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Characterization of DNA-Protein Interactions Involved in Marek's Disease Virus-Mediated Rous Sarcoma Virus Long Terminal Repeat Promoter Transactivation

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Major professor

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CHARACTERIZATION OF DNA-PROTEIN INTERACTIONS INVOLVED IN MAREK'S DISEASE VIRUS-MEDIATED ROUS SARCOMA VIRUS LONG TERMINAL REPEAT PROMOTER TRANSACTIVATION

By

Wei Sun

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ABSTRACT

CHARACTERIZATION OF DNA-PROTEIN INTERACTIONS INVOLVED IN MAREK'S DISEASE VIRUS (MDV)-MEDIATED ROUS SARCOMA VIRUS (RSV) LONG TERMINAL REPEAT (LTR) PROMOTER TRANSACTIVATION

By

Wei Sun

MDV-mediated RSV-LTR transactivation has been used as an *in vitro* model system to investigate the molecular mechanisms involved in MDV-enhanced avian leukosis virus (ALV) tumorigenesis. Previous studies have suggested a 28 bp region in the RSV-LTR promoter is critical for MDV-mediated RSV-LTR promoter transactivation. Also, a unique DNA-protein complex was generated with DNA sequence from this 28 bp region in MDV-infected cells. In this study, further characterization of the MDV-unique DNA-protein complex has revealed that the cellular DNA-binding proteins in the 28 bp region, rather than the DNA sequence from this region, serve as direct target for MDV-specific factor(s). Two cellular factor DNA-binding sites were found in the 28 bp region: a previously reported EFI (enhance factor type I) binding site and an *ets*-like DNA-binding site. Because this newly discovered binding site is similar to *ets* factor binding site core sequence, it has been termed as *ets*-like-element (ELE). Primary study of this DNA-binding site has revealed a unique feature of ELE factor binding activity. In order for ELE factor to recognize its own DNA-binding site, a protein-protein interaction with EFI factor seems required. It suggested that the interaction between EFI and ELE factors might cause a structure change in ELE factor which could lead to the release of ELE DNA-binding activity. The involvement of both EFI

and ELE factors can result in two cellular DNA-protein complexes comformation. The small complex (complex I) is an intermediate form of cellular DNA-protein interaction, which was composed of EFI factor alone. While the larger complex (complex II) was formed by both EFI and ELE factors. The formation of complex II is correlated with characteristically high RSV-LTR promoter activity, which suggests that complex II is functionally important for RSV-LTR promoters. In MDV-infected cells, formation of the larger cellular DNA-protein complex (complex II) was important for generation of a unique MDV DNA-protein complex (complex III), where complex II may serve as a target for MDV-specific factors. We also noticed a coincidence between loss of the unique MDV DNA-protein complex and reduction of MDV-mediated RSV-LTR promoter transactivation, which suggests that formation of the unique MDV DNA-protein complex may functionally relate with MDV-mediated RSV-LTR promoter transactivation. As pointed out from these studies, MDV-specific factor can recognize cellular DNA-binding proteins in the RSV-LTR 28 bp region. The protein-protein interaction between MDV-specific factor and cellular DNA-binding proteins can be used to isolate the MDV-specific regulatory protein. Further study of this MDV-specific factor will help to elucidate the mechanism of MDV serotype II specific-enhancement of ALV-induced lymphoid leukosis (LL), and even faciliate to create new generation of MDV vaccines without the augmentation of LL.

To my dear wife, Xinguang Li,

for her love, understanding, and support,

and

To my parents, Quan Sun and Huifang Song,

for their love.

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LIST OF ABBREVIATIONS

AGP	agar gel precipitation
AIDS	acquired immunodeficiency syndrome
ALV	avian leukosis virus
ALSV	avian leukosis/sarcoma virus (group)
ASV	avian sarcoma virus
bp	base pairs
CAT	chloramphenicol acetyl transferase
CD4	cluster of differentiation (antigen) No. 4
CD8	cluster of differentiation (antigen) No. 8
C/EBP	CCAAT enhancer binding protein
CEF	chicken embryo fibroblasts
CEK	chicken embryo kidney
cDNA	complementary DNA
CHX	cycloheximide
CMI	cell-mediated immunity
$CsCl_2$	cesium chloride
kDa	kilo dalton
DEF	duck embryo fibroblast
DR	direct repeat
E	early

EBV	Epstein-Barr virus
EFI	enhancer factor I
ELE	ets-like-element
EV	endogenous virus
gB	glycoprotein B
gC	glycoprotein C
HCMV	human cytomegalovirus
HHV-6	human herpesvirus 6
HIV	human immunodeficiency virus
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
IFA	immunofluorescent assays
IN	integrase
IRL	internal repeat long
IRS	internal repeat short
kbp	kilobase pairs

L	late
LL	lymphoid leukosis
LAT	latency associated transcript
LTR	long terminal repeat
MATSA	MDV-associated tumor surface antigens
MD	Marek's disease
MDV	Marek's disease virus
NF-kB	nuclear factor kappa B
NK	natural killer (cell)
Oct-1	octamer binding factor-1
ORF	open reading frame
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
RSV	Rous sarcoma virus
RT	reverse transcriptase
SDS	sodium dodecyle sulfate
SRE	serum response element
SRF	serum response factor
SV40	simian virus 40
TBP	TATA-box protein
TFIIB	transcriptional factor IIB
TFIID	transcriptional factor IID

TFIIH	transcriptional factor IIH
TK	thymidine kinase
TRL	terminal repeat long
TRS	terminal repeat short
UL	unique long
US	unique short
VP16	(MDV) virion protein 16
vMDV	virulent Marek's disease virus
vvMDV	very virulent Marek's disease virus
VZV	varicella-zoster virus

Chapter 1

Literature review

I. Introduction

Marek's disease virus (MDV), an avian herpesvirus, is the pathogen of Marek's disease (MD) which is characterized by T-lymphoma and peripheral nerve demyelination in chickens (Calnek and Witter, 1991). Because MDV is highly contagious and often fatal or leads to condemnation, MD was a serious threat to the poultry industry before development of live-virus vaccines. In order to boost chicken's immune-response against pathogenic MDV, non-pathogenic strains of MDV were used as vaccines (Churchill et al., 1969; Okazaki et al., 1970). Since the emergence of very virulent (vv) MDV, polyvalent vaccines which combined two or three non-pathogenic strains of viruses from different MDV serotypes were developed (Witter, 1982; Calnek et al., 1983; Witter and Lee, 1984). With efficient control of vvMDV infection by using polyvalent vaccines, however, a side effect was also observed (Witter, 1985). The serotype 2 MDV which was commonly used in polyvalent vaccines could interact with avian leukosis virus (ALV, an avian retrovirus), and resulted in an enhanced ALV pathogenesis (Bacon et al., 1989).

In the past decade, both in vitro and in vivo methods have been employed in ALV/MDV interaction studies. Results from these studies provided pathological and molecular biological evidence of direct ALV/MDV interactions. Because of the multiple-stage nature of ALV tumorigenesis, study of the pathological changes in ALV/MDV co-infected chickens was important to determine the role of MDV in different stages of ALV pathogenesis. Therefore, in this review, both pathology studies in ALV/MDV co-infected chickens and molecular biology studies of ALV-LTR promoter transactivation will be

summarized and discussed. As implied from these studies, there may be more than one way that MDV enhances ALV pathogenesis.

ALV/MDV interactions in chickens also present a valuable model system for retrovirus/herpesvirus interaction studies applicable to humans. Co-infections with human herpesviruses and retroviruses have been frequently found in patients afflicted with acquired immunedeficiency syndrome (AIDS), and is considered a possible risk factor in development of AIDS (Spector et al., 1984; Koening et al., 1986; Salahuddin et al., 1986). Many in vitro studies have confirmed the interactions between human immunodeficiency virus (HIV) and several human herpesviruses, such as herpes simplex virus (HSV) (Mosca et al., 1987), human cytomegalous virus (HCMV) (Drew et al., 1984), and human herpesvirus type 6 (HHV-6) (Josephs et al., 1986). Comparing results from ALV/MDV interaction studies in chickens to human cases, one common feature appears to be that many herpesviruses enhance retrovirus-pathogenesis by modifying retrovirus LTR promoter activities. Evidences of human retrovirus/herpesvirus interactions will be also described in this review.

II. Marek's disease virus

1. History of MD and MDV studies

The most common lymphoproliferative disease of chickens is MD, which was first reported by a Hungarian scientist, Joseph Marek, in 1907 (Marek, 1907). The first described symptoms of MD were paresis and paralysis that were caused by mononuclear infiltration of peripheral nerves and spinal nerve roots. As observations were added to Marek's early description, lesions were also found in gonad, iris, various viscera, muscle, and skin (Calnek and Witter, 1991). This so-called acute MD with unusual high mortality and preponderance of visceral lymphomas reflects the neoplastic features of the disease. It was not until the late 1960's that the etiologic agent of MD was determined to be a highly cell-associated avian herpesvirus, termed Marek's disease virus (MDV) (Churchill and Biggs, 1967; Nazerian et al., 1968).

Prior to development of MDV vaccines, MD constituted a serious economic threat to the poultry industry due to heavy annual losses (Purchase, 1985). A few birds that develop signs may recover from the clinical disease (Biggs and Payne, 1967) but, in general, mortality is nearly equal to morbidity. Losses in affected flocks were estimated to a range from a few birds to 25 or 30% and occasionally as high as 60% (Purchase, 1985).

In the 1960's and early 1970's, some significant research had contributed to a better understanding of MD and led to virtual control of the disease by vaccination. The first breakthrough was a successful transmission of the disease experimentally by Biggs and Payne in 1963. Subsequently, two groups in the UK and USA discovered that a herpesvirus

was the etiologic agent of MD, and successfully propagated MDV in cell culture (Churchill et al., 1967; Nazerian et al., 1967; Solomon et al., 1968). Perhaps one of the most important developments was identification of attenuated strains of oncogenic MDV in tissue culture systems (Churchill et al., 1969). Attenuated MDV were applied as a vaccine against MD (Churchill et al., 1969a&b). At the same time, an antigenically related but non-pathogenic herpesvirus was isolated from turkey, called herpesvirus of turkey (HVT). Although superseded by other vaccines, MDV vaccine was the first effective cancer vaccine in any species. Since vaccines are not 100% effective, MD losses still occur but are no longer as serious as previously described.

In addition to its great economic importance in agriculture, MDV offers a superb model for studying herpesvirus oncology due to several unique advantages. Importantly, MDV can constantly reproduce lymphoma in its natural host. Furthermore, a large spectrum of virus strains ranging from very virulent to non-oncogenic and non-pathogenic have been isolated. Also, well-characterized genetic lines of chickens have been produced, which range from extremely susceptible to remarkably resistant to the disease. Finally, MD has been successfully prevented by vaccination. However, a pattern of evolution in the virulence of MDV strains has been reported (Calnek and Witter, 1991). Selection pressure caused by administration of MDV vaccine has generated some highly virulent strains of MDV which accounted for most losses from MDV outbreaks in vaccinated flocks. Therefore, development of new generation vaccines using molecular biology approaches has been promoted. The efforts to uncover the molecular mechanisms of MDV oncogenicity and immunoprotection will contribute to both science and the poultry industry.

2. MDV vaccine

Development of successful vaccines for control of MD is a remarkable achievement both in agriculture (because prior to vaccination MD had become the most costly poultry disease) and basic cancer research (because this was the first time an important neoplastic disease had been so successfully controlled by vaccination in any species) (Calnek and Witter, 1991).

Three classes of viruses are capable of protecting chickens against MD: attenuated serotype 1 MDV, HVT, and naturally non-pathodenic isolates of serotype 2 MDV (Churchill et al., 1969; Okazaki et al., 1970; Schat and Calnek, 1978). Polyvalent vaccines mainly composed of serotype 2 and 3 viruses have been described (Witter, 1982). Although all vaccine types are protective, HVT has been used most extensively because it is economical to produce and cell-free virus extracted from infected cells is more convenient for storage and handling.

Outbreaks associated with vvMDV strains in flocks vaccinated with HVT can often be controlled by vaccination with polyvalent vaccines composed of all three viral serotypes, or of serotypes 2 and 3 (Calnek et al., 1983; Witter and Lee, 1984). The improved efficiency of a bivalent vaccine against challenge from very virulent strains was confirmed (Schat et al., 1982; Vielitz and Landgraf, 1985), although the interval between vaccination and exposure to the virulent field virus affects vaccine efficacy. Early exposure is probably one of the most important causes of excessive MD in vaccinated flocks, because it takes 7 days to establish protective immunity in vaccinated birds (Basarab and Hall, 1976).

Although bivalent or trivalent vaccines are extremely efficacious, concern was raised

when Witter (1985) reported a high incidence of lymphoid leukosis (LL) were found in flocks vaccinated by polyvalent vaccines. LL, a B-cell lymphoma, is caused by ALV infection. High rates of LL in vaccinated chickens suggested that non-pathogenic MDVs in polyvalent vaccines may enhance ALV pathogenesis. Details of this interaction will be presented in the " Retrovirus and herpesvirus interactions" section.

3. Pathology of MDV

MDV is a highly contagious agent which spreads horizontally by direct or indirect contact with infected birds, or via an airborne route (Sevoian et al., 1963; Payne, 1985). It is generally conceded that vertical transmission, if it occurs at all, is so rare as to be of no significance (Calnek and Hitchner, 1973).

A. MDV infection

With cell-free MDV virus, enveloped virion enters the cell by conventional absorption and penetration, which occurs within 1 hour. The time to appearance is 5 hours for viral antigens, 8 hours for DNA synthesis, 10 hours for nucleocapsid production, and 18 hours for enveloped virion production post infection (Hirai et al., 1980). Viral DNA replication occurs during the S phase of cell replication (Lau and Nonoyama, 1980). More commonly, instead of infected by cell-free virus, most cells are infected by contact with other infected cells, accomplished through formation of intracellular bridges (Kaleta and Neumann, 1977).

Three general types of virus-cell interactions are recognized in MDV infection: **1)** productive infection; **2)** latent infection; **3)** transforming infection (Payne, 1985; Schat,

1985a; Calnek and Witter, 1991).

In productive infections, replication of viral DNA occurs, antigens are synthesized, and in some cases virus particles are produced. There are two types of productive infection: fully-productive and semi-productive infections. Fully-productive infection occurs only in the FFE of chickens, and results in development of large numbers of enveloped, fully-infectious virions (Calnek et al., 1970). Semi-productive infections are observed in B lymphocytes and some epithelial cells in chickens, as well as in most cultured cells. In semi-productive infection, viral antigens are produced but most of the viruses are non-enveloped and non-infectious. In all types of cells, productive infection is lytic and leads to intranuclear inclusion body formation and cell destruction. Therefore, cytolytic infection is another term used in place of productive infection (Calnek, 1986).

The second type of virus-cell interaction in MDV infection, latency, has been observed only in lymphocytes, predominantly in T-cells but also in some B-cells (Shek et al., 1983). Latency is characterized by persistence of the viral genome in cells without expression of most viral antigens or production of virions. Only about five copies of the viral genome are typically present (Ross, 1985). Latency occurs in all three serotypes of MDV (Shek et al., 1982). Latent infection can persist for a lifetime in the chicken, and MDV viruses can be rescued by co-cultivation with permissive cells (Calnek, 1985).

The third type of MDV infection, transforming infection, only occurs with infection of oncogenic strains of serotype 1 MDV and is limited to T-lymphocytes in the absence of ALV. Transformed T-cells, in general, are activated T-helper cells which are $CD4^{+}CD8^{-}$ (Schat et al., 1991). In this type of infection, viral genomes persist in transformed cells with

highly methylated DNA and only a limited portion of the genome may be transcribed (Kanamori et al., 1987). Viral antigens and virions are not typically observed in transferred T-cell cultures (Silver et al., 1979).

B. MDV pathogenesis

The pattern of MDV infection, which occurs in genetically susceptible chickens with oncogenic strains of MDV, can be sequentially divided into four phases (Calnek and Witter, 1991): **1)** early lytic infection which causes primarily degenerative changes and is largely restricted to B cells, **2)** latent infection primarily restricted to activated T cells, **3)** a second phase of cytolytic infection coincident with permanent immunosuppression, and **4)** a proliferative phase of transformed MDV lymphocytes.

As a horizontally transmitted disease, primary MDV infection usually occurs via the respiratory tract. MDV virions are probably phagocytosed by phagocytic cells, and then transferred through B-lymphocytes from lung to lymphoid organs. **1)** Cytolytic infection of MDV initially affects lymphoid tissues, primarily B lymphocytes and a few T lymphocytes. Only activated T helper cells are targets for MDV invasion, likelydue to cell-mediated immune response. Cytolytic infection can be detected in spleen, bursa of Fabricius, and thymus, peaking at 3-6 days post infection. Ultimately there can be atrophy of the bursa and thymus. In this stage of MDV infection, chickens of susceptible and resistant strains are equally susceptible to infection (Calnek, 1973; Sharma, 1973; Witter et al., 1973). **2)** At about the 6th or 7th day post-infection, there is a switch to latency coincident with the development of immune responses, especially cell-mediated immunity (CMI) (Buscaglia et al., 1988). Most latently infected cells are activated T lymphocytes (Calnek et al., 1984).

Latently infected T-cells are dispersed to various organs and tissues via the circulatory system (Payne, 1985). Virus load in infected chickens is significantly reduced at this stage. However, by eliminating of MDV infected cells, permanent immunosuppression is created through loss of infected lymphocytes following cell-mediated immune response. **3)** Genetically resistant birds do not progress past the second stage (latency). However, genetically susceptible birds develop a second wave of cytolytic infections after 2 or 3 weeks, and coincident with permanent immunosuppression (Calnek, 1973; Calnek and Hitchner, 1969; Lee et al., 1981). The lymphoid organs are again involved, as well as tissues of epithelial origin in various visceral organs. The extent of infection during this phase is an important factor which influences the incidence of tumor formation. The most widespread and intense infections occur in genetically susceptible chickens. **4)** The molecular mechanisms in MDV mediated T-cell transformation are still not clear.

C. Gross lesions

Clinical signs of MD usually appear at 3 to 4 weeks post infection. Nerve lesions are the most constant finding in affected birds. Depending on the strain of MDV, lymphoid tumors may occur in one or a variety of organs. Besides neoplastic lesions, MD induces severe atrophy of the bursa of Fabricius and thymus as well as degenerative lesions in the bone marrow and various visceral organs (Jakowski et al., 1970). These are the results of intense cytolytic infections, which can result in death of chickens at an early age, before lymphomas have developed.

There are two pathological forms of MD: classical and acute (Payne, 1985). The classical form of MD is characterized by impairment of neural function and cytolytic

infection which is caused by mild-strain serotype 1 MDV infection. The infected chicken displays visible signs of nerve function impairment, beginning with partial and ending with complete paralysis. A common characteristic of classical MD is uneven gait or one leg stretched forward and the other leg stretched backward (Purchase, 1985). This classical form of MD was historically considered the most common in the field leading to the name "R? paralysis". Presently, however, there is a higher prevalence of the acute form of MD which is caused by virulent or very virulent strains of serotype 1 MDV infection (Payne, 1985). The acute form of MD is marked by T-lymphoma formation. Lymphomas are frequently found in different visceral organs in acute forms of MD, and sometimes occur even without gross nerve lesions (Calnek and Witter, 1991).

4. Biology of Marek's disease virus

A. Virion structure

The MDV virion, like other herpesvirus, consists of four elements: **1)** a protein core wrapped with DNA; **2)** an icosahedral capsid with 162 capsomeres surrounding the core; **3)** an amorphous tegument surrounding the capsid; and **4)** an outer envelope with external glycoprotein spikes on its surface (Schat, 1985a).

In MDV infected tissue cultures, most virions are hexagonal naked particles or nucleocapsids, 85-100 nm in diameter, and are usually found aggregated in the nucleus and occasionally in the cytoplasm. Only a few enveloped particles, approximately 130-170 nm in diameter, are observed budding from inner nuclear membranes of the infected cell (Nazerian et al., 1968; Hamdy et al., 1974). However, in MDV infected chickens, large

numbers of cytoplasmic enveloped virions (270-400 nm in diameter) are observed in feather follicle epithelium (FFE) which is the only location capable of producing fully infectious cell-free MDV virions.

B. Serotypes and pathotypes of MDV

Based on agar gel precipitation (AGP) and indirect immunofluorescent assays (IFA), MDV is classified into three serotypes which are correlated with their biological properties (Bulow and Biggs, 1975a). This serotypic classification has been confirmed through the use of type-specific monoclonal antibodies (Ikuta et al., 1982; Lee et al., 1983). Serotype 1 MDV includes all oncogenic viruses and their attenuated derivatives. Serotype 2 MDV is naturally occurring non-pathogenic MDV isolated from chickens. Serotype 3 MDV is the non-pathogenic herpesvirus of turkey (HVT) (Bulow and Biggs, 1975b).

With regard to their oncogenic potentials, serotype 1 MDV is subdivided into different pathotypes: mild MDV (mMDV), virulent MDV (vMDV), and very virulent MDV (vvMDV) (Witter, 1983 and 1985). Mild strains such as CU2 can cause tumors only in a few very susceptible chickens (Schat et al., 1985b). Virulent stains can cause high incidence of MD in genetically susceptible but not in resistant birds, e.g. JM and GA strains. Very virulent strains, such as MD5 and RB1B, can cause high incidence of MD in all chickens except vaccinated birds from genetically resistant lines, or birds protected by bivalent or trivalent vaccines (Witter, 1985).

C. MDV isolation and cultivation

MDV is present in a cell-associated form in many tissues of MDV-infected chickens, for example, kidney, liver, blood, and lymphoma (Philips and Biggs, 1972). The FFE is the

only tissue that produces cell-free MDV. Primary isolates of MDV can be propagated on chicken embryo fibroblast (CEF), duck embryo fibroblast (DEF), and chicken embryo kidney (CEK) cells. MDV produces cytopathic plaques, a characteristic of herpesviruses, within a few days after inoculating onto tissue culture monolayer (Churchill, 1968; Solomon et al., 1968; Nazerian, 1970). Attenuated virus can be produced from continuous cultivation.

Lymphoblastoid cell lines have been developed from MD lymphomas (Akiyama et al., 1973). These cells grow continuously in cell culture, have T-cell markers (e.g. CD4) and MDV-associated tumor surface antigens (MATSA). Most lymphoblastoid cell lines can be termed "producer" cell lines because a small proportion (1-2%) of their cells can enter into semi-productive infection (Powell et al., 1974). Virus can be readily recovered from most cell lines by co-cultivation with primary CEF.

Recently, a chemically immortalized chicken embryo fibroblast cell line, OU2, has been used for MDV infection. The MDV OU2.2 cell line was generated by infection with MD11 strain low passage viruses. Like lymphoblastoid cell lines, MDV OU2.211.1 cells are capable of transferring MDV infection to primary CEF monolayer and inducing T-lymphoma formation in chickens. Interestingly, MDV can undergo transition from latent infection to lytic infection when these cells grow from subconfluent to confluent in tissue culture (Abujoub and Coussens, 1995 and 1997).

5. Molecular biology of MDV

Comparing with other herpesviruses, research on MDV gene expression and regulation are still far behind. This is partly due to the cell-associated nature of MDV

infection in tissue culture systems. However, information from well-characterized herpesviruses, especially herpes simplex virus (HSV), has greatly facilitated MDV studies.

A. Common themes of herpesvirus gene regulation

In this section, the review will cover common features of herpesvirus gene regulation, primarily through current understandings of HSV-1 gene regulation.

Analysis of genomic diversity among herpesviruses revealed at least three common features of herpesvirus gene regulation.

First, herpesviruses share patterns of gene expression during the lytic phase of viral infection, which means their genes are all regulated in a cascade fashion (Wagner, 1992). There are three kinetic groups of genes in herpesvirus: immediate-early (IE, or alpha α) genes, early (E or beta β) genes, and late (L or gamma γ) genes.

Second, herpesviruses are all able to establish and maintain a latent state of infection at a specific physiological site within an immunocompetent host. The latent phase of infection leads to a close and lengthy association between viral and host cell genomes (Roizman and Sears, 1996). In the latent phase of infection, only a limited group of viral genes are expressed. The mechanism of the switch between lytic and latent phase gene expression by herpesvirus is still unclear.

The third common feature of herpesviruses is that herpesvirus genomes are promoter rich. Generally, the expression of a given protein is mediated by a specific promoter mapping at that gene (Honess, 1984). Therefore, it is an exception rather than a rule that herpesviruses express long multigene transcripts. This means that there is no strict constraint

on genomic order of genes. Therefore, during virus evolution, the content of viral genes rather than the position of these genes in the genome has been selected.

Herpes simplex viruses (HSV) is the first human herpesvirus discovered, and one of the most intensively investigated of all viruses (Roizman and Sears, 1996). The number of predicted HSV-1open reading frames is 72, and proteins from about 50 of these genes are readily detected (McGeoch et al., 1985 and 1988) in infected cells. Regulation of the three kinetic group genes in HSV lytic infection,is presented in Figure 1.1 (Roizman and Sears, 1996).

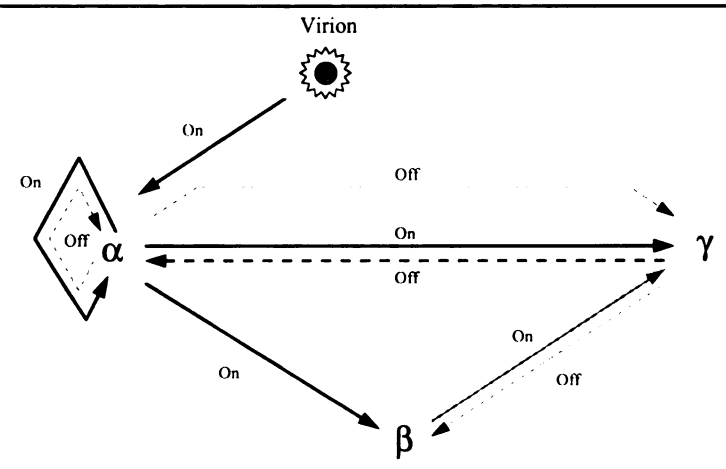


Figure 1.1. The cascade gene regulation of three HSV-1 kinetic groups (α, β and γ).

The α genes are the first group to be expressed. There are five α proteins, ICP0, ICP4, ICP22, ICP27, and ICP47. The α genes are expressed immediately upon infection and their transcription does not require de novo viral protein synthesis. The synthesis of α proteins reaches peak rates at approximately 2 to 4 hours post-infection, the α proteins continue to accumulate at nonuniform rates until late in infection (Honess and Roizman,

1974; Ackermann et al., 1984). All α proteins, with the exception of ICP47, have been shown to have regulatory functions, and α gene products are required for subsequent activation of β and γ genes, as well as autoregulation of α genes themselves (Roizman and Sears, 1996).

The β genes are the next group of genes expressed. Without α proteins, β gene expression is very inefficient. The appearance of β proteins signals the onset of viral DNA synthesis, and most viral proteins involved in viral nucleic acid metabolism are encoded by β genes (Roizman and Sears, 1996).

The γ genes encode all structural proteins and one IE gene transactivator (VP16, viral protein 16) (Roizman and Sears, 1991). Some γ genes are expressed late in infection only after viral DNA synthesis. The others are expressed relatively early in infection and are less influenced by viral DNA synthesis.

B. MDV genome structure

MDV DNA is a linear, double-stranded molecule with a molecular weight of 108-120 x 10^6 daltons (Cebrian et al., 1982; Hirai et al., 1980; Lee et al., 1971). This molecular weight is equivalent to a size of 166-184 kilobase pairs per genome. The density of MDV DNA in neutral $CsCl_2$ is 1.705 g/ml, close to that of chicken cell DNA (Adldinger and Calnek, 1973). Interestingly, it has been demonstrated that MDV genomic DNA alone is infectious both in vitro and in vivo (Kaaden, 1978).

Primary investigation of MDV genomes by restriction enzyme analysis revealed that the digestion patterns of several strains within serotype 1 were very similar (Hirai et al., 1979 and 1981; Kaschka-Dierich et al., 1979; Ross et al., 1983). However, viruses among

serotype 1, 2 and 3 have unique restriction enzyme patterns (Hirai et al., 1979; Kaschka-Dierich et al., 1979; Ross et al., 1983). In spite of antigenic similarities among three serotypes, there was little homology between the three serotypes as determined by reassociation kinetics experiments under stringent hybridization conditions (Hirai et al., 1979; Kaschka-Dierich et al., 1979; Ross et al., 1983; Lee et al., 1979).

Despite significant differences in DNA sequence among three MDV serotypes, the structure of all MDV genomes can be divided into unique long and unique short regions (U_L, U_S), flanked by terminal repeat and inverted repeat regions (TR $_L$IR $_L$ IR $_S$TR $_S$ respectively) (Figure 1.2) (Cebrian et al., 1982; Fukuchi et al., 1984). The genome structure of MDV

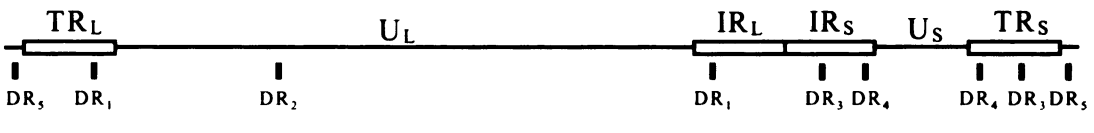


Figure 1.2. The genome structure of MDV.

belongs to the *Herpesviridae* group E genome family (Cebrian et al., 1982). Based on the lymphotropic feature of MDV which is similar to that of Epstein-Barr virus (EBV), MDV was originally classified as a γ-herpesvirus (Roizman and Sears, 1991). However, the genome structure and gene arrangement of MDV are more similar to that of α-herpesviruses, such as herpes simplex virus (HSV) and varicella-zoster virus (VZV) (Buckmaster et al., 1988; Roizman et al., 1992; Karlin et al., 1994). The gene co-linearity between MDV and HSV has led to re-classification of MDV as an α-herpesviruses (Roizman, 1992).

In addition to terminal repeats flanking U_L and U_S regions, there are five direct repeat (DR) sequences. These DR sequences are located within the internal or terminal repeat regions (Hirai, 1988), Figure 1.2. DR1 is a tandem direct repeat of a 132 bp repeat unit located within the TR_L and IR_L of *BamHI* D and H, respectively (Maotani et al., 1986). Copy number of the 132 bp repeat unit within TR_L and IR_L regions of oncogenic MDV strains is one to three each, while that of attenuated derivatives is 3 to 100 (Maotani et al., 1986). These findings suggested that low copy number of 132 bp repeats may be necessary or sufficient for induction and maintenance of oncogenic transformation by MDV, while high copy number of DR1 repeats is associated with adaption to tissue culture and attenuation with respect to oncogenicity in vivo. DR2, DR3 and DR4 are 1.4 kilo bp, 178 bp and 200 bp repeats, respectively (Fukuchi et al., 1984; Hirai et al., 1984). These three repeats have not been associated with oncogenicity. DR5 is a putative terminal direct repeat at each end of the MDV DNA molecule. DR5 may contain signals for cleavage of replicative-form genomes to yield virion DNA.

C. MDV gene expression

Like other herpesviruses, MDV gene expression is regulated in a cascade fashion (Maray et al., 1988). Three major kinetic classes of MDV genes are expressed: IE, E and L genes. The close relationship of MDV genome structure with that of HSV-1 genome (Buckmaster, 1988) has significantly facilitated MDV gene expression studies. Many HSV-1 gene homologies have been found in the MDV genome, in all three kinetic classes. Despite their overall similarities, however, not all details concerning the precise mechanism involved in control of MDV lytic cycle processes are the same as HSV-1. Unique MDV

proteins that likely contribute to these differenceshave been found (Silva and Lee, 1984; Hong and Coussens, 1994). This review will be focused on MDV regulatory proteins.

IE genes are expressed immediately after infection. This group of genes does not require de novo viral protein synthesis and hence IE mRNAs are synthesized in the presence of metabolic inhibitors such as cycloheximide (CHX). By CHX treatment, numerous IE transcripts have been detected in cells lytically infected with MDV and MDV lymphoblastoid cell lines (Maray et al., 1988; Schat et al., 1989). However, all these reports are based only on Northern hybridization analysis, without exact genes and gene products being identified.

Recently, several MDV IE genes have been reported and three of these are homologues to HSV-1 ICP4, ICP27 and ICP22 (Anderson et al., 1992; Ren et al., 1994). A MDV unique IE gene, pp14, has been identified within the BamHI I2 fragment of the MDV genome (Hong and Coussens, 1994). The pp14 gene encodes a product with a molecular weight of 14 kDa and is expressed in cells lytically infected with oncogenic MDV strains, or their attenuated derivatives, as well as in latently infected and transformed cell lines. The function of pp14 is unclear at this time.

Early genes are the next group expressed in MDV lytic infection, and their synthesis requires the activity of at least one IE protein. Early gene encoded proteins are required for nucleotide metabolism and viral DNA synthesis. Therefore, in the presence of drugs that block viral DNA synthesis, such as phosphonoacetic acid (PAA), early gene expression is enhanced rather than reduced.

Several MDV early genes homologous to those of HSV-1 have been identified.

These include thymidine kinase (TK), DNA polymerase, and origin binding protein (UL9) (Buckmaster et al., 1988; Wu et al., 1996). A unique MDV gene, phosphoprotein 38 (pp38), has also been identified within the BamHI H fragment and spans the junction of MDV U_L and IR_L regions. MDV pp38 was originally identified as an MDV serotype 1 specific antigen (Silva and Lee, 1984). Recently, however, it was reported that MDV serotype 2 and 3 contain pp38-related polypeptides that have epitopes or polypeptides homologous to MDV serotype 1 pp38 (Cui et al., 1992).

Expression of herpesvirus late genes usually requires both viral protein synthesis and viral DNA replication. Late genes encode structural proteins required for virion assembly and an IE gene transactivator, VP16 (Roizman and Sears, 1991). Seven MDV late genes homologous to HSV-1 have been identified, including gB, gC, gD, gE, gI, gK and VP16 (Silva et al., 1984; Ikuta et al., 1983; Buckmaster et al., 1988; Chen and Velicer, 1992; Coussens and Velicer, 1988; Isfort et al., 1987; Ross et al., 1989 and 1991; Brunovskis and Velicer, 1992).

III. Avian leukosis virus

1. History of avian retrovirus research

Avian retrovirus research began in 1908 when Vilhelm Ellerman and Oluf Bang demonstrated that chicken leukemia could be transmitted to recipient birds by cell suspensions and cell-free filtrates from tissues of a chicken with myeloblastosis. On the basis of biological properties and genome structure, the avian leukosis/sarcoma viruses fall into two groups; the slowly or weakly transforming viruses, ALV, that mainly cause lymphoid leukosis with a long clinical-latency period, and the rapidly transforming avian sarcoma viruses (ASVs). Most ASVs are replication-defective, producing infectious progeny only in the presence of a helper virus, while the slow-acting ALVs are replication-competent. Early studies generally agreed that avian retroviruses were multipotent; that is, they induced a spectrum of diseases according to different virus dosage and various genetic background of the host animal. For example, studies with cloned virus stocks isolated from replication-competent ALVs could cause a variety of neoplasms in addition to lymphoid leukosis, including nephroblastoma, erythroblastosis, and sarcomas (Smith and Moscovici, 1969; Biggs et al., 1973; Purchase et al., 1977).

The existence of both vertical and horizontal ALV transmission in flocks of chickens was established by Burmester and colleagues in the early 1950s (Burmester et al., 1957). Later, the role of immunity and immunological tolerance in the spread of ALV infection was clarified (Rubin et al., 1961 and 1962). Congenitally infected chickens frequently are immunologically tolerant to the virus, develop viremia that persists for life, and shed large

amounts of virus in secretions and excretions. These congenitally infected shedders become the principal source of horizontally transmitted virus.

Lymphoid leukosis, the most common retrovirus-induced neoplasm in birds, is induced by ALV infection. ALV, however, contains no oncogene was found in ALV genome, and the mechanism of ALV tumorigenesis remained obscure until 1981, when Hayward et al. found the ALV integration sites in the proto-oncogene c-myc locus among bursal lymphomas. In this case, presence of an ALV promoter in front of c-myc gene coding region, resulted in an enhanced expression of c-myc gene. The high level of c-myc gene expression presumably accounts for B-cell transformation, which results in a high proliferation rate of ALV-infected cells. Preneoplastic lesions generated by ALV-transformed B-cells are necessary but not sufficient for malignant tumor formation.

A second c-onc gene, *Blym,* unrelated to c-myc or any other known v-onc gene, was isolated from bursal lymphomas (Cooper and Neiman, 1981). Since lymphoma- genesis seems to be a multi-step process, it has been suggested that activation of these two genes may be involved in different stages of tumor formation (Hayward et al., 1981; Cooper and Neiman, 1981; Goubin et al., 1983).

2. Pathology of ALV

A. ALV pathogenesis

Under both natural and experimental conditions, ALV-induced LL can be found 14 weeks post neonatal infection, and ALV-infected chickens reach peak mortality at 20 to 24 weeks of age (Purchase, 1987). Chickens are usually infected by ALV congenitally or

shortly after hatching (Crittenden, 1981). It is likely that mating and vaccination procedures play a role in disseminating the virus. Primary amplification of virus results in a viremia, which occurs at the same time as many non-neoplastic lesions. Establishment of viremia may be delayed by maternal antibody. The virus may persist in white blood cells for prolonged periods (Mass et al., 1982). Preneoplastic changes can first be observed in individual bursa follicles as early as 4 weeks after experimental inoculation at one day of age. By 7 weeks, most chickens may have one or more abnormal follicles (preneoplastic lesions) (Cooper et al., 1968). Many follicular lesions regress, a result of active immune response. However, there is always a few aggressive-growth follicles which can survive and progress to malignant tumor. Metastasis of the proliferating B-cells to other organs may occur. Under field and laboratory conditions, where low virus doses transform only a few target cells, tumors are usually monoclonal. On the other hand, experimental conditions using high doses of virus result in multiple transformation events, and neoplasms are polyclonal (Smith et al., 1980).

B. ALV gross lesions

The target cell for ALV-mediated transformation is a post-bursal stem cell (Purchase and Gilmour, 1975). Any treatment that destroys the target cell prior to transformation effectively prevents the development of LL (Peterson et al., 1966). Chickens dying of LL have gross or microscopic tumors in the bursa in almost every case. Death usually results from organ dysfunction. Grossly visible tumors may develop in the bursa, liver, ovary, spleen, kidney, and other visceral organs, with the most visible tumors occuring in liver. Tumors are usually soft, smooth, and glistening, and on the cut surface are creamy white

(Purchase, 1987).

C. Factors affecting the pathogenic response

Most ALV strains are capable of generating more than one kind of tumor. The types of tumor produced under specified conditions are characteristic of virus isolate or strain, and are referred to as the "oncogenic spectrum" of the virus. Conditions that affect the oncogenic spectrum include the virus strain, inoculation dose, inoculation route, age of host, genetic make-up of the host, sex of the host, and various environmental factors (Payne, 1987).

3. ALV genome structure and viral replication

ALV virions consist of two identical subunits of a single-stranded RNA molecule (Kung et al., 1976; Mangel et al., 1974). A genetic map of the non-acute retrovirus is depicted in Figure 1.3a. ALV genomes contain four viral genes: **1)** *gag* encodes group-specific antigens which are major components of the nucleocapsid, and some of these proteins are involved in packaging RNA genomes into the virion; **2)** *pro* encodes the protease (PR) responsible for the cleavage of the *gag* and *pol* polyproteins, and sometimes part of *env*; **3)** *pol* encodes two enzymes, reverse transcriptase (RT) and integrase (IN), involved in the synthesis of viral DNA and its insertion into the host genome; **4)** *env* specifies the envelope glycoproteins essential for viral attachment to the host cell. These proteins determine the host range and interference patterns of the virus. The RNA genome is bounded by a short repetitive sequence, r. The regions next to r at the 5' and 3' end of the genome are designated, respectively, u5 (unique 5' sequence) and u3 (unique 3' sequence).

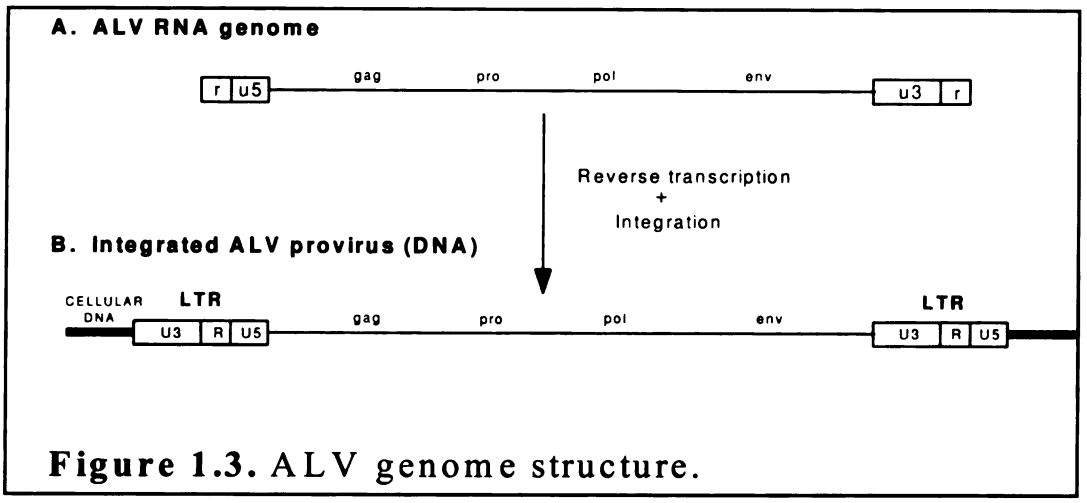


Figure 1.3. ALV genome structure.

In ALV, these terminal sequences do not contain any protein-coding information, but do contain critical transcriptional regulatory signals.

Upon infection, the RNA genome is first converted into double-stranded DNA by reverse transcriptase. During this process, the terminal sequences of the RNA genome, namely R, U3 and U5, are duplicated at both ends of the DNA, Figure 1.3b. The duplicated sequences are usually referred to as long terminal repeats (LTR). These sequences appear to be required for the insertion of viral DNA into the host genome. Only a fraction of the linear DNA is then circularized and inserted at random sites into the host genome. The insertion is aided by a specific cleavage at the LTR-LTR junction by an endonuclease activity associated with the integrase. Cleavage is followed by a recombination event with the host genome. Insertion sites in the host genome do not appear to be sequence specific but, also, are not totally random but are distributed throughout the host genome. Analysis of "favorite" virus integration sites indicate that chromosome structure rather than DNA sequence is the more critical factor determining site of integration. Retroviruses target transcriptionally-active regions in chromosomes, a process called semi-random integration.

The integrated viral DNA, referred to as a provirus, is transcribed by host RNA polymerase II to generate genome-sized RNA for viral replication and spliced mRNA for producing viral antigens. Virion assembly proceeds by incapsidation of the genome by unprocessed precursors of the *gag, pro* and *pol* genes, association of the nucleocapsids with the cell membrane, release of the virion by budding, and processes the precursors to the finished products. The entire cycle of viral replication is summarized in Figure 1.4, and includes attachment, penetration, reverse transcription, transferring to nucleus, viral integration, viral gene expression, viral protein synthesis, virion assembly, budding and proteolytic processing of capsid proteins.

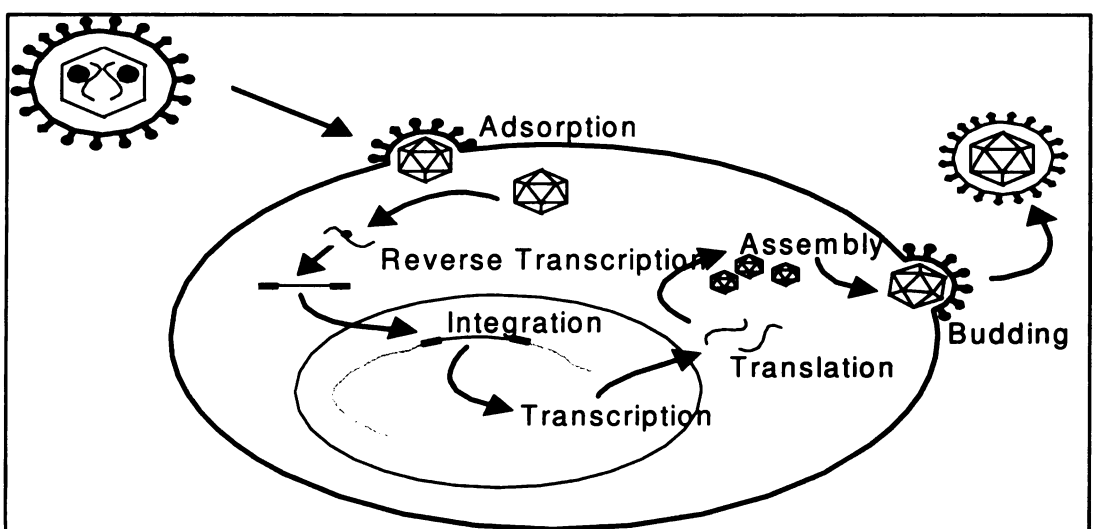


Figure 1.4. ALV Life Cycle.

4. Mechanisms of oncogene activation

ALVs do not carry oncogenes of their own. As a result, ALV can induce tumors only

after a long clinical-latent-period in its host (Tsichlis and Lazo, 1991; Fan, 1994; van-Lohuizen and Berns, 1990). Close examination of the virus-cell relationship in such tumors reveals a striking result: virtually all tumors have a provirus inserted in a similar portion of the genome, specifically, within the proto-oncogene c-myc (Hayward et al, 1981). In the majority of tumors, the myc-associated provirus is similarly located. The provirus almost always lies within an intron between the first (noncoding) and the second exon in the same transcriptional orientation, Figure 1.5. The effect of this insertion is to bring the two coding exons of c-myc under transcriptional control of the 3' LTR. Transcripts encoding c-

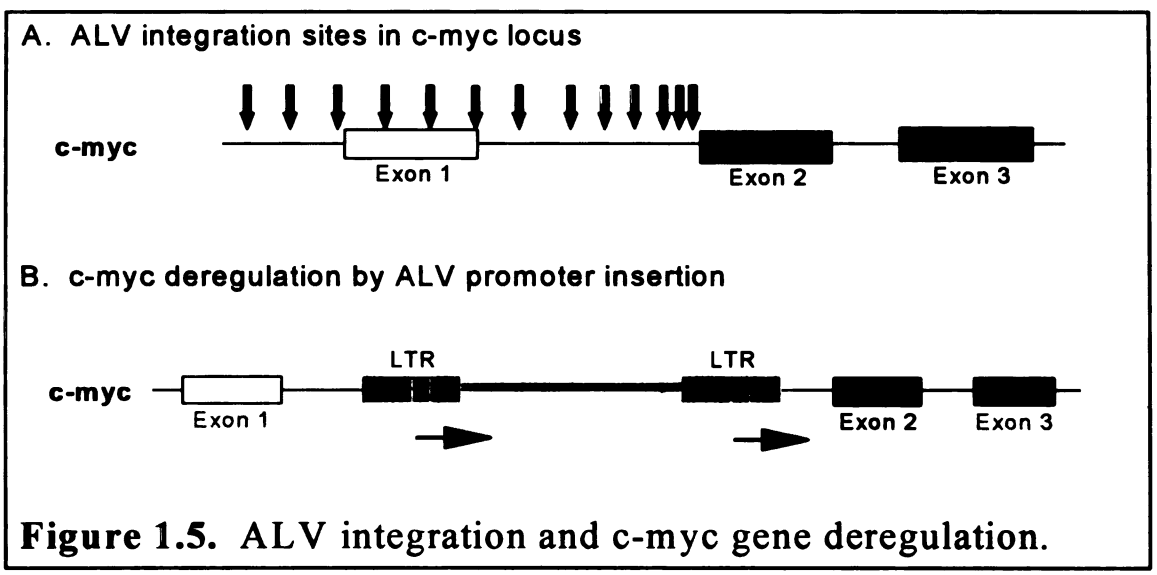


Figure 1.5. ALV integration and c-myc gene deregulation.

myc are found at higher levels than normal and are structurally different in that they containthe ALV R-U5 sequence derived from the LTR at their 5' ends. Thus, synthesis of these transcripts must be initiated at the promoter sequence within the 3' LTR. This mechanism of activation of c-myc by ALV is usually referred to as promoter insertion. Interestingly, the promoter insertion mechanism observed in ALV-induced lymphomas is

relatively uncommon for other retroviruses. More common mechanisms of oncogene activation by retrovirus include enhancer insertion, leader insertion, and terminator insertion (Coffin, 1996).

It should be noted that insertion of a provirus in such a position as to create this sort of damage is quite rare on a per-cell basis. However, considering the total number of infected cells in the target organ, this type of insertion is more frequent when the whole organ is considered..

Curiously, structural analysis further reveals that a significant portion of these proviruses carry deletions at their 5' ends, usually extending into the 5' LTR (Neel et al., 1981; Payne et al., 1982; Fung et al., 1981). In the extreme case, a solo LTR is present in association with the c-myc locus (Westaway et al., 1984). In ALV pathogenesis, there is an interval of several months between the earliest presumed c-myc activation (in bursal follicular hyperplasia) and the development of the malignant tumor. Generally, only one out of a large number of hyperplastic follicles survives attacks by host immune response. Most follicles regress, and many cells transformed by c-myc activation are eliminated. Obviously, the regression of hyperplastic follicles and the lag of tumor formation reflect an extensive selection among a variety of ALV-transformed clones. Two hypotheses have been proposed. The first hypothesis states that deletion of viral genes or disruption of virus transcription by the 5' LTR, would facilitate tumor progression by removing immunogenic surface viral antigens (Neel et al., 1981). However, B-lymphoma tumors and cell lines derived from them, which release viruses (presumably due to the presence of a second intact provirus inserted at a distinct chromosomal site), do exist (Payne et al., 1982; Fung et al., 1981). The

second hypothesis is that transcription promoted by the 5' LTR may interfere with promotion of the c-myc gene by the downstream 3' LTR, a phenomenon similar to that described as "promoter-occlusion" in procaryotes (Adhya and Gottesman, 1984). Indeed, plasmids with both the 5' and 3' LTRs linked to test genes in an in vitro transient expression assay revealed that presence of the 5' LTR-promoted transcription did down-regulate 3' LTR-promoted transcription, supporting with the second hypothesis (Cullen et al., 1984).

Although correlation between disturbances in the c-myc gene and induction of B-lymphomagenesis is now well established, the role of the c-myc gene product in this process remains obscure. In the early 1980's, a second cellular gene, *ChBlym-1,* was found in ALV induced B-lymphomas which also transformed NIH-3T3 cells (Cooper and Neiman, 1980 and 1981). This finding was consistent with the concept that development of lymphoid leukosis is a multistage process. *Blym-1* activation has been demonstrated in Burkitt's lymphoma, a B-lymphoma also caused by c-myc activation following Epstein-Barr virus (EBV, a human herpesvirus) infection (Diamond et al., 1983). *ChBlym-1* has been cloned and sequenced. It encodes a small 8 kDa polypeptide with partial homology to the amino-terminal domain of secreted forms of the transferrin family proteins (Goubin et al., 1983). The role of *Blym-1* in lymphomagenesis and its relation to c-myc activation are unknown.

5. Rous sarcoma virus long terminal repeat (RSV-LTR) promoter

The RSV-LTR is highly related by sequence to ALV-LTRs, only a few small deletions and several point mutations differentiate it from the ALV-LTR (Ruddell, 1995).

Evidence from promoter functional studies indicate that RSV-LTR and ALV-LTR are highly related. A recombinant virus that contained the Schmidt-Ruppin (SR) strain RSV-LTR on an otherwise leukosis virus genome induced a pattern of B-cell lymphoma analogous to that observed with ALV (Hughes et al., 1986). This result suggested that the modest sequence variations between these two viruses do not affect their disease spectrum. The functional similarity between these two promoters supports the theory that ALVs are the progenitor viruses that gave rise to the acute retroviruses (like RSV) through recombination with host oncogenes (Bishop, 1983; Varmus, 1983). RSV-LTR serves as an attractive model system for studying transcriptional activation because of its strong promoter activity, details of RSV-LTR promoter are actually better known than for ALV-LTR promoters. Therefore, in this review, RSV-LTR will be used to present general concepts of ALSV group gene regulation.

The major function of the LTR is to provide signals recognized by cellular transcription machinery for efficient expression of the provirus (Majors, 1990). Transcription of the provirus is initiated at the site of U3-R junction (or cap site). Transcripts often proceed through the 3' LTR into flanking cellular DNA. Sequences within the 3' end of R provide signals for mRNA cleavage and poly(A) addition. All retrovirus genomes are synthesized by RNA polymerase II, the same enzyme responsible for synthesis of cell mRNA. The retrovirus promoter, LTR, contains recognition sequences that are clearly identifiable with cellular transcriptional factors. However, these binding sites often exist in complex combinations (ref.).

Transcriptional activity of RSV-LTR promoter depends on two types of elements:

the core promoter and the enhancer. The core promoter includes a TATA box at -30 bp upstream of transcription start site and an Inr (initiation recognition) site right on the transcription start site. The RSV-LTR enhancer has been functionally defined by deletion mutagenesis (Luciw et al., 1983; Laimins et al., 1984; Cullen et al., 1985a) and enhancer trap experiments (Weber and Schaffner, 1985) to encompass a region from -229 to -54 bp upstream from the start site of transcription. Within this region, four sequence-specific DNA-binding factors have been described, and these are called EFI (enhancing factor I) (Sealey and Chalkley, 1987), EFII (Sealey and Chalkley, 1987), EFIII (Boulden and Sealy, 1990), and EFIV (Houtz and Conklin, 1996), Figure 1.6.

EFI. EFI recognizes two different inverted CCAAT motifs in the RSV-LTR, one

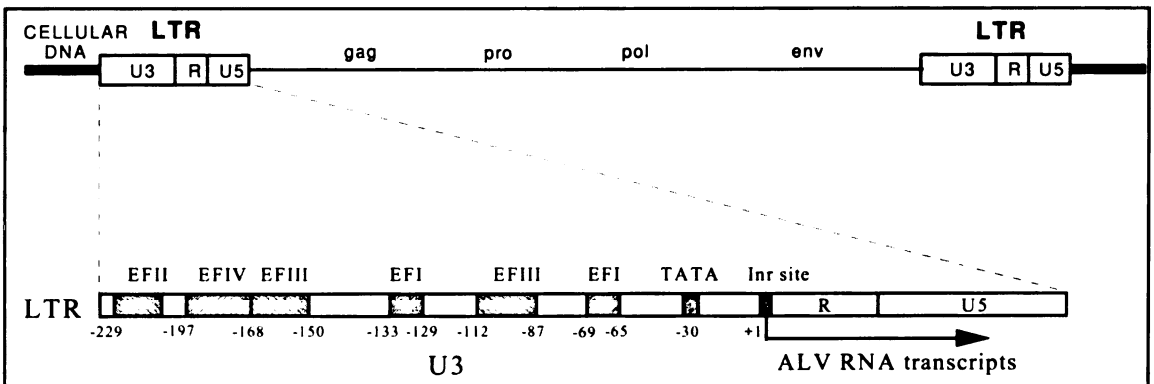


Figure 1.6. RSV-LTR promoter and transcriptional factors DNA-binding sites.

occurring at -129 bp to -133 bp (Sealey and Chalkley, 1987) and the second at -65 bp to -69 bp with a lower binding affinity (Faber et al., 1990). EFI factor is thus a member of the CCAAT transcription factor family. EFI factor can bind to the DNA binding sites of another CCAAT family member, CBF (core binding factor), with greater affinity. However, the

affinity associated with C/EBP (CCAAT enhancer binding protein) binding-site is 10-fold lower than recognizing its own binding site (EFI) (Faber et al., 1990). This suggests that EFI factor is more closely related to CBF than C/EBP. Even two forms of EFI DNA binding activity exist in nuclear extracts of avian cells, both forms of EFI give rise to the same mobility shift in gel retardation assays (Ozer et al., 1990). However, the two forms can be separated chromatographically under buffer conditions that stabilize the two DNA binding activities (Ozer et al., 1990). One form requires two heterologous components (EFIa)(EFIb) for high affinity DNA binding, whereas a second form is not dependent on EFIb for binding. The second form of EFI may be composed solely of EFIa, perhaps as a multimeric complex (Ozer et al., 1990). A cDNA for EFIa was first isolated from a rat liver cDNA expression library (Ozer et al., 1990), but has now been isolated from a chicken liver expression library (xxxx, 1994). The 1489 base pair EFIa cDNA encodes a 322-amino acid protein which is nearly identical to two previously described human DNA binding proteins, YB-1 (Didier et al., 1988) and dbpB (Sakura et al., 1988). With a combination of SDS-PAGE fractionation and mobility gel shift assay, EFIa and EFIb have been estimated in the range of 43-60 kDa and 41-48 kDa, respectively (Faber et al., 1990).

EFII. EFII cis-element is a 38-bp sequence located from -229 to -192 of the RSV-LTR transcription start site. Within this region, at least eight nucleotides (-201 to -208) are required for maximal enhancer activity (Sealey et al., 1987; Sears et al., 1992). Three heat-stable protein complexes, referred to as EFIIA, EFIIB, and EFIIC, have been identified and specifically bind the EFII DNA sequence in vitro. All three forms of EFII appear to recognize the same nucleotides within EFII binding sites (Sears et al., 1992). C/EBPβ is a

major component of these three EFII DNA binding complexes. Three different forms of C/EBPβ, p42, p35, and p20, can bind the EFII DNA sequence as homodimers and heterodimers. Dimerization experiments suggest that EFIIa is a homodimer of p20 C/EBPβ; EFIIb is primarily composed of a p20/p35 heterodimer with minor amount of p20/p42 heterodimer and p35 homodimer; EFIIc is composed of p20 and/or p35 heterodimerized with a novel 60 kDa protein (Sears et al., 1994).

EFIII. EFIII factor recognizes two sites, -175 to -150 and -112 and -87, which contain a common sequence motif known as the CArG box. By its sequence-specific DNA-binding properties and antibody recognition, EFIII has been shown to represent the avian homolog to the serum response factor (SRF) (Boulden et al., 1990).

EFIV. EFIV factor interacts with a 30 bp region from -197 to -168 relative to the transcriptional start site (Houtz et al., 1996). Two copies of "GCAACATG" and sequences in between are important. Gel shift competition assay suggests that the EFIV protein(s) may be related to members of the C/EBP (CCAAT/enhancer binding protein) family of transcription factors that interact with different regions of the RSV-LTR (Houtz et al., 1996).

III. Interaction between retrovirus and herpesvirus

Retroviruses and herpesviruses are two common pathogens found in most animals, including human beings. In the early 1970's, the synergistic effect generated by co-infection with these two kinds of viruses, ALV and MDV in this case, was reported in avian system. ALV and MDV have been shown to affect the pathogenesis of both viruses in the avian systems (Peter et al., 1973; Campell et al., 1978 and 1979). In the mid 1980's, retrovirus/herpevirus interactions received new attention as many reports described a very high frequency of human retrovirus and herpesvirus co-infection in patients afflicted with AIDS (Koening et al., 1986; Salahuddin et al., 1986; Nelson et al., 1988). Several epidemiological studies suggested that herpesvirus infection can either increase an individual's susceptibility to infection by human immunodeficiency virus (HIV), or accelerate the progress of AIDS (Drew et al., 1984; Spector et al., 1984; Salahuddin et al., 1986). In this section, the review will focuse on summarizing the latest research progress from both human and avian systems. At least one common feature of these interactions is that all these herpesviruses affect with retrovirus-pathogenesis by modifying retrovirus LTR promoter activities.

1. Human retrovirus and herpesvirus interactions

AIDS, a chronic and lethal disease caused by HIV-1, and HIV-2 infection, is characterized by a profound deficiency in T-cell mediated cellular immune responses due to a drastic reduction in $CD4^+$ lymphocytes. The period between initial infection and the

development of the disease can span many years. During this time period, only very low levels of cells containing the HIV-1 provirus can be detected in blood. While in the highly infected secondary lymph nodes, replication is easily detected. Although infected cells were shown to contain proviral DNA, the HIV-1 provirus is not expressed. These data suggest that, in a high percentage of infected cells, HIV infections are latent (Embretson et al., 1993). The long incubation period, called clinical latency, as well as the high proportion of latently infected cells, has led to speculation that progression of AIDS might be affected by extracellular cofactors. AIDS patients have increased incidence of heterologous virus infections, herpesviruses in particular. Epidemiological studies have indicated that herpesvirus infection is one of the risk factors for concurrent or subsequent HIV-1 infection (Quinnan et al., 1984; Holmberg et al., 1988).

There are at least three mechanisms by which heterologous virus infections can influence the life cycle of HIV-1. The first is through a direct effect of herpesvirus gene products on the transcription and replication of HIV-1 provirus. This enhancement, which would result in an increase in HIV-1 expression and consequent release of infectious HIV-1 virions, requires simultaneous infection of cells with latent HIV-1 by a herpesvirus. Herpesviruses are known to express potent transcriptional transactivators. HSV-1, EBV, HCMV, VZV, and HHV-6 have all been demonstrated to activate HIV-LTR directed gene expression in vitro (Rando et al., 1987). The second mechanism is indirect; the stimulation of HIV-1 expression is mediated either directly by cytokines released from cells as a response to a herpesvirus infection, or by stimulation of resting T-cells with viral antigens. It was shown that activated T-cells not only support more productive HIV-1 infection than

resting T-cells (Zack et al., 1990), but activation can also stimulate the expression of latent HIV-1 provirus. Finally, there are a few instances where infection with herpesvirus was shown to alter cell tropism of HIV-1. It was shown that the phenotypic mixing between HIV-1 and HSV-1 can alter cell tropism of HIV-1 and allow infection of cells that are otherwise not infectable by HIV-1 (Zhu et al., 1990).

A. HIV-1/HSV-1

Clinically, the high incidence of HSV-1 infection in AIDS patients has been well established, although the connection between the progression of AIDS and HSV-1 infection is still difficult to assess. Even if the in vivo encounter between HIV-1 and HSV-1 is rare, HSV-1 is currently the best characterized herpesvirus and represents a valuable model for studies on interactions between HIV-1 and the herpesvirus group.

HSV-1 was the first herpesvirus shown to activate HIV LTR-directed gene expression, and activation occurs at the level of transcription (Gendelman et al., 1986; Mosca et al., 1987a and 1987b; Davis et al., 1987; Kenney et al., 1988; Horvat et al., 1989). It has been reported that there are several pathways mediating HSV-1 induced HIV-LTR transactivation. **1)** Like other viruses, HSV-1 can transactivate HIV-LTR by inducing high levels of NF-*k*B which is a typical outcome of early inflammatory response upon HSV-1 infection (Mosca et al., 1987a). **2)** HLP-1 (histone-like protein type 1), another cellular factor which binds to three LBP-1 (leader binding protein type 1) binding sites in HIV-LTR, is induced in later stages of HSV-1 infection (Vlach and Pitha, 1992). Transactivation of HIV-1 LTR was observed in HSV-1 caused by HLP-1 induction (Vlach and Pitha, 1992). **3)** A 150 kDa HSV-1 origin protein (designated TAR150) was found in HSV-1 infected

cells with the binding site located within the DNA TAR region (Ostrove et al., 1987). Functionally, the binding of TAR150 does not seem to have significant effect on the activation of HIV-1 LTR. **4)** Two out of five HSV-1 IE genes (ICP0 and ICP4) have been shown to modulate transcriptional activities of HIV-1 LTR, while one of HSV-1 IE gene (ICP27) products affect mRNA levels at the post-transcriptional level (Everett et al., 1991). **5)** An interesting feature of ICP0 is its cooperation with Tat, a HIV-encoded transactivation protein, during transactivation of HIV-1 LTR. ICP0 can recruit Tat to the vicinity of HIV-1 promoter, thereby providing the alternative binding site for Tat with resulting synergistic transactivation (Chapman et al., 1991).

B. HIV-1/HCMV

A high percentage of persons with AIDS are infected with HCMV, and many are infected with multiple strains of HCMV (Drew et al., 1984; Spector et al., 1984). Cells frequently infected with HCMV, macrophages, endothelial cells, and glial cells, are also sites of HIV infection (Koenig et al., 1986). In vivo, HIV and HCMV have been shown to co-infect cells in the brain, retina, and lung (Nelson et al., 1988), and thus the potential exists for direct interactions between these viruses during their replication cycle.

Infection of a host with HIV and a second virus that induces immunoregulatory abnormalities, such as HCMV or EBV, might lead to decreased immune surveillance and enhanced replication of both viruses (Carney et al., 1981; Rinaldo et al., 1977; Tosato, 1987). Infection with HCMV may also result in tissue inflammation, attracting HIV-infected macrophages to the affected organ and spreading the HIV infection. Antigenic or mitogenic stimulation of the target cells for HIV could also accelerate the HIV infection. In this regard,

HCMV antigens can induce cytokines capable of activating HIV in chronically infected promonocyte and T-cell clones (Clouse et al., 1989). It has also been shown in tissue culture systems that Fc receptors induced on the cell surface of HCMV-infected fibroblasts facilitate the entry of antibody-coated HIV into these cells (McKeating et al., 1990). If this mechanism were to operate in vivo, it might allow HIV infection of CD4-negative cells.

As early as 1987, it was reported that HCMV infection, or recombinant constructs encoding the major HCMV IE genes IE1/IE2 or IE2 alone, could activate HIV-LTR promoter in transient expression assays (Davis et al., 1987; Mocsa et al., 1987a; Rando et al., 1987). Later, other studies have observed that there is also an opposite effect with co-infection of HCMV, which leads to a marked reduction of HIV expression (Henderson et al., 1991; Koval et al., 1991; Levy et al., 1990). The differential effects of HCMV on HIV-1 replication depend on cell type, multiplicity of infection, and relative permissivity of the cells for the two viruses (Koval et al., 1991). While it is generally agreed that the IE2 86 kDa protein is the major activator of the HIV-LTR, and that the function of the IE1 72 kDa protein remains debatable. Since the activation of the HIV-LTR by IE2 expression construct varied considerably in different cell types, it is believed that the interactions of the HCMV IE proteins with the HIV-LTR are complex and most likely involve protein-protein interactions with other cellular factors.

C. HIV-1/HHV-6

One major problems with herpesviruses mentioned above, which serving as possible co-factorsr in development of AIDS, is that none of those viruses infect $CD4^+$ T cells as their primary target. Thus, the in vivo relevance of these viruses to HIV-1 activation is unclear.

Recently, a T-cell tropic HHV-6 with close association to AIDS was discovered (Lusso, 1995). HHV-6 was first isolated in 1986 from the peripheral blood lymphocytes of patients with lymphoproliferative disorders and AIDS (Salahuddin et al., 1986).

Even though clinical data on the effects of HHV-6 in AIDS patients is still controversial, in vitro studies have shown that HHV-6 has drastic effects on HIV-1 replication and pathogenesis. The major target cell for HIV and HHV-6 is $CD4^+$ T cells, both in vitro and in vivo. A wide range of effects has been observed in lymphocyte cultures that are dually infected with HHV-6 and HIV. First, in 1989, Lusso et al. showed that the HCMV-HIV co-infected T-cells underwent on accelerated cell death program and increased HIV-1 viral expression as compared to HIV-1 only infected cells. Secondly, HHV-6 co-infection expanded the host range of susceptible cells for HIV. It has been shown that HHV-6 can induce expression of CD4 molecules in $CD4^-CD8^+$ cytotoxic T lymphocytes, thus rendering them susceptible to HIV infection (Lusso et al., 1991). Cytotoxic T-cells play an important role in the anti-viral immune response, therefore, elimination of these cells by HIV and HHV-6 infection would further disrupt the immune system. It has been shown that natural killer (NK) cells, which are important in immune surveillance, can also become targets for HIV-1 once infected with HHV-6 (Lusso et al., 1993). Apparently, HHV-6 infection induces expression of CD4 molecules in NK cells. However, interaction between HIV-1 and HHV-6 can also lead to the suppression of HIV. Experiments performed with different strains of HHV-6 and HIV revealed suppression of HIV replication in co-infected cells (Carrigan et al., 1990). Thus, HHV-6 can effect HIV-1 replication and pathogenesis by either enhancing or suppressing its replication depending upon virus strains and type of

host cell.

In order to understand the mechanism of interaction between HIV and HHV-6, it is important to identify the HHV-6 genes and the HIV target sites that are responsible for either activation or suppression of HIV gene expression. In vitro studies have shown that HHV-6 infection of T-cells results in an increase of HIV-LTR-controlled gene expression (Horvat et al., 1989; Ensoli et al., 1989). Ensoli et al. (1989) has also demonstrated that NF-*k*B enhancer is the cis-element on the HIV-LTR responsible for HHV-6 induced transactivation. Compared to other cellular factor binding to HIV-LTR, NF-*k*B seems to play a major role in HHV-6 mediated transactivation of HIV-LTRs. A HHV-6 encoded factor, B701, transactivates HIV-LTR promoters interacts with the NF-*k*B enhancer sequence (Geng et al., 1992). The B701 gene encodes 143 amino acids that share no significant sequence homology to other viral transactivators (e.g., ICP4 or ICP0). Transfection of the B701 construct into monocytes induced TNF-α transcription at very high levels, while other control plasmids had no effect (Neipel et al., 1991). This suggests that B701 can augment its effect by inducing TNF-α from mononuclear cells, which in turn can activate the HIV-LTR.

2. Avian retrovirus and herpesvirus interaction (ALV/MDV)

As mentioned above, the first observation of interactions between retrovirus and herpesvirus came from enhanced pathogenesis of both ALV and MDV in co-infected chickens. For a long time, it was believed that MD associated tumors were caused by dual infection with MDV and ALV (Peters et al., 1973; Frankel et al., 1974). Subsequently, it

was demonstrated that MD symptoms could be induced by MDV in ALV-free chicken (Calnek and Payne, 1976). Even it was clear that MDV was the etiological agent of MD, these studies showed that dual infection with MDV and ALV could dramatically affect the pathogenesis of both viruses. Recently, this phenomenon was re-emphasized because an enhanced ALV pathogenesis was observed when birds were vaccinated with bivalent vaccines against vvMDV infection (Witter, 1985).

A. Biology of ALV/MDV interaction

As shown by Bacon et al. (1989), it was the serotype 2 MDV in bivalent vaccines that accounts for enhanced lymphoid leukosis (LL) in ALV-infected chickens. Chickens developed similar levels of viremia and neutralizing antibodies to ALV, regardless of the presence of serotype 2 MDV. This suggested that the mechanism of LL enhancement did not result from differences in susceptibility or immune response caused by MDV coinfection. Therefore, MDV was presumed to directly influence the process of ALV-induced LL formation (Bacon et al., 1989). However, MDV-induced LL enhancement was influenced by the genetic constitution of the host, since only four out of eight chicken lines used in a trial developed LL enhancement. No disruption of LL resistance was found in a LL-resistant laboratory chicken line even with serotype 2 MDV co-infection.

One possible genetic difference, that may contribute to susceptibility of chickens to MDV -induced LL enhancement, is the presence or absence of ALV endogenous virus (EV). Because of the immune tolerance generated by EV, EV is known to increase susceptibility of chickens to LL in ALV single infection (Crittenden and Fadly, 1985; Crittenden et al., 1987). Recently, results from studies conducted to determine the influence of EV on

enhancement of LL by serotype 2 MDV revealed that enhancement of LL was noted only in chickens harboring EV (Fadly, 1992). These results clearly suggest that EV plays an important role in susceptibility of chickens to enhancement of LL by serotype 2 MDV.

B. MDV-enhanced ALV pathogenesis

It is important to know the process of ALV-induced lymphomagenesis in order to understand the role of MDV co-infection in enhanced-ALV tumorigenesis. The natural history of ALV induced lymphomas involves a multistage process which includes ALV infection (tumor initiation), hyperplastic transformation (tumor promotion), bursal lymphoma development (tumor progression), and metastasis to visceral organs (tumor metastasis)(Ewert and De Boer, 1988; Bishop, 1991) (Figure 1.7). It takes about 14-25 weeks to complete the whole process. The target of ALV transformation is B-cells which presents in the bursa of Fabricius during late embryonic development and the first 2 weeks of age.

Tumor initiation is characterized by ALV genome semi-random integration in the host genome upon infection (Shih et al., 1988). Only a small portion of infected cells have ALV integrated into the c-myc locus (Varmus et al., 1985). This small population of cells is able to go on to promotion stage. Tumor promotion occurs with abnormal expression of the c-myc gene where c-myc gene expression is controlled by integrated viral promoters, (ALV-LTR), instead of the c-myc promoter. Deregulation of c-myc gene expression leads to a higher rate of cell proliferation (Teich et al., 1985). Although most of the cells that traffic the bursa by the 6th week of age are infected with ALV, only several of them become transformed. These transformed cells will appear as hyperplastic follicles in bursa at 8

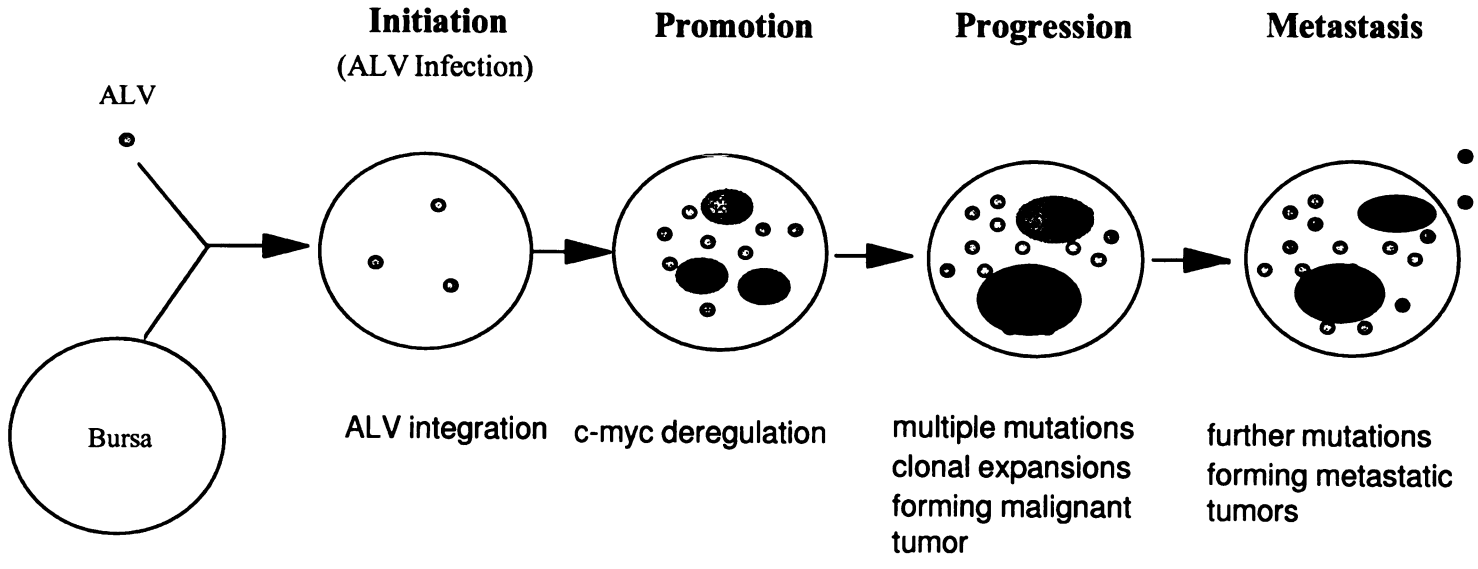


Figure 1.7. Four stages of ALV tumorigenesis.

weeks of the age (Ewert and De Bour, 1988). Tumor progression may result from multiple processes of mutation and clonal expansion, however, details of these processes are still not known. Only 1 or 2 lymphomas (malignant tumors) will be developed in an individual bursa and other follicles will regress with age. Tumor metastasis is the final stage of the disease, and is characterized by the spread of lymphomas from bursa to other visceral organs. Development of metastatic tumor will likely require an accumulation of more mutations. The mechanism responsible for metastasis is totally unknown. The last two stages of lymphoma formation occur in 14 to 25 weeks of age. Despite the unknown aspects of the later stages of ALV induced lymphoma, it is clear that ALV is directly involved in the first two stages of tumor formation, namely tumor initiation and tumor promotion.

In the case of ALV and MDV co-infection, the final target cells of ALV and MDV are different, however, infection of B-lymphocytes appears to be a common feature in life-cycles of both viruses (Calnek et. al., 1991; Payne, et al., 1991). Potential co-infection of a single B-cell will offer the opportunity for direct interaction between ALV and MDV. Evidence from two in vivo studies showed that MDV genomes were detected in almost all ALV-induced hyperplastic follicles (March et al., 1995) and B-lymphomas derived from co-infected birds (Fynan et al., 1992). This suggests that ALV and MDV could simultaneously infect a single B-lymphocyte in vivo, which also implies that co-infected cells with ALV and MDV were essential for MDV-enhanced ALV tumorigenesis.

The outcome of ALV/MDV direct interactions was first demonstrated in vitro where co-infection of primary chicken embryo fibroblasts (CEFs) resulted in an increase of ALV virion production (Pulaski et al., 1992), suggesting that MDV co-infection can enhance

ALV replication. Lately, this suggestion was confirmed by an in vivo study (Marsh et al., 1995). In this study, co-infection of ALV and MDV resulted in a significantly higher number of ALV-induced hyperplastic follicles. Because the number of hyperplastic follicles is correlated with the intensity of ALV infection (Purchase, 1987), a higher number of hyperplastic follicles means a higher intensity of ALV infection. Since same amount of ALV viruses were used in both ALV single infection (control) group and ALV/MDV co-infection group, the higher intensity of ALV infection indicates a higher level of ALV replication in ALV/MDV co-infected birds. This result suggests that MDV, at least, acts as an enhancing agent during ALV tumor initiation. The MDV-enhanced tumor initiation will consequently accelerate ALV-induced tumorigenesis, which results in a higher number of hyperplasticfollicles (Marsh et al., 1995), an earlier onset of malignant tumor (Bacon et al., 1989), and a higher number of metastatic tumors (Fynan et al., 1992). Therefore, MDV-enhanced ALV-replication (ALV tumor initiation) is, at least, one of mechanisms responsible for MDV enhancd ALV tumorigenicity, which is summarized in Figure 1.8.

C. MDV-enhanced ALV replication

Due to the nature of ALV genome structure, ALV replication is predominantly controlled by viral promoters (LTR promoter) located at the 5' end of the ALV genome. Therefore, enhanced ALV replication during ALV/MDV co-infection suggests an up-regulated ALV-LTR promoter activity. This enhanced LTR promoter activity was also observed in study of ALV gene expressions. Since LTR promoter also regulates ALV viral gene expression, the modification of LTR promoter regulation should also result in a change in ALV gene expression. Enhanced ALV gene expression was observed with 5-fold increase

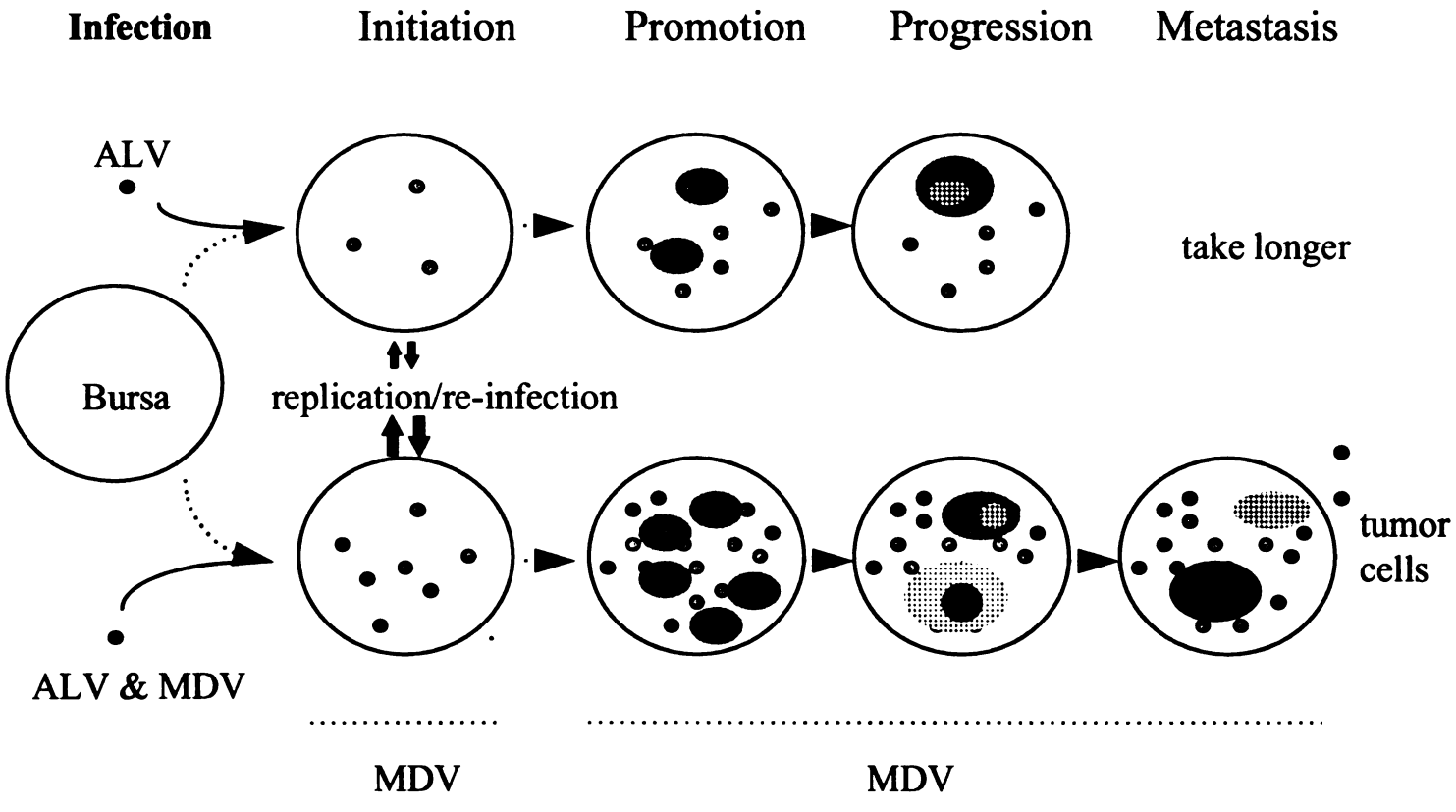


Figure 1.8. Effects of MDV-enhanced ALV replication in ALV tumorigenesis.

of ALV transcripts and 10-fold increase of ALV antigens in ALV/MDV co-infected CEFs (Pulaski et al., 1992). Together, this enhanced ALV-LTR promoter activities suggests that either MDV-encoded viral factors or MDV-induced cellular factors are responsible for ALV-LTR promoter up-regulation.

In order to elucidate the molecular mechanism of MDV-mediated ALV-LTR up-regulation, an in vitro system was established (Tieber et al., 1990). As mentioned previously, ALV is a group of avian retroviruses which are absent of v-onc genes in their genome and are able to induce lymphoid leukosis after a long-time of incubation in birds. Because strains of ALV were first isolated as helper viruses (replication competent virus) together with Rous sarcoma virus (RSV, replication deficient virus but contain v-onc gene), strain of ALV has been named Rous associated virus (Rav), e.g., Rav-1, Rav-2, etc.. Since RSV was derived from ALV by replacing a part of viral genome with a host oncogene, the promoter regions of these two viruses were not disrupted and are almost identical. Compared to ALV-LTR promoter, RSV-LTR promoters were better studied and also served as a model promoter in transcriptional regulation studies. Therefore, we choosed RSV-LTR as the model promoter to investigate mechanisms involved in MDV-enhanced ALV-LTR promoter up-regulation in *in vitro* studies.

In a transient expression assay, reporter-plasmids with RSV-LTR promoters which were inserted upstream of chlorophenical actyle transferase (CAT) gene were transfected into either CEFs or MDV-infected CEFs. RSV-LTR promoter activities were measured by CAT assay with transfected CEF cell lysate (Tieber et al., 1990). RSV-LTR demonstrated a significantly higher level of promoter activity in the presence of MDV serotype 2 infection

(Tieber et al., 1990). However, no significant transactivations were observed with infection of serotype 3 MDV. All these results generated from the *in vitro* system perfectly matched results observed in ALV/MDV co-infected cells (Pulaski et al., 1992). Therefore, MDV-mediated RSV-LTR transactivation presented a reliable *in vitro* system for study MDV-enhanced ALV-LTR up-regulation.

In the following study, deletion mutation was used to investigate cis-acting elements within RSV-LTR promoter which respond to MDV transactivation. As described earlier, there are four distinct enhancers found in RSV-LTR promoter, namely EFI, EFII, EFIII, and EFIV. Transient expression assays with RSV-LTR promoter deletion mutants, revealed a 28 bp region (-137 to -109 upstream of transcription start site which includes the EFI binding site) that was essential for MDV-mediated RSV-LTR transactivation (Banders et al., 1994). Nevertheless, DNA-sequence from this region also formed a MDV unique DNA-protein complex (complex III) in mobility shift assay (Banders et al., 1994). The formation of MDV-unique DNA-protein complex suggests that there is an interaction between MDV-specific regulatory protein and the 28 bp region of RSV-LTR promoter. Since two cellular DNA-protein complexes have also been detected by DNA-sequence from in this region, it suggests the existence of cellular factor DNA-binding sites in the 28 bp region. Therefore, the MDV-unqiue DNA-protein complex could be formed either by directly bind to the DNA sequence or indirectly associate with the 28 bp region through a cellular DNA-binding protein. Theoratically, there could be at least three types of interaction between MDV factor and the RSV-LTR DNA sequence, namely the independent-binding model, the compete-binding model and the indirect-binding model, which are illustrated in Figure 1.9. In the

independent-binding model (Figure 1.9-I), MDV factor could have a DNA-binding site independent from cellular factor binding site. However, if MDV factor recognizes the same DNA sequence as cellular factor, the binding of MDV factor will compete with the binding of cellular factor, which is presented in compete-binding model (Figure 1.9-II). In addition to these two direct-binding models, MDV factor could also form the unique DNA-protein complex by indirect binding (Figure 1.9-III), namely protein-protein interaction, which MDV factor would interact with an existed cellular DNA-binding protein instead of RSV-LTR DNA sequence.

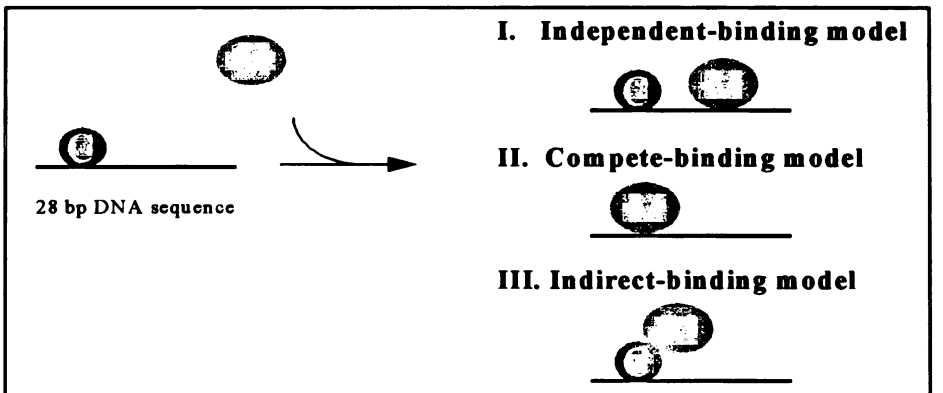


Figure 1.9. Models of MDV unique DNA-protein complex formation.

To date, the only reported transcription factor binding site in the 28 bp region was the EFI site (Sealey and Chalkley, 1987; Faber et al., 1990; Ozer et al., 1990). Further sequence analysis of the 28 bp region revealed two potential ets transcription factor binding sites, and two pentanucleotide repeat elements (PREs, GGTGG) which are similar to Tax response elements (TRE, GGTGG) in human T-cell leukemia virus type I (HTLV-I) LTR promoter. MDV-specific factors which target the 28 bp region could bind directly to DNA

near EFI or ets cellular factor binding sites, or perhaps could displace these factors by binding to the same sequence. However, results from other retrovirus/herpesvirus interaction studies strongly suggest that herpesvirus factors can transactivate retrovirus LTR promoters without directly recognizing any specific-DNA sequence in the promoter. In the other words, herpesvirus transactivators would associate with cellular factors bind to the LTR promoters rather than bind directly to the DNA sequences of the promoter.

Therefore, our hypothesis for the interaction between MDV-specific factor and the 28 bp region from RSV-LTR promoter is that MDV-specific (MDV-encoded or MDV-induced) factor(s) may target to the 28 bp region by recognizing cellular DNA-binding protein, which has been described in Figure 1.9-III as the "indirect-binding model".

Understanding the interactions between MDV factors and cellular factors involved in RSV-LTR transactivation will allow us to determine the identity of these MDV factors, study the role of these factors in MDV life cycle, and even create serotype 2 MDV vaccines without the side-effect of enhanced LL formation in the future.

There were four specific aims, as follows:

1) Map MDV factor target site by site-direct mutagenesis study of RSV-LTR promoter.

2) Determine the critical binding site involved in MDV-unique DNA-protein complex formation by mobility shift competition assay.

3) Elucidate the role of cellular DNA-protein complex in formation of MDV-unique DNA-protein complex.

4) Study the cellular factor binding sites and cellular DNA-protein complexes formation in RSV-LTR PRE region.

Chapter 2

Materials and Methods

I. Cells, virus and cell nuclear extracts

Primary Line 0 chick embryo fibroblast (CEF) cells were prepared as described (Glaubiger et al., 1983; Pulaski et al., 1992). Primary CEF cells ($5x10^7$ to $1x10^8$) were seeded on 150-mm culture dishes and incubated for 24 hrs at 37 ^oC in an atmosphere of 95% air and 5% CO_2. Secondary CEF cells ($1x10^6$) were plated on 60-mm culture dishes in preparation for infection or transfection. CEF cells were infected with approximately $1x10^5$ plaque forming units (PFU) of MDV stain SB-1 as described (Pulaski et al., 1992).

Nuclear extracts were prepared from MDV serotype 2 SB-1 strain infected CEF cells and uninfected CEF as described by Coussens et al. (1991) except three more protease inhibitors were used added to buffers just before use. These protease inhibitors are: 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/liter leupeptin and 10 mM benzamidine. Protein concentrations were determined by standard lowery assays and 550 nm spectrophotography. Extracts were aliquoted and stored at -70 ^oC until used. Typical protein concentration in extracts was 1.0 to 5.0 ug/ul.

II. Antibody generation

Polyclonal antibody for EFIa was generated from a 20 amino acid synthetic peptide which derives from chicken EFIa (5-25 aa) (Grant and Deeley, 1993). Both peptide synthesis and antibody production were performed by Biosynthesis Inc.. Antibody generated

from this 20 aa peptide not only detected a denatured 52 kDa protein in Western blot assay, but also recognized a native EFI factor in mobility super shift assay (Figure 6).

III. Plasmid constructs and in vitro mutagenesis

Plasmid pCAT-Sph (pCS) (Banders et al., 1994) contains a 137 bp fragment upstream of RSV-LTR transcription start site. Within this fragment, there are an intact PRE region, two EFI sites, one EFIII site, and a RSV-LTR core promoter. pCS was normally used as a wild type RSV-LTR report construct in our studies. Plasmid pCAT-Sph~ps (pCS~ps) (Banders et al., 1994) was constructed by removing the 28 bp PRE region from pCS with PvuI and partially SphI digestion. pCS~ps loses both high level of promoter activity and MDV-mediated RSV-LTR transactivation.

In order to study the potential cellular factor binding sites in PRE region, *Altered Sites in vitro Mutagenesis System* (Promega Corporation) was used to introduce site-direct mutation in pCS. First, the entire RSV-LTR promoter was isolated by SphI digestion from pCS, and inserted into the SphI site of pALTER-1 phagemid. The generated construct, pALTER-Sph (pAS), was then used as a template for site-direct mutagenesis. Besides the RSV-LTR insertion, pAS contains two genes for antibiotic resistance. One of these genes, for tetracycline resistance, is always functional. The other, for ampicillin resistance, has been inactivated. An oligonucleotide was applied which restores ampicillin resistance to the mutant strand during the mutagenesis reaction. This oligonucleotide was annealed to the

single-stranded DNA (ss DNA) template at the same time as the mutagenic oligonucleotide, and subsequent synthesis and ligation of the mutant strand links the two. The DNA was transformed into a repair minus strain of E. coli (BMH71-18 mut S) and the cells were grown in the presence of ampicillin, yielding large numbers of colonies. A second round of transformation in JM109 or a similar host ensured proper segregation of mutant and wild type plasmids. Mutation was confirmed by sequencing. Then, mutated RSV-LTR promoter fragment was cut by SphI digestion, and inserted into pCAT-basic SphI site. Orientation of this insertion was checked by endonuclease digestion. With different mutagenic oligonucleotides, six RSV-LTR promoter mutants were generated by this method. There are pCS-EFIx, pCS-1x, pCS-2x, pCS-3x, pCS-4x and pCS-Mx. Except for the mutated sequences, all inserts and vectors between pCS and pCS mutants are identical.

IV. Transfection and Chloramphenicol Acetyl-transferase (CAT) Assay

All plasmid transfections for transient expression assays were performed by electroporation with a Gene-Pulser Electroporator (Bio-Rad, Richmond, CA.). Primary cultured CEF cells were removed from plates by using 0.05% trypsin, then CEF were washed twice with PBS. After final washing, cells were resuspended with HBS 2x buffer (50 mM HEPES, pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4) at a density of $7.5x10^6$ cells/ml. About 0.8 ml of cell suspension ($6x10^6$ cells) was mixed with 0.5 - 3 ug of plasmid DNA and incubated on ice for 15 to 20 minutes. Cell mixtures were transferred into an electroporation

cuvette and electroporated with a single pulse at 350 mV and 960 uF with a capacitance extender. After electroporation, cells were incubated at room temperature for 10 minutes and then plated equally into three 60 mm tissue culture plates with 3 ml of Leibovitz-McCoy medium (Life Technologies, Inc., Gaithersburg, NY.). Generally, cells were harvested 48 hours post-transfection. In the case of studying MDV-mediated RSV-LTR transactivation, cells were infected with MDV serotype 2 SB1p31 viruses at 24 hours post-transfection with $3x10^4$ plaque forming units each 60 mm plate. MDV-infected CEF cells were harvested 24 hours after infections. Harvested cells were lysised by three cycles of freezing and thawing in 0.1 M Tris.HCl (pH7.8). Protein concentration of cell extracts were determined by Lorry Assay. CAT activities were assayed by using same amount of total protein for all samples in each experiment. Also, transfection efficiencies were verified in most cases by slot-blot hybridization with pCAT-basic probe.

V. Oligonucleotides and probe labeling

All oligonucleotides for mobility shift assays and site-direct mutagenesis were synthesized at the Michigan State University Macromolecular Synthesis, Structure and Sequencing Facility on an Applied Bio-systems 380B DNA synthesizer. Synthetic oligonucleotides were dissolved in TE pH 8.0 buffer and quantitated by GeneQuant II RNA/DNA Calculator (Pharmacia Biotech). Complementary oligonucleotides were annealed by first 10 min. 95^0C denaturation and then 1 hour 37^0C incubation.

The upper strand sequences of PRE oligonucleotides and all PRE-mutated oligonucleotides are listed in Figure 3. Some transcriptional factor consensus sequences used in this study are listed below: ATF, 5'-CGATGGTCTGACATGGATT-3' (upper strand); C/EBP, 5'-TGCAGATTGCGCAATCTGCA-3'; and HTLV-1 LTR ets-1 5'-TCCGGGAAGCCACCGGAACCACCCATTTCCTCC-3'.

In preparation for mobility-shift assays, double-stranded (ds) oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase for 30 minutes at 37 C. Unincorporated radiolabelled nucleotide was removed by spun column chromatography through Sephadex G-50 (pharmacia-LKB, Piscataway, NJ). Final probe concentrations were adjusted to approximately 1 ng/ul.

VI. Gel shift assay and super shift assay

Mobility-shift assays were performed essentially as described by (Garner and Revzin, 1981; Fried and Crothers, 1981). Cell extracts were combined with buffer D (Dignam et al., 1983) to provide a constant volume of 10 ul. Probe mixes were prepared by combining equal amounts of probe solution (1 ng/ul), 1 M KCL, 0.1 M $MgCl_2$, and Poly(dI-dC) at 1 ng/ul (Boehringer-Mannheim). Probe mix volumes were adjusted so that 10 ul of probe mix could be added to each reaction. Final concentrations in each reaction were: 10 mM HEPES, pH 7.9; 10% glycerol (v/v); 100 mM KCL; 0.1 mM EDTA; 0.25 mM DTT; 5 mM $MgCL_2$; 0.05 ug/ul Poly(dI-dC); and 1 ng of end-labeled probe DNA. Reactions were initiated by

addition of probe mix to extract proteins on ice. Complexes were allowed to form at 37 ^{O}C for 15 to 30 minutes.

In mobility shift competition assay, unlabelled oligonucleotides were used as competitor, generally with a concentration 100-folds higher than probe. All mobility-shift reaction products were analyzed by 6% polyacrylamide gel in Tris-borate EDTA (TBE) buffer (Garner and Revzin, 1981). Bands of unbound and protein-bound probe were detected by auto-radiography of dried gels. We used ATF consensus sequences oligonucleotides as non-specific competitor, and used unlabelled probe oligonucleotides as specific competitor in all mobility shift assays.

VII. Southwestern blot assay

DNA-binding proteins were identified by a previously described procedure (Gitlin et al., 1991). Briefly, the proteins in 20 ug of either CEF or CEF/MDV(SB1) cell extracts were separated by 10% SDS-PAGE and electrotransferred onto nitrocellulose membrane. The membrane undergo three 45-min. washes with renaturation buffer (10 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.1 mM dithiothreitol [DTT], 2.5% Nonidet P-40, 5% nonfat dry milk, 10% glycerol) and then was rinsed briefly with binding buffer (10mM Tris.HCl pH7.5, 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.125% nonfat dry milk, 8% glycerol). The membrane was placed into a heat-sealable bag with binding buffer containing 5 mM $MgCl_2$ plus poly(dI)-poly(dC) (50ug/ml) and ^{32}P-labelled oligonucleotide probe (5 x 10^6 cpm/ml).

Following incubation for 12 to 16 hrs at room temperature with continuous gentle rocking, the membrane was washed six times (45 min each) with wash buffer (10 mM Tris.HCl pH7.5, 50 mM NaCl), air dried, and then exposed to X-ray film.

Chapter 3

Results

I. Two cellular factor binding sites in RSV-LTR PRE region are essential for generating MDV-mediated LTR transactivation as well as LTR basal promoter activity

In previous deletion studies, a 28 bp region (from -109 to -137) in RSV-LTR promoter was found to play an essential role in the MDV-mediated RSV-LTR transactivation, as well as maintaining RSV-LTR basal promoter activities (Banders et al., 1994). This MDV-responsive sequence contains two GGTGG pentanucleotide repeat elements (PRE), so called PRE region. The important roles of PRE region in both MDV-mediated transactivation and basal promoter activities have suggested that the PRE region contains both a MDV factor target site and cellular transcriptional factor DNA-binding sites. In order to determine whether or not MDV regulatory protein(s) and cellular factor(s) bind to the PRE region independently, we have generated a set of RSV-LTR promoter mutants by site-direct mutation and tested these constructs in transient expression assays.

In RSV-LTR PRE region, besides a previously described EFI (enhancer factor I) binding site (Sealey et al., 1987; Faber et al., 1990; Ozer et al., 1990), there are several potential DNA-binding sites (Figure 3.1a). Among these sites, two sites are similar to the core sequences recognized by members of the *ets* transcription factor family (5'-GGAA-3'); and two sites are identical to Tax response elements (TRE, 5'-GGTGG-3') within HTLV-LTR promoters. Our working hypothesis was that these cellular factor binding sites were important for MDV-mediated RSV promoter transactivation. To test this

hypothesis, site-specific mutations were introduced to each of these sites. Mutation have also been prepared in sequences between these sites (Figure 3.1a).

This promoter mutant library allowed us to investigate the relative importance of all potential transcription factor binding sites in the RSV-LTR PRE region with respect to basal and MDV-activated expression. In transient expression assays, RSV-LTR promoter activities were measured in the presence or absence of MDV serotype 2 SB1 strain low-passage viruses. Cells transfected with pCAT-Sph plasmid, containing a wildtype RSV-LTR promoter, were used as a positive control to demonstrate both high level basal promoter activity and MDV-mediated promoter transactivation (Figure 3.1b, Lane pCS). Cells transfected with pCS~ps in which the entire PRE region had been deleted (Lane pCS~ps) served as a negative control in this assay. Site-directed mutations of either the EFI binding site (Lanes EFIx and 1x) or the downstream ets-like-element (ELE) site (Lanes 4x and 2x) abolished MDV-mediated RSV-LTR transactivation. In contrast, mutations between the EFI and ELE sites had no measurable effect on MDV-mediated RSV-LTR transactivation, suggesting that both EFI and ELE sites are important in maintaining MDV-mediated RSV-LTR transactivation. Mutations in either the EFI binding site (Lanes EFIx and 1x) or the ELE site (Lanes 4x and 2x) also diminish basal RSV-LTR promoter activity, while replacement of sequences between the EFI and ELE sites had little effect on promoter strength. Since both MDV-mediated RSV-LTR transactivation and RSV-LTR basal promoter activity requires these two cellular factor binding sites, this suggests that MDV activation protein(s) and cellular transcriptional factor(s) may share the same two binding sites in the PRE region.

II. Critical DNA-binding site involved in MDV-unique DNA-protein complex formation

Direct interaction between the MDV-specific factors and the RSV-LTR PRE region were first suggested by formation of a unique DNA-protein complex in mobility shift assays with MDV infected cell lysates (Banders et al., 1994). Despite evidence that EFI and ets binding sites within the PRE region were important in MDV-mediated transactivation and basal promoter activity. The nature of interactions at these sites remained unclear. Mobility shift competition assays were employed to further study the role of these cellular DNA-binding sites in forming unique MDV-factor DNA-protein complexes.

Two sequence-specific cellular DNA-protein complexes were identified (complex I and II) by mobility shift assay using labelled PRE probes and uninfected CEF cell nuclear extracts (Figure 3.2). Cellular DNA-protein complexes were eliminated by increasing amounts of unlabelled PRE competitor suggesting that these complexes were formed by sequence-specific interactions (Figure 3.2, lane 3-7).

For the study of the MDV-unique DNA-protein complex formation, cellular nuclear extracts were prepared from CEF cells infected with MDV serotype 2 strain SB1 as described in materials and methods. A unique DNA-protein complex, complex III, was visible in reactions between PRE probes and extracts from MDV-infected cells (Figure 3.3b, lane 2). Complex III could be eliminated by a specific competitor (lane 3), and was not disrupted with a non-specific competitor (lane 4), confirming that complex

III represented a sequence-specific DNA-protein interaction. In contrast, competitors with mutations in the PRE EFI site lost their ability to interfere with complex III formation (lanes 5 and 6). This result suggests that EFI factor is required for the formation of the unique DNA-protein complex. Other competitors with mutations in the ELE site alone only influenced formation of complex II (lanes 9 and 10). Replacement of sequences between the EFI and ELE sites did not adversely affect formation of any complexes (lanes 7 and 8). This result suggests that the EFI binding site is a major cellular DNA-binding site responsible for interacting with an as yet unknown MDV transactivator. However, it was not clear if the EFI site alone could generate the unique DNA-protein complex (complex III), or if a more complex interaction between closely spaced sites was involved.

III. Dependence of both EFI and ELE binding sites in forming a unique MDV DNA-protein complex (complex III)

To address the question of whether an EFI site alone was sufficient to generate the unique MDV DNA-protein complex (complex III), oligonucleotides with a single EFI binding site were used as probes in mobility shift assays (Figure 3.4). A small cellular DNA-protein complex (complex I) was generated with single EFI site probes (lane 2). This cellular DNA-protein complex could be eliminated by a sequence-specific competitor (lane 3), and could not be disrupted with a non-specific competitor (lane 4),

comfirming that complex I represented a sequence-specific DNA-protein complex derived from the EFI binding site. No unique MDV DNA-protein complex (complex III) was formed with a single EFI site. In contrast, a second probe with a single ELE binding site could not generate any sequence-specific DNA-protein complex (lanes 5 to lane 8). However, when both EFI and ELE binding sites were presented on oligonucleotide probes, unique MDV complexes (complex III) were formed as were both cellular DNA-protein complexes (complex I and complex II) (lane 9 to lane 12). These results suggest that MDV-specific factors could not directly bind to EFI binding sites in forming the unique MDV complex. Furthermore, both EFI and ELE sites appear to be required in formation of complex III, suggesting that these two cellular factors together may serve as a target for MDV-specific factors.

IV. Detection of similar PRE DNA-binding proteins in MDV-infected cell and un-infected cell extracts

Consistant with mobility shift studies presented above, southwestern blot assays failed to detect any MDV-specific DNA-binding protein that could bind directly to RSV-LTR PRE region sequence. This result also suggests that cellular factors, not DNA sequence, might mediate interactions with MDV-specific factors. In this experiment, nuclear proteins from uninfected and MDV infected cells were first separated by SDS-PAGE and then transferred to a nitrocellulose membrane. After renaturization of the

denatured proteins, double-stranded PRE probe was used to detect PRE DNA-binding proteins. PRE probes detected two major DNA-binding proteins in uninfected cells (Figure 3.5, lane 1) and in MDV-infected cells (lane 2). Since the two DNA-binding proteins detected in MDV-infected cells were also present in un-infected cells, MDV-specific regulatory proteins might associate with RSV-LTR promoter PRE regions through interaction with cellular DNA-binding proteins rather than by direct binding to PRE DNA sequences.

V. Importance of EFI and ELE factor binding sites in complex I and complex II formation

Since the preponderance of evidence suggests that MDV-specific factors may enhance RSV-LTR function by interacting with cellular PRE DNA-binding proteins, identification of these cellular proteins and understanding their interactions is critical to further characterization and isolation of the MDV-specific factor(s).

To determine how cellular factor binding sites are involved in formation of the two cellular DNA-protein complexes (complex I and II), mobility shift competition assays were employed. In these assays, a series of single-site mutated PRE oligonucleotides were used as competitors to investigate the importance of each potential binding site, Figure 3.6a. Two predicted cellular factor binding sites, the EFI binding site and a potential *ets* transcription factor binding site (ELE site), were found to be

responsible for complexes observed in mobility-shift assays (Figure 3.6b). Although the importance of EFI site has been discussed by Sealey et al., the *ets*-related (termed an *ets*-like-element (ELE, AAGG)) binding site has not been previously reported. In competition mobility-shift assays, competitors with a mutation in the EFI site lost their ability to compete for both complex I and complex II (lane 5 and lane 6), suggesting that the EFI site was important in formation of both complexes. In contrast, competitors with mutations in the ELE site only lost their ability to compete for formation of DNA-protein complex II (lane 9 and lane 10), suggesting that an intact ELE site was only involved in formation of complex II. Taken together, these results suggest the EFI site is solely responsible for formation of complex I while both EFI and the *ets*-related binding site are important in formation of complex II.

VI. Involvement of EFI factor in both complex I and complex II formation

Direct evidence of EFI involvement in both complex I and II formation was obtained from mobility super shift assays using a polyclonal antibody developed from a synthetic polypeptide which encodes 20 amino acids (5 to 25aa) in the N-terminal portion of chicken EFIa factor (Grant and Deeley, 1993). This polypeptide derived antibody could detect a 52 kDa protein in western blot assays (Figure 3.7a), consistent with the estimated size of EFIa factor of 50 kDa to 60 kDa (Faber et al., 1990). In super shift assays, as expected, pre-immune serum did not disrupt specific formation of any cellular

DNA-protein complexes (Figure 3.7b, lane 3). With addition of EFIa specific antiserum, both complexes I and II were eliminated under blocking conditions in which antiserum was added before PRE probe (lanes 4 and 5). In contrast, addition of EFI-specific antiserum did not disrupt or change mobility of either complex I or II under conditions in which antiserum was added after PRE probes (lanes 6 and 7). These results confirmