inverted repeats (TR_L, IR_L, IR_S, and TR_S) (Cebrian et al., 1982; Fukuchi et al., 1984). MDV repeat regions have attracted attention by many investigators for several reasons: First, genes encoded in herpesvirus repeat regions are more varied and tend to be specific to individual virus (Buckmaster et al., 1988). Second, abundant transcripts of immediate-early genes are detected in these regions (Maray et al., 1988; Schat et al., 1989). Third, and perhaps most important, transcripts derived from repeat regions may be related to MDV oncogenicity (Bradley et al., 1989 a; b). Previous studies have revealed that serial in vitro passage of virulent MDV in primary chicken embryo fibroblast (CEF) cells results in loss of MDV tumorigenicity. This attenuation was strongly correlated with amplification of a specific 132 bp repeat sequence located within the MDV TR_L and IR_L (BamHI-H and D fragments, respectively) (Chen and Velicer, 1991; Fukuchi et al., 1985; Maotani et al., 1986; Silva and Witters, 1985). Bradley et al.

(1989 a; b) reported that a 1.8-kb BamHI-H gene family is transcribed rightwardly from the expanded region. Expression of these transcripts in cells infected with oncogenic MDV, but not in attenuated MDV suggested an association of this gene family with MDV tumorigenicity. This hypothesis was supported by antisense inhibition of MDV-induced lymphoblastoid cell line proliferation, using an oligonucleotide complementary to the putative splice donor sequence of the 1.8 kb gene family (Kawamura et al., 1991). A cDNA library from this BamHI-H gene family was developed by Peng et al. (1992) with identification of four cDNA clones. Two cDNAs (1.69 and 2.2 kb) were reported as nonspliced transcripts, while two other cDNAs (1.5 and 1.9 kb) were recognized as single spliced transcripts spanning MDV BamHI-H and BamHI-I_2 fragments. Recently, Peng et al. (1994) reported that two transcripts from this region (1.69 and 1.5 kb) can induce prolonged proliferation and reduced serum dependence of primary chicken embryo fibroblasts (CEF) in transfection assays. Peng et al. (1994), therefore, suggested that one function of the BamHI-H gene family may be cell-growth control. Additional transcripts that initiate or terminate within or near the 132 bp repeat region have also been described (Chen and Velicer, 1991). In addition, a 38 kDa phosphoprotein (pp38) expressed both in MDV lytically infected cells and transformed lymphoblastoid cell lines was localized to the BamHI-H region, left of the 1.8 kb gene family and transcribed in a leftward direction (Chen et al., 1992; Cui et al., 1991).

The *meq* gene, a basic-leucine zipper gene and partial homolog of the *fos/jun* oncogene family, has been mapped to the rightward region of BamHI-I_2 and is only expressed in MDV transformed lymphoblastoid cell lines but not in cells lytically infected with MDV strain GA (Jones et al., 1992). However, differences between reports on size,

number, and direction of transcripts derived from MDV repeat regions have made these regions the most controversial and apparently complicated regions of the MDV genome.

Recently, we reported an immediate-early transcript derived from the MDV IR_L region (Hong and Coussens, 1994). With cDNA cloning and sequencing, two cDNAs (C1 and C2) were identified. Both cDNAs are derived from spliced mRNAs spanning the BamHI-H and I_2 fragments. Combined with data of cDNA clones reported by Peng et al. (1992), we found that at least three distinct cDNAs, which utilize different splice donors but share a common acceptor site, are transcribed across the BamHI-H and I_2 fragments of MDV. Despite abundant transcription as detected by Northern blot analysis, only two small open reading frames (ORFs) were found within each cDNA (C1 contains ORF1a of 83 amino acids and ORF2 of 107 amino acids, while C2 may encode 76 amino acids for ORF1b and the same 107 amino acids for ORF2 as in C1). To understand the function of these spliced transcripts, particularly with regarding to the potential importance of this region in viral oncogenicity, we focused on identification of a possible protein product encoded by these transcripts. In western immunoblot analysis, a 14 kDa polypeptide (p14) was identified by antisera against Glutathione-S-Transferase (GST)-ORF1a and GST-ORF1b fusion proteins. This polypeptide is expressed in cells lytically infected with both oncogenic and attenuated MDV, as well as in cells latently infected and transformed by MDV. In this report, we further characterized the properties of this 14 kDa MDV specific protein.

MATERIALS AND METHODS

Cell culture and Viruses.

Primary and secondary duck embryo fibroblast cells (DEF) were prepared and maintained as described (Glaubiger et al, 1983). Secondary DEF cells were infected with cell-associated MDV serotype-1, strain GA passage 7; serotype-2, strain 281 MI/1 passage 16; and serotype-3, herpesvirus of turkey, passage 7. To detect glycosylation of p14, DEF cells infected with MDV strain GA were cultured in medium containing 2.0 ug tunicamycin (TM) per ml for 1 hr, then changed to fresh medium containing same concentration of TM for additional 4 hrs before lysis.

Fusion protein antibody production.

DNA fragments containing ORF1a and ORF1b were cloned into pGEX-3X vector to induce Glutathione S-transferase (GST) fusion proteins. The respective GST-ORF1a and GST-ORF1b fusion proteins were prepared and purified as described previously (Hong and Coussens, 1994). Rabbit anti-GST-ORF1a and GST-ORF1b sera were induced and titered as described (Hong and Coussens, 1994).

Western immunoblot analysis.

Cultured cells were lysed, separated on 0.1% sodium dodecyl sulfate(SDS)-12.5% polyacrylamide gels and transfered to nitrocellulose filters. Immune detections were performed with an Amersham ECL Western blot kit according to manufacturer's specification. Anti-ORF1a and -ORF1b sera were used at a 1:200 dilution and incubated in room temperature for 1 hour. The secondary antibody of donkey anti-rabbit

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immunoglobulin conjugated with horseradish peroxidase was added and incubated for another 1 hour at room temperature. Proteins were detected by chemiluminescence reagent and exposed to X-ray film.

For "Cross-Absorption" experiments, anti-ORF1a serum was pre-absorbed with purified GST-ORF1b fusion protein and anti-ORF1b serum was pre-absorbed with GST-ORF1a fusion protein prior to western blot detection of p14.

Subcellular fractionation.

Fractionations of MDV infected DEF cells were conducted essentially as described (Ramsay et al., 1986). Hypotonic buffer containing 5 mM KCl, 1 mM $MgCl_2$, and 25 mM Tris hydrochloride (pH 7.5) was added to cell culture plates, and cell were incubated on ice for 10 minutes. Then an equal volume of hypotonic buffer containing 1% NP40 was added, and cells were incubated for additional 5 minutes on ice. Cell lysates were transfered to eppendoff tubes and centrifuge at 1000 X g for 5 minutes to separate nuclei. Nuclei were washed once with hypotonic buffer containing 0.5% NP40 before solubilization in lysis buffer (25 mM Tris hydrochloride, 150 mM NaCl, 0.5% NP40, 0.5% sodium deoxycholic acid, 0.2% sodium dodecyl sulfate). Both nuclear and cytoplasmic fractions were subjected to western blot analysis.

Immunoprecipitation

Immunoprecipitation analysis was carried out essentially as described by Glaubiger et al. (1983). For competition immunoprecipitation, cell lysates from DEF infected with MDV strain GA, passage 7 were mixed with normal rabbit serum, anti-ORF1a serum or anti-ORF1b serum, and then immunoprecipitated by protein A-agarose (Sigma, St. Louis,

MO). Each supernatant was collected and subjected to western blot analysis.

For ^{32}P labeled immunoprecipitation, uninfected DEF cells and MDV strain GA infected DEF cells were cultured in 60 mm plates and pre-incubated in phosphate-free Dulbecco modified Eagle medium (Gibco-BRL, Gaithersburg, MD) for 1 hour. 500 µCi ^{32}P orthophosphate (DuPond, Wilmington, DE) was added to each plate and the cells were continuously labeled for 4 hours prior to harvesting. Cell lysates were immunoprecipitated with anti-ORF1a or anti-ORF1b sera as described (Glaubiger et al., 1983). Immunoprecipitates were washed, suspended in sample buffer, and analyzed by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE). Prestained protein markers (Bio-Rad, Richmond, CA) were used as molecular weight standards to estimate protein size.

Phosphatase assay.

After the final washing of immunoprecipitation, MDV immunoprecipitates were divided into two parts. One part was treated with 20 IU calf intestinal phosphatase (CIP) for 1 hour at 37°C. The reaction was terminated by washing the treated immunoprecipitates and resuspended in sample buffer (Morrison et al., 1989). The CIP treated sample was analyzed on same SDS-polyacrylamide gel as with other immunoprecipitates.

Similarly, unlabeled cell lysates from MDV infected cells were treated with CIP for 1 hr at 37°C, run on the SDS-PAGE along with untreated cell lysates. then transferred to nitrocellulose membrane for western blot analysis.

Indirect immunofluorescence.

Uninfected DEF and MDV strain GA infected DEF cells were grown on glass coverslips. Cells were fixed by acetone/methanol (1:1) for 3 minutes and extensively washed with phosphate buffer saline (PBS), then preincubated with 3% bovine serum albumin (BSA) for 1 hour at room temperature. Anti-ORF1a and -ORF1b sera were added to the cells in a 1:20 dilution and incubated for 1 hour at room temperature. Goat-anti-rabbit IgG conjugated with phycoerythrin (PE) (Sigma, St. Louis, MO) was utilized as secondary antibody in a dilution of 1:20. Slow-Fade (Molecular Probes, Eugene, OR) was added prior to mounting to glass slides to prevent quenching of fluorescence. For double staining, monoclonal anti-MDV/gB antibody (1:200 dilution) (generously provided by Dr. L. Lee, USDA, Avian disease oncology laboratory, East Lansing, MI) with a secondary goat anti-mouse IgG antibody conjugated with fluorescein-5'-isothiocyanate (FITC) (Coppel, Durham, NC) (1:20 dilution) was utilized as the secondary set immune reaction. Confocal fluorescence images were acquired using the Meridian INSIGHT-PLUS Laser Scanning Confocal Microscope (Meridian Instrument, Okemos, MI). Fluorescent labels were excited using the 488 nm argon laser line. Emissions of phycoerythrin and FITC were acquired using 580/30 nm and 530/30 nm band pass filters, respectively. Emissions were detected through integration on a cooled-CCD camera, primarily used 100X Olympus Splan APo (1.4 NA) objective, 2.5 X photoeyepiece.

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RESULTS

Antigenic relatedness of ORF1a and ORF1b

Antisera raised against ORF1a and ORF1b detected polypeptides of similar size on western blots (Hong and Coussens, 1994). Since ORF1a and ORF1b only differ in their first exon sequences (39 bp or 13 amino acids for ORF1a, 18 bp or 6 amino acids for ORF1b), it was of interest to determine whether these two antisera can cross-react with both ORF1a and ORF1b derived polypeptides, and whether they detect the same polypeptide. Therefore, a competition immunoprecipitation was conducted prior to western blot analysis. While cell lysates immunoprecipitated with normal rabbit serum has no effect on p14 detection by anti-ORF1a serum (Figure 1B, Lane 3), p14 was significantly reduced in cell lysates which have been immunoprecipitated with anti-ORF1b serum prior to western blot analysis by anti-ORF1a serum (Figure 1B, lane 4). Also, cell lysates immunoprecipitated with anti-ORF1a antiserum itself were used as a positive control for clearance (Figure 1B, lane 5). Similarly results were obtained in a complementary experiment where p14 detected by anti-ORF1b was almost completely blocked in cell lysates immunoprecipitated with anti-ORF1a serum (Figure 1C, lane 4), while normal serum had little or no effect on availability of p14 (Figure 1C, lane 3). Cell lysates immunoprecipitated with anti-ORF1b were used as positive control for clearance (Figure 1C, lane 5).

In cross-absorption experiments, anti-ORF1a serum was pre-absorbed with GST-ORF1b fusion proteins, while anti-ORF1b serum was pre-absorbed with GST-ORF1a fusion proteins prior to western blot analysis. Both antisera lost their specific binding activity for p14 in western blot analysis (data not shown).

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p14 is a MDV serotype 1 specific antigen.

Protein data base searches failed to identify any significant homology between p14 and any known protein sequence (Hong and Coussens, 1994), suggesting that p14 represent a MDV specific antigen. Previous identification of p14 was limited to cells lytically infected with oncogenic MDV serotype-1 (GA and Md11) (Hong and Coussens, 1994), we therefore extended our study to two other MDV serotypes. Serotype-2 represents naturally occurring, nononcogenic strains of MDV, while serotype-3 represents nononcogenic herpesvirus of turkey (HVT) (Schat, 1985) . Western blot analysis of DEF cells infected with all three serotypes along with uninfected DEF was performed using anti-ORF1a (Figure 2A) and anti-ORF1b (data not shown) sera. Anti-MDV gB Z45 (kindly provided by Dr. L. Velicer, Michigan State University), which detects MDV gB antigen in cells infected with all three serotypes of MDV, was used as a positive control to ensure presence of viral proteins in all cell lysates(data not shown). Interestingly, p14 was only detected in lysates of cells infected with serotype-1 MDV (Figure 2A, lane 2). No homolog of MDV p14 was detected in cells infected with MDV serotypes-2 or -3 (Figure 2A, lane 3 and 4, respectively). Therefore, p14 represents a protein specific to serotype-1 MDV (oncogenic and attenuated).

MDV p14 is a phosphoprotein.

The 14 kDa polypeptide identified previously is significantly larger than sizes of 9.6 kDa and 10.3 kDa calculated from ORF1a and ORF1b DNA sequence, respectively (Hong and Coussens, 1994). Post-translation modification could be the reason of slow migration. To determine whether the 14 kDa polypeptide identified in this study is

phosphorylated, we immunoprecipitated lysates from ^{32}P-labeled uninfected DEF and MDV strain GA infected DEF cells with anti-ORF1a serum (Figure 2B, lane 1 and 2). Immunoprecipitates from MDV infected cell were divided into two parts. One part was subjected to treatment with calf intestinal phosphatase (CIP) (Figure 2B, lane 3) (Morrison et al., 1989). Results of SDS-PAGE analysis demonstrate that p14 is phosphorylated (Figure 2B, lane 2). Significant reduction in intensity of p14 bands in CIP treated sample indicates sensitivity of phosphoprotein p14 to phosphatase (Figure 2B, lane 3). Based on the phosphorylation, we propose that this MDV-specific polypeptide be designated as pp14. However, when cell lysates from MDV infected cells were treated with CIP, western blot revealed only slight shift (1-2 kDa) of p14 relative to untreated p14 (data not shown).

In addition, treatment of MDV infected cells with TM, an inhibitor of N-glycosylation, prior to western blot analysis has little or no effect on the apparent size of p14 (data not shown).

pp14 is predominately located in the cytoplasm of MDV infected cells.

Protein subcellular localizations are defined by their functions. To determine if pp14 is transported to nucleus, as most regulatory protein located, we prepared subcellular fractions from MDV strain GA infected DEF cells as described (Ramsay et al., 1986) and assayed fractions for pp14 by immunoblotting (Figure 2C). Surprisingly, the 14 kDa polypeptide was detected exclusively in cytoplasmic fractions (Figure 2C, lane 3), same as that detected in whole cell lysates (Figure 2C, lane 2). An anti-chicken hMG14/17 antibody (kindly provided by Dr. M. Bustin, NIH) which detects a predominately chicken

nuclear protein hMG14/17, was used as a positive control to confirm the presence of nuclear proteins (data not shown).

To confirm the intracellular localization of pp14, we extended our analysis by employing indirect immunofluorescence. As shown in Figure 3, pp14 specific staining was localized to the cytoplasm of infected cells, primarily in areas near the nucleus. To confirm the immune-signals detected by anti-ORF1a serum were truly in MDV infected cells, we chose MDV gB as a positive control and conducted double-stained immunofluorescence. All cells staining with anti-ORF1a or anti-ORF1b (Phycoerythrin) also displayed anti-gB binding activity (FITC) (data not shown). Pre-immune serum and various combinations of antibodies were used as negative controls (data not shown).

DISCUSSION

The cross-reaction of anti-ORF1a and anti-ORF1b sera suggested that the 14 kDa polypeptide(s) detected by these two sera are highly related and may represent the same polypeptide. The results from cross-absorption indicate that the unique amino acids encoded by each first exon in C1 and C2 cDNA, if translated, do not comprise strong epitopes. Although we temporarily designate the proteins as a single polypeptide, p14, It is possible that there are two distinct proteins differing in only a few amino acids. Subtle changes in amino acid sequence may result in secondary structure and protein function changes, thus providing a way for these differently spliced transcripts to execute distinct functions. Peptide mapping and/or amino acid sequence analysis will be required to further clarify this question.

Extensive use of serotype-2 and serotype -3 as live virus vaccines to prevent MDV serotype 1 induced tumor formation indicates that antigens in these three serotypes share common epitopes (Glaubiger et al., 1983; Isfort et al., 1986). However, lack of analogues in serotype-2 and -3 indicates p14 is a MDV serotype-1 specific antigen. This finding is consistent with p14 coding sequences mapping within IR_L regions of the MDV genome. IR_L regions do not share extensive homology among the three MDV serotypes (Buckmaster et al., 1988; Igarashi et al., 1987).

According to computer analysis, ORF1a has two potential casein kinase consensus phosphorylation sequences (amino acids 5 and 66), and four potential histone kinase consensus phosphorylation sites (amino acids 9, 42, 49, and 63). ORF1b has one casein kinase phosphorylation consensus sequence (amino acid 59), and three potential histone kinase phosphorylation sequences (amino acid 35, 42, and 56) (Hong and Coussens,

1994). Extensive phosphorylation or other post-translation modification can change protein secondary structure and surface charges, resulting in slow apparent mobility of proteins (DeCaprio et al., 1989). The immunoprecipitation results clearly demonstrate that p14 is a phosphoprotein (pp14), therefore, supporting our previous hypothesis that phosphorylation of p14 may be one factor contributing to the discrepancy between calculated and apparent sizes of p14. Protein phosphorylation is a well known method for mediating activity of proteins involved in regulation of gene expression and other regulatory functions (review, Hunter and Karin, 1992). Since p14 is derived from an immediate-early (IE) gene and IE genes typically encode virus transcription factors, phosphorylation of p14 was not unexpected. However, comparing of CIP treated and untreated p14, only slight difference was observed in western blot analysis. In addition, although computer analysis indicating several potential N-glycosylation sites within ORF1a and ORF1b, treatment of infected cells with TM had little or no effect on migration of p14. Together, these results indicate that neither phosphorylation nor glycosylation is completely responsible for the discrepancy between calculated and apparent size of p14.

Nuclear transport of transcription factors is a commonly used mechanism of regulation in eukaryotic cells. If pp14 functions primarily to regulate viral gene expression as do most other herpesvirus IE gene products, one might expect a significant proportion of pp14 to be localized in the nucleus of infected cells. However, western blot analysis of subcellular fractions and immunofluorescence detection both revealed that pp14 is predominately a cytoplasmic protein. It is possible that pp14 is transported to the nucleus in limited amounts. Additionally, lack of a consensus nuclear transport signal in pp14

suggests that another factor would have to be involved in transport to the nucleus.

Expression of pp14 in MDV transformed tumor cell lines (Hong and Coussens, 1994) and as a MDV serotype-1 specific antigen suggest that this protein may be associated with MDV tumorigenicity. Although pp14 was detected in both oncogenic and attenuated serotype 1 strains (Hong and Coussens, 1994), subtle changes or modifications of protein content in attenuated strains may dramatically affect protein function. It is of considerable interest that pp14 is encoded by spliced transcripts which the 132-bp expansion region is removed. Properties of pp14 present in this report provide clues for further investigation of this serotype-1 MDV specific IE gene product. Functional and biochemical analysis of this unique protein are in progress and will be an important issue for understanding a potential role of pp14 in MDV-induced tumorigenicity.

Acknowledgement

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We thank Drs. M. Bustin, L. Lee, L. Velicer for kindly providing the antibodies used in these studies. We also thank R. Southwick for excellent technical assistance and assistance with graphic preparation.

Figure 1. Antigenic relatedness of ORF1a and ORF1b. A). Schematic representation of MDV genome structure with enlarged restriction map of BamHI-H and I_2 regions. A dark line represents the region from which C1 and C2 cDNAs, as well as other MDV transcripts (Bradley et al., 1989, a; b; Chen and Velicer, 1991; Peng et al., 1992; Hong and Coussens, 1994), are derived. Grey shaded boxes represent the 132 bp repeat region. Locations of C1 and C2 cDNAs are shown with approximate introns and the open reading frames (black boxes). ORF1a of C1 is 83 amino acids. ORF1b of C2 is 76 amino acids. ORF2 in both C1 and C2 is 107 amino acids. **B**). Western blot analysis by anti-ORF1a serum. Lane 1 is lysate from uninfected DEF cells, lane 2 is lysate from DEF cells infected with MDV strain GA, lanes 3 , 4 and 5 are MDV GA infected DEF cell lysate which have been immunoprecipitated with normal rabbit serum (NRS), anti-ORF1b, and anti-ORF1a sera, respectively, prior to electrophoresis and transfer. All lysate were resolved in 12.5% SDS-PAGE gels and transferred to nitrocellulose membrane, followed by immune-detection using anti-ORF1a serum. **C**). Western blot analysis by anti-ORF1b serum. Lanes 1, 2, and 3 are the same as described for panel B. Lanes 4 and 5 are MDV infected DEF cell lysates which have been immunoprecipitated with anti-ORF1a and anti-ORF1b sera, respectively, prior to electrophoresis and transfer.

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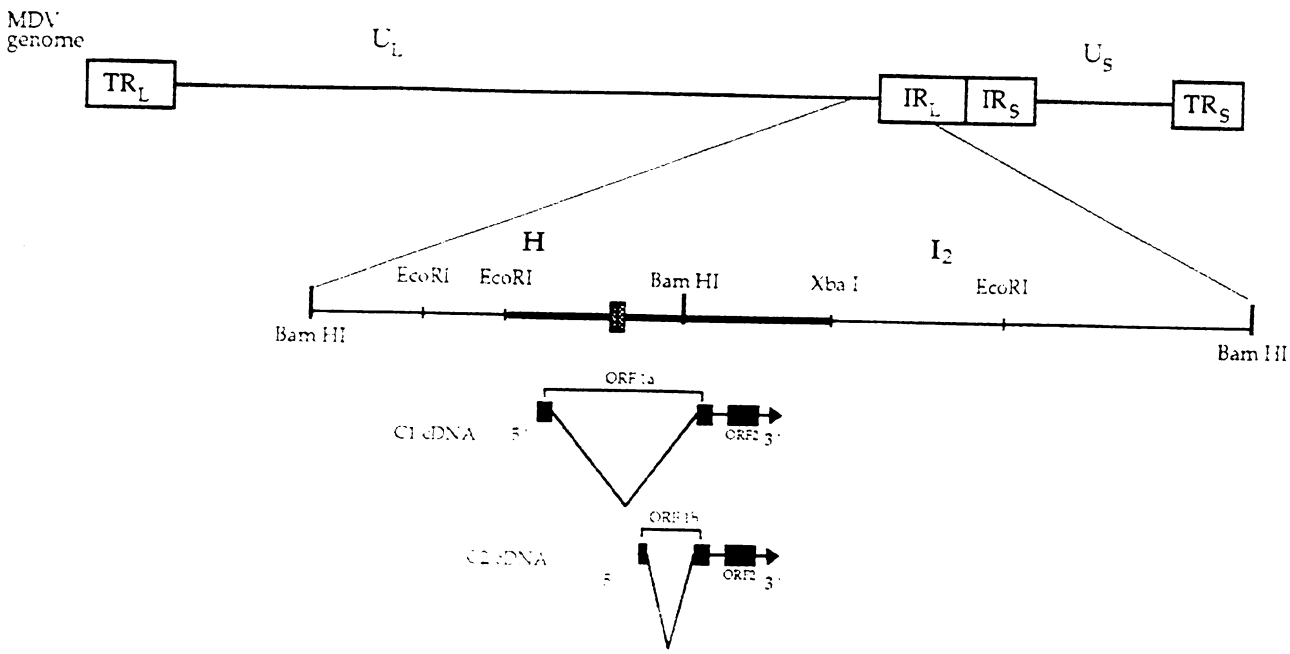
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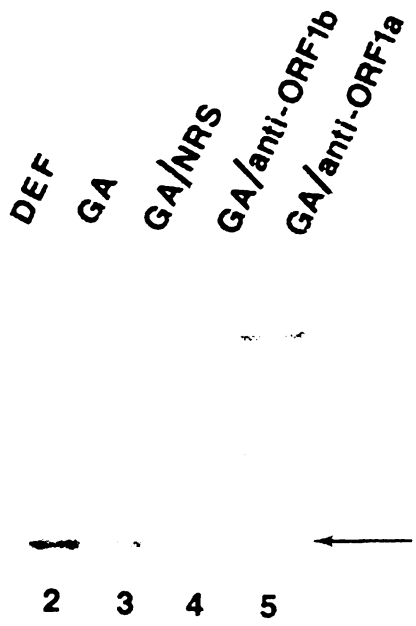
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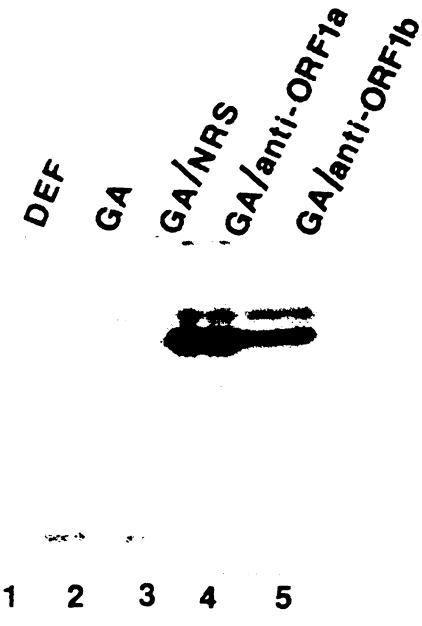
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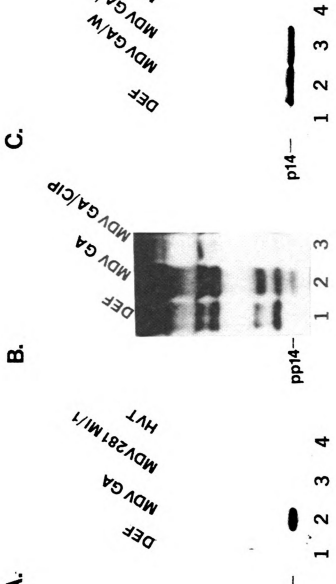
1 2 3 4 5

Figure 2. Characterization of p14. A). Western blot analysis of p14 serotype specificity. Lane 1: lysate from uninfected DEF, lane 2: lysate from DEF infected with MDV strain GA (serotype-1), lane 3: lysate from DEF infected with MDV strain 281 MI/1(serotype-2), lane 4: lysate from DEF infected with HVT (serotype-3). proteins were detected by anti-ORF1a serum. **B**). Immunoprecipitation of p14 as phosphoprotein (pp14). Uninfected DEF cells and DEF cells infected with MDV strain GA were preincubated in phosphate-free Dulbecco modified Eagle medium (Gibco-BRL, gaithersburg, MD) for 1 h. 500 µCi ^{32}P orthophosphate was added to each 60 mm plate and cells were continuously labeled for 4 hrs prior to harvesting. Cell lysates were Immunoprecipitated with anti-ORF1a serum as described (Glaubiger et al., 1983). Immunoprecipitates were analyzed by 12.5% SDS-PAGE. Lane 1: lysate from uninfected DEF cells, lane 2: lysate from DEF infected with MDV strain GA, lane 3: same as lane 2, except cell lysate was digested by calf intestinal phosphatase (CIP) following immunoprecipitation. **C**). Western blot analysis of subcellular fractions. Lane 1: whole cell lysate of uninfected DEF, lane 2: whole cell lysate of DEF infected with MDV strain GA, lane 3: cytoplasmic fraction of MDV GA infected DEF cells, lane 4: nuclear fraction of MDV GA infected DEF.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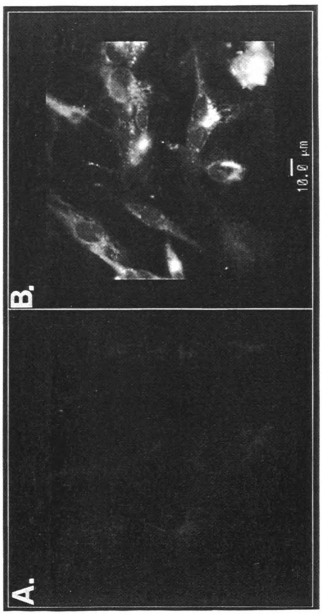
MDV GA
MDV281 MI/1
HVT
p14—
1 2 3 4
B.
DEF
MDV GA
MDV GA/CIP
pp14—
1 2 3
C.
DEF
MDV GA/W
MDV GA/C
MDV GA/N
p14—
1 2 3 4

Figure 3. Localization of pp14 by indirect immunofluorescence. Primary DEF cells and DEF cells infected with MDV strain GA, prepared as described by Glaubiger et al. (1983), were grown on glass coverslips. Cells were fixed by acetone/methanol (1:1) for 3 minutes and washed with PBS extensively, then preincubated with 3% bovine serum albumin (BSA) for 1 h at room temperature. Anti-ORF1a serum was added to the cells in a 1:20 dilution and incubated for 1 hour at room temperature. Goat anti-rabbit IgG conjugated with phycoerythrin (PE) (Sigma, St. Louis, MO) was used as secondary antibody in a dilution of 1:20. Slow-Fade (Molecular probes, Eugene, OR) was added prior to mounting to glass slides to prevent quenching of fluorescence. Confocal fluorescence images were acquired using the Meridian INSIGHT-PLUS Laser Scanning Confocal Microscope (Meridian Instrument, Okemos, MI). Fluorescent labels were excited using the 488 nm argon laser line. Emissions were acquired using 580/30 nm band pass filter with 100X Olympus Splan APo (1.4 NA) objective, 2.5 X photo eyepiece and detected through integration on a cooled-CCD camera. Panel **A**). uninfected DEF cells. Panel **B**). DEF infected with MDV strain GA.


A.
B.
10.0 µm

Chapter IV

DISCUSSION

1. SUMMARY OF RESULTS AND CONCLUSIONS

MDV genes are expressed in a sequential order as immediate-early (IE), early (E) and late (L) genes (Maray et al., 1988; Nazerian and Lee, 1976; Schat et al., 1989). Expression of IE genes is required for expression of early and late genes. Transcripts from MDV immediate-early genes are clustered in repeat regions, similar to locations of other herpesvirus IE genes (Roizman and Sears, 1990; Schat et al, 1989). Recent molecular biology studies reveal that MDV genomic structure and gene arrangement are closely related to herpes simplex virus and varicella zoster virus. Many HSV gene homologs have been found in MDV unique long and unique short regions, including TK, gB, gC, pK, gD, gI, gE etc (Buckmaster et al., 1988; Coussens and Velicer, 1988; Chen and Velicer,1992; Ross et al., 1989; 1991; Velicer and Brunovskis, 1992). Recently, ICP4 and VP16 homologs in MDV were also identified in a location similar to their homologs in HSV (Anderson et al., 1992; Yanagida et al., 1992). Therefore, the original hypothesis was to identify that additional MDV IE genes, possibly an ICP0 homolog, were present in the MDV IR_L region.

MDV IR_L region has been intensively studied due to its potential relationship with MDV oncogenicity. This relationship was derived from a correlation between amplification of a specific 132 bp repeat sequence located in IR_L and TR_L regions with attenuation of MDV. However, transcripts of different sizes, numbers and directions originated from this region have been reported (Bradley et al., 1989; Chen and Velicer, 1991; Iwata et al., 1992; Schat et al., 1989; Maray et al., 1988; Peng et al.,1992). The significance of this controversy can not be assessed without identification of gene products in IR_L region. By Northern blot analysis, a 1.6 kb transcript which hybridized

to the MDV BamHI-I_2 fragment was identified in MDV-infected DEF cells treated with CHX (Hong and Coussens, 1994). The 1.6 kb transcript was further mapped to a BamHI-XbaI subfragment of I_2, suggesting the presence of an immediate-early transcript in this region. Subsequently, viral genomic DNA of this region was sequenced. Despite abundant transcription, no continuous open reading frame (ORF) was identified in viral genomic DNA sequence. Suspecting a spliced transcript, led to construction of a cDNA library which was screened with BamHI-I_2 specific probes.

To enhance IE gene expression, mRNA isolated from MDV-infected cells treated with CHX was used in cDNA library construction. An oligo(dT)-tailed plasmid was employed in cDNA synthesis to minimize priming from internal poly(A) tracts, which are present in BamHI-H and I_2 genomic sequences. By cDNA cloning and sequencing, two cDNAs (C1 and C2) were identified as spliced transcripts spanning BamHI-H and I_2 fragments. Both cDNA clones were completely sequenced in both directions. C1 and C2 use the same splice acceptors and 3' ends, but differ at their 5' ends and utilize different splice donors. Combining data from cDNA clones identified by Peng et al. (1992) and our results, we conclude that there are at least three spliced transcripts derived from the MDV IR_L region. These three cDNAs use different splice donors, but share the same splice acceptor. Two cDNAs, C1 and cDNA 4 reported by Peng et al. (1992), contain introns which encompass the 132 bp repeat region, suggesting that expansion of the 132 bp repeat during attenuation may not affect the final transcript. Despite abundant transcription, as detected by Northern blot analysis, cDNA sequence analysis revealed only two small potential ORFs in each cDNA. C1 cDNA contains ORF1a of 83 amino acids and ORF2 of 107 amino acids, while C2 cDNA contains ORF1b of 76 amino acids and the same

ORF2 as C1 cDNA. Lack of continuous ORFs within these cDNA sequences raises questions regarding the function of these abundantly expressed spliced transcripts.

Using GST-ORF1a and GST-ORF1b fusion proteins, antisera against these two potential ORFs were prepared. Both sera detected a 14 kDa polypeptide (p14) in DEF cells infected with MDV strain GA by western blot analysis. Since ORF1a spans a 2.3-kb intron which includes the 132 bp repeat region amplified during attenuation, we were interested in determining the effect, if any, of the expanding repeat region on expression of p14. Although the 132 bp repeat region was expanded by 0.6 to 5.4 kb (about 5-40 copies of 132 bp repeat) in Md11 high passage (Silva and Witter, 1985), the same 14 kDa polypeptide was detected in DEF cells infected with both MDV strain Md11 high passages (passage 83) and low passage (passage 16). These results suggested that amplification of the 132 bp repeats may not be as critical in MDV attenuation as was previously thought. Since amplification of the 132 bp repeats is just one of the several phenomena observed in attenuation, other changes, such as a deletion in the BamHI-L region or reduced expression of gC protein, may collaborate to affect MDV pathogenesis (Bulow and Biggs, 1975; Churchill et al., 1969; Wilson and Coussens, 1991). Subtle differences in p14 secondary structure or amino acid content between oncogenic and attenuated strains are also possible and remains to be elucidated.

The 14 kDa protein is expressed in a lymphoblastoid cell line MSB-1, which are latently infected and transformed by MDV. Expression of p14 in tumor cell lines without IUdR induction indicated that p14 is constitutively expressed and may play some role in maintenance of the transformed state or latent infection of tumor cell lines. However, absence of homology with any known oncogene and expression of p14 in attenuated

MDV suggests that this role may be indirect or require collaboration with other viral or cellular factors.

All potential ORFs (1a, 1b, and 2) were searched against Swiss-Protein data base without finding significant homology. Limited homologies were found with mouse zinc finger protein ZFP-27 (*mkr4*) and *myc* proto-oncogene protein. These similarities were not in conserved or functional regions, such as zinc finger motif or *myc*-box regions. Therefore, we assumed that p14 represents a MDV specific antigen.

Extensive use of nononcogenic serotype-2 and serotype-3 MDV as live virus vaccines to prevent MDV serotype-1 induced tumor formation indicates that antigens in these three serotypes share common epitopes (Glaubiger et al., 1983; Igarashi et al., 1987). To determine if any proteins analogous to p14 exist in MDV serotype-2 or -3 infected cells, western blot analysis was conducted on lysates from cells infected by all three serotypes and detected by antisera to ORF1a and ORF1b. Absence of homologs in serotype-2 and -3 indicated that p14 is not only MDV specific, but also serotype-1 specific. In view of the facts that p14 coding sequence is located within MDV IR_L and IR_L regions share little homology among the three MDV serotypes, this finding is not entirely unexpected.

With primer extension and sequence analysis, the 5' end and upstream regulatory region of p14 has been defined. The regulatory region contains two potential TATA boxes, two CCAAT sequences, two Sp1 sites, an Oct-1 motif, and a putative origin of MDV replication. Preliminary data from transient assay of this region in a CAT reporter plasmid indicates this region is a bidirectional regulatory region shared by p14 and pp38 (Abujoub and Coussens, Personal communication). A similar bidirecitional regulatory

region has been reported between the HSV ICP4 and ICP22/47 genes (Preston et al., 1988; Wong and Schaffer, 1991). Regulatory elements shared by HSV ICP4 and ICP22/47 not only regulate the IE genes in both direction, but also have a stimulatory effect on replication functions mediated by the HSV origin located in the middle of the regulatory region (Wong and Schaffer, 1991). Reflecting on our data in MDV, how this bidirectional promoter-enhancer region regulates expression of p14 and pp38, whether it has any effect on MDV origin function will be interesting issues to further investigate.

The apparent size of p14 is significantly larger than the sizes of 9.6 kDa and 10.3 kDa calculated from ORF1a and ORF1b sequence, respectively. Computer analysis of C1 and C2 DNA sequence indicated several phosphorylation and N-glycosylation sites in both ORF1a and ORF1b. To determine if post-translational modification is one reason for slow migration, immunoprecipitation of lysates from ^{32}P -labeled uninfected DEF and DEF cells infected with MDV strain GA was performed. The results demonstrated that p14 is expressed as a phosphoprotein (designated as pp14) and, therefore, is sensitive to calf intestinal phosphatase (CIP) treatment. Protein phosphorylation is a well known mechanism to mediate protein activities involved in regulation of gene expression and other regulatory functions (review, Hunter and Karins, 1992). However, western blot analysis of MDV infected cells treated with CIP revealed only a slight shift of pp14 compared with that of untreated cells. In addition, TM treatment has little or no effect on pp14 apparent size. Together, neither phosphorylation nor glycosylation is completely responsible for the discrepancy between calculated and apparent size of pp14.

Characterizing pp14 as a phosphoprotein represents the third such identification of a phosphorylated protein encoded in MDV repeat regions, after the identification of

pp38 and *meq* (Chen et al, 1992; Cui et al, 1991; Jones et al., 1992; Brunovskis et al., 1993). While the *Meq* gene has a partial homology with the *fos/jun* oncogene family, pp14 and pp38 are both MDV serotype 1 specific antigens. Expression of these three proteins in MSB-1 cells suggests they may be associated with MDV tumorigenicity. Whether these proteins are functionally synergistic or antagonistic will be investigated.

Phosphorylation of pp14 supports the postulation of pp14 as a regulatory protein, which may be primarily located in the nuclei of infected cells. Surprisingly, both western blot analysis of subcellular fractions and indirect immunofluorescence detection revealed that pp14 is predominately a cytoplasmic protein. Whether p14 is transported to the nucleus in limited amounts, regulates gene expression by interaction with another protein factor, or serves other functions will be the subject of further experiments.

All experiments were conducted using both anti-ORF1a and anti-ORF1b sera. Both sera detected the same size protein with the same properties. However, whether these two antisera detected the same polypeptide and whether the two antisera cross-react with both ORF1a and ORF1b derived polypeptides are unknown. To answer these questions, competition immunoprecipitation combined with western blot detection, and cross-absorption experiments were conducted. Results indicated that these two antisera block each other's binding. Therefore, it is most likely that the proteins recognized by anti-ORF1a and anti-ORF1b sera represent the same protein or two polypeptides with high antigenic relatedness. However, without data from amino acid sequence or polypeptide mapping, it is impossible to know whether slightly different forms of pp14 exist in serotype 1 infected cells.

2. FUTURE RESEARCH DIRECTIONS

Identification of pp14 in MDV IR_L region adds important information to studies of MDV gene expression, particularly regarding association of pp14 with MDV tumorigenicity and latency. Many questions remain to be answered and future research should focus on both structural and functional analysis of this MDV specific antigen.

Partial amino acid sequence or peptide mapping could clarify the question of whether protein(s) detected by anti-ORF1a and anti-ORF1b sera represent the same or two different proteins. This will also provide a clue to further investigate other polypeptide(s), possibly encoded by different spliced transcripts derived from this region and to understand the function of these altered splicing patterns. Studying the structure of pp14 at the amino acid level may also identify any subtle but critical changes, or modifications in pp14 expressed in attenuated MDV strains. Although pp14 has been demonstrated to be a phosphoprotein, the precise phosphorylation sites are unknown. The biochemical properties of this protein should be addressed in order to further additional functional assays.

Analysis of functions of pp14 should focus on two aspects: 1) a potential role in regulation of heterologous gene expression; 2) a potential role in transformation of MDV infected cells. Cloning of C1 and C2 cDNAs into eukaryotic expression vectors has been started. Transient assay by co-transfection of these two expression clones with CAT reporter plasmids containing MDV early and late gene promoters will test a regulatory function of these genes. With the establishment of an *in vitro* expression system, transformation assay can be processed to assay any effect of pp14 on growth rate, proliferation properties, or transformation of primary cells, such as CEF, DEF or

lymphocytes, in regarding the tumor association of pp14. Oligonuclotides complementary to C1 or C2 sequence can be used to test antisense-inhibition in MDV transformed cell lines.

It would be important to extend the experiments to *in vivo* studies. The function of pp14 can be further defined by construction of "knock-out" virus with selective genes (i.e. neo^r and gpt) inserted into pp14 region. A chicken embryo fibroblast cell line, OU2, has been recently developed for growth of MDV in our laboratory. OU2 cells can be used to produce recombinant virus or transfection with C1 and C2 expression plasmids to provide complementary cells. Recombinant viruses can be used to infect susceptible birds to further define the specific function of pp14.

In respect that a number of transcripts detected in MDV latently infected cell lines and expression of some MDV specific genes (pp14, pp38, and *meq* gene), MDV latency is more like EBV than HSV. In EBV latency, at least nine virus-encoded proteins are expressed, including six nuclear proteins and three membrane proteins. These proteins have the functions for initiation and maintenance of virus latency, immortalization and transformation of infected cells (Kieff and Liebowitz, 1991). Since pp14 is expressed in MDV latently infected and transformed cell lines, it would be of interest to investigate if pp14 has a role in MDV latency.

In addition, functional relatedness between pp14 and pp38, or *meq* would be an important part of studying the mechanism of MDV-induced oncogenicity. MDV transformation is most likely a multi-step progress involving more than one gene products. *In vitro* expression systems may be utilized to analyze any collaboration of these three genes on MDV transformation by cotransfection of gene expression plasmids.

Functions of the bidirectional regulatory region between pp14 and pp38 should be pursued. Characterization of regulatory elements in this region is in progress in this laboratory by site specific mutation and transient assay. It would be of interest to find out how pp14 and pp38 genes are regulated and whether their expression are mutually exclusive or coordinated. To address this issue, plasmid, such as pCAT 4 (or 5), containing both CAT and β-gal reporter genes in opposite directions, can be used for testing regulation in both directions.

Although a putative origin of replication in this bidirectional promoter region was reported several years ago (Bradley et al, 1989), there is no direct evidence of origin function from this region. To study the origin function of replication, a plasmid including the origin and flanking regions must be established for *in vitro* replication assays. Since enhancement of origin function by transcription regulatory elements is a common phenomenon among DNA viruses (de Villier et al., 1984; De Lucia et al., 1986; Stenlund et al., 1987; Wides et al., 1987; Wysokenski et al., 1989), mutations on different regulatory elements flanking the origin can be conducted to test effects of these mutations on origin replication function.

Studies on pp14 structure and function will add important information to our knowledge of MDV gene expression and regulation, particularly in understanding of MDV oncogenicity.

APPENDIX

APPENDIX

SUPPLEMENTARY DATA

MDV viral genomic DNA sequencing.

A BamHI-XbaI subfragment (2.3 kb) of MDV BamHI-I_2 was sequenced as described in chapter II. (Figure 1 and 2). Failure to detect any extended (> 100 amino acids) ORFs lead to cDNA isolation. The genomic sequence was used for alignment and analysis of C1 and C2 cDNAs which have been detailed in chapter II.

Fusion protein cloning and identification.

As described in chapter II, DNA fragments of ORF1a and ORF1b were cloned into pGEX-3X for expression of fusion proteins. An illustration of the vector and data from identification of fusion protein clones are presented in Figure 3. To confirm specificity of antiserum to ORF1a encoded protein, GST-ORF1a fusion protein was cleaved by Factor Xa to remove vector GST proteins. Cleaved ORF1a protein was then detected by anti-ORA1a serum (Figure 4).

Effect of phosphorylation and glycosylation on pp14 migration.

Immunoprecipitation data indicated p14 is a phosphorylated protein (pp14). Phosphorylation may be one factor contributing to slow migration of pp14. To further investigate how post-translational modifications might affect the apparent size of pp14, two treatments were conducted: 1) MDV-infected cell lysates were treated with calf intestinal phosphatase (CIP) to remove phosphate. 2) MDV-infected cells were treated with tunicamycin (TM), an inhibitor of N-linked glycosylation. TM and CIP treated cell lysates were compared with untreated cell lysates to identify any size discrepancy of pp14

by western blot analysis (Figure 5).

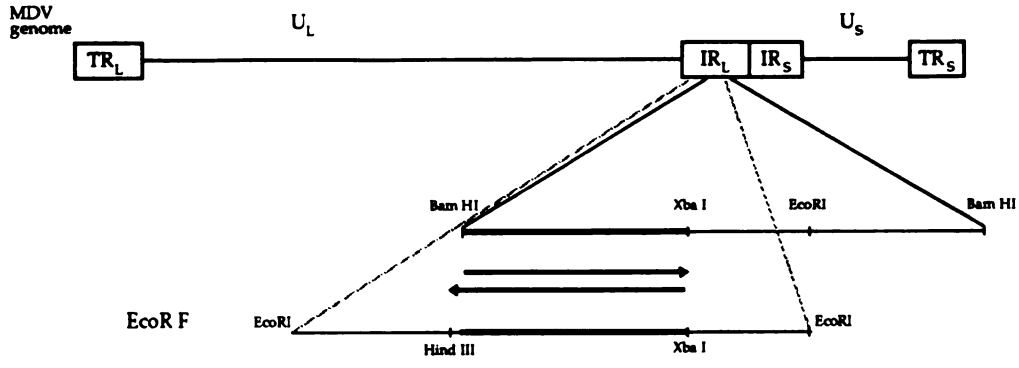
Double-stained immunofluorescence on MDV infected cells.

As detailed in chapter III, monoclonal anti-MDV/gB antibody was employed as a positive control to confirm that immune signals detected by anti-ORF1a serum are truly in MDV-infected cells. Data from double staining immunofluorescence which shows signals detected by both antibodies (anti-ORF1a and anti-gB) are primarily present in the same cells (Figure 6).

Figure 1. Location of BamHI-XbaI subfragment in MDV genome and computer analysis of the viral genomic DNA sequence ORFs. A). MDV genome structure and location of the region sequenced. Dark lines represent sequenced fragments from the MDV BamHI library and EcoRI library. Sequence was determined for both strands. **B).** Translation of the viral DNA sequence in all six reading frames by Genepro program. Upper vertical bars indicate methionine or start codons. Lower vertical bars represent stop codons.

Figure 1.

A.


MDV genome
U_L
U_S
TR_L
IR_L
IR_S
TR_S
Bam HI
Xba I
EcoRI
Bam HI
EcoR F
EcoRI
EcoRI
Hind III
Xba I

B.

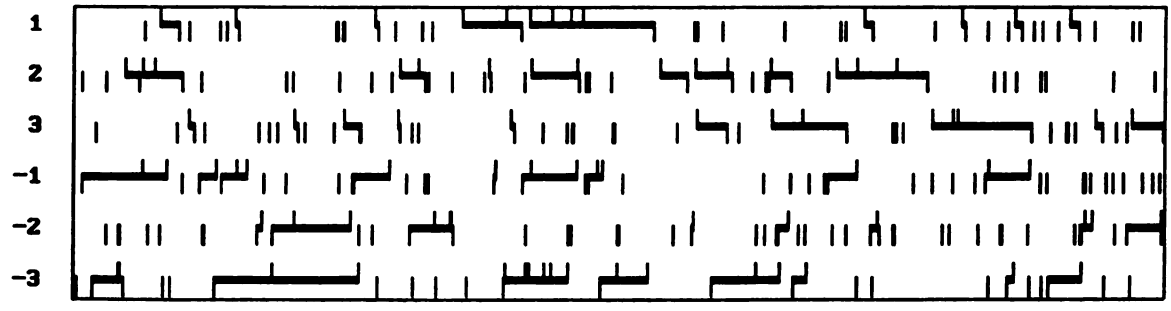

1
2
3
-1
-2
-3

Figure 2. Nucleotide sequence of the BamHI-XbaI region in MDV BamHI-I_2 fragment.

```
1     TTTCACTCTC CCCTAAAAAA AAAAAGGAGG TTTGGTTACC CAGGTAGTGC CCCCCCAGAA
                                                                BamHI
61    AGAATAATCA CTGCTTCGAA CGGAGCTCGA TTCATCATCC GGGATTATGC TTTGGGGGAT
121   CCAATTTTCG CAGAGGTAAA TATCCATGCG TGATCTATTT GTGGTGGCAA TGTGGACTTT
181   TCTATGCCTC AGGAAGAACA TATCTACTTT GTTGGATTTT GTGAGTGAGA ATTAAGAACG
241   TGTATATGAA GCAGGACTGA AAAAAAACTA TTAAGTTCTA CTAACAAAGT GTGCCGGTGT
301   ACCTACCATC CGTGTTGATT ACAGCTGTGA TATCGTTGCA GACCCCGGAT GCATTGACTG
361   CGGACCCACT TCCATCTCGA AACGGATACT GCGACAACTA GGAACGGAAC ATCAAGTTTT
421   GGTGATCGCA TCAGAAGTTT TTGTGAGAGG GCTCGCTCCT TAATTTCCAA CTTTGTAACA
481   TGGCGTAGTA GAAACGAGAG CTGTGAGGTT CTGGCAGAGA TTCCAGCAAG AGAAAGAAGT
541   GGAATTCGAC CCTCTCGAAT CCAGTATAAA TAGTAGCTAG GCGGATAATG AGTCGCTGTT
601   TGCACATTAT CAAACGTACG CATTAGTAAC TGCAGAAAGA CGCTGCGTAT AGTTATGTAT
661   TCTTAGAATA CGTCTGTATA TACGCACGAA CATATAAGTC TGTAAGAATG TAATGCTTCG
721   TACAGATCAC TGTTTATTGA AGTTCAACGG TATGAAATTT GAGTATACCT AGAATTAGCA
781   GGATAATTGG CATCTCAATT TCTCGAGGCT TTTTTTTTTT GCACATTGAC ATCTACCGGA
841   AAAGTGTGTA TGCGATTCGC TTACCCTTCC CCAACTTTCT CTGTCGGTCG TGGTATATTG
901   AGGCCGGTGT ATGTAGAGAG TCTACATCTT CGATCTCCTT CGGATCACAT GGAGCGGAGA
961   TGTTGTAGGG TTCGAGAGGG GTGAGACCTA AACATCAGTC GCATGCATGT GGAACACGAT
1021  TGGCCGTGTT GTAGCATACA AGCAGTACAC ATGGCGAAAG TTTGCCGTCC GCCTGTTCGG
1081  TGTGACGTCA TGTTTAGGTT TGAGCATGTA AGAAAAATGG AACTGTTAAC TCTAAAAAGA
1141  AGTATCTCGC CCCATTTGTA TCATTCGGCG GTGGGAAATA TAGGTAATAG AAAAATCATA
1201  CCTACGTCAG GCTTCTTCGT TCGCGATCGC AGAAGTGTCT GGAGACGCGC AAAGAAGGTC
1261  TGGCGCATTC CGATATAGTT TGCAGCCAAT GCTTGGTCCG CGGAATCGGG ATCGGAGCCG
1321  ATTATCGATA ATACGGAAGC AAAGGGGATA ACTTCCTTGT TTACATAGAA TGTATGACCG
1381  GTACACGACA ACACGGAAAT CCTGTCTCAA AGTCTTTGTT TGCGGAAATA GAACGGATAT
1441  GACGCTTCTG TAAACGATTG CGGAAGTAGC GGTCGCCGAG ACGAACGCGG ACGTGTAGCG
1501  GGGACGTTCA TTGTCTTTGT TTTTCTAATT ATTTTGAATG TATGTATATT TTTCAGCCTC
1561  ATCCTGTAAA TCGGTCGAGC ATTAAAAGGT TACGGATATT GGTTCACTGT ATGCGTGTTC
1621  ATTTTATCTT TCGTATACTC CCCTTCGCGT ACGGTACGAT CAAGGTTAAA AAAAGTTACT
1681  GCAAATGTAC AGTAACCGCC GTAAATTAAC TTGCGGTGTC GGTTGCTTCA TGCGTCTCGA
1741  TATATGTTCT GTGCGCTTCT CAACTAGCTG GCGGCATCTA CAATTTTTGT CTGTTTGTCG
```

1801 TTTGCCACTA AACTCAATGA TTTCCGGACC AGTAGCGGCG GGGGAGGGGG TGTGTTATAT

1861 TTCGAGAGCG TTCGGCCGGG TTTGATCCTG GAATTATGAA ACCGTGCCGA CTGCGTTTTT

1921 ATTTTTGTTG TTGTTATTCT ATGGTTCCAA TGGCGCGAAA TGGGGTAGTT TTTTTTTTGC

1981 GGCAGCTCCT CAGGATTTAT ACGGAATTGT TTATAACGTC ATTGGGTATT GAAGGTATTT

2041 CCTTTTATTA TAAGTTTTTT TTAAGTCGCT GTTCCCATGA GAAATTTTTG ATAACCGATA

2101 TTGCTTACTC ATAGCTGAGT GCCCGGAATT TTAGCTTAAG TGAAGTCACC TTATAGGTGC

2161 AGGTGCCTTT GAAGTGTCTG GGGCCCTGAT AGACAGATGC CCTTTCTTAA GTGCTGATTT

2221 ATCATCTCAT TACCCCTCGC CTCAGCATAA ATATGCGATC GGTGTGACGA TTACTCAGAA

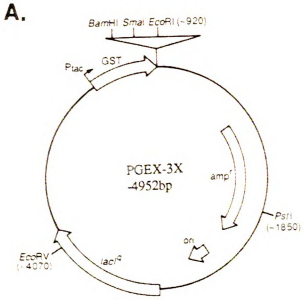
2281 CCGCTCTTCC TAATTAGCAG AACCAGCGAA TTATACTTTA GAGGCCTTTA ATGTCGAGAG

XbaI

2341 GCCCTGTAAA TTTTACCGCC CCCGAACACT TTCCAACTAA CTGTCGATTT GTCATCTAGA

(Figure 2. continued)

Figure 3. Fusion protein expression vector and fusion protein clone identification. **A).** Plasmid pGEX-3X with multiple cloning sites labeled. ORF1a was cloned in SmaI sites; while ORF1b was cloned into BamHI and SmaI sites. **B).** Cells expressing fusion protein clones were identified by SDS-PAGE stained with Coomassie brilliant blue. T means total cell lysates; F means glutathione-sepharose purified fusion proteins. Lane 1 and 2 are GST-ORF1a fusion proteins. Lane 3 and 4 are GST-ORF1b fusion proteins. Lane 5 and 6 (G) are GST proteins. Lane 7 (M) is prestained protein marker.


A.
BamHI SmaI EcoRI (~920)
GST
Ptac
PGEX-3X
~4952bp
ampr
PstI
(~1850)
ori
lacIq
EcoRV
(~4070)

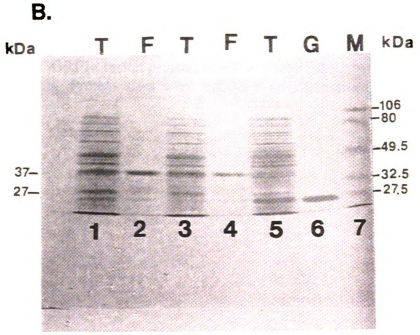

B.
kDa
T F T F T G M
kDa
106
80
49.5
37–
27–
32.5
27.5
1 2 3 4 5 6 7

Figure 3.

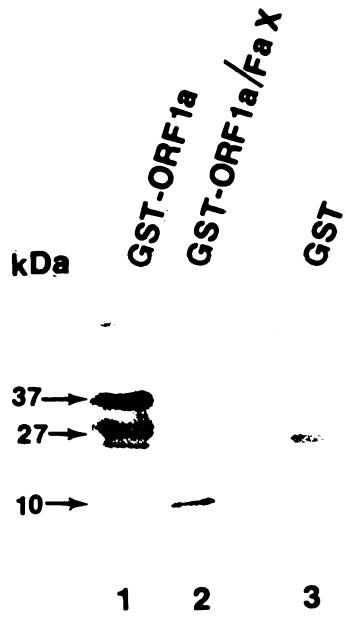


Figure 4. Western blot showing antibody specificity to ORF1a protein. GST-ORF1a fusion proteins were bound to glutathione sepharose 4B and cleaved by Factor Xa. The cleaved ORF1a protein was analyzed by western blot using anti-ORF1a serum. Lane 1 is uncutted GST-ORF1a fusion protein, lane 2 is Factor Xa cleaved ORF1a protein, lane 3 is GST protein. Protein sizes were estimated by comparison with prestain protein marker.

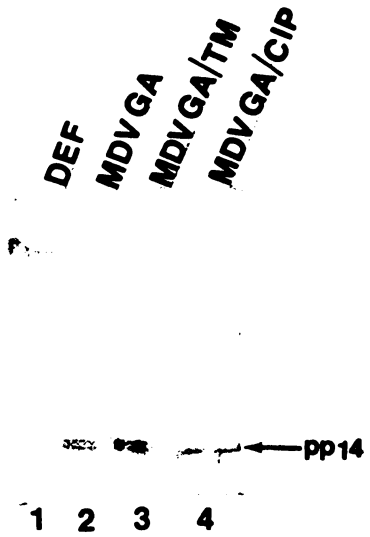


Figure 5. Western blot analysis of post-translation modification. Lane 1 is cell lysate from CEF, lane 2 is cell lysates from CEF infected with MDV strain GA, Lane 3 is cell lysate from MDV infected cells which treated with TM, while lane 4 is cell lysate from MDV infected CEF and digested with CIP.

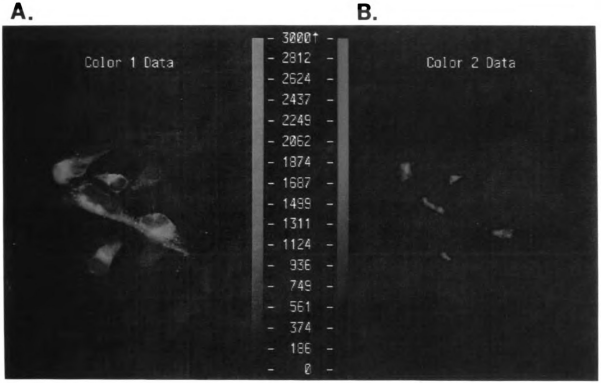


Figure 6. Double-stained immunofluorescence of MDV infected CEF cells. Anti-ORF1a with secondary antibody conjugated with PE and monoclonal anti-MDV/gB antibody with secondary antibody conjugated with FITC were utilized to conduct double staining. **A**). Fluorescent labels were identified by 530/30 nm band pass filter to show anti-gB-FITC stain.. **B**). 580/30 nm band pass filter was used to detect anti-ORF1a-PE stain.

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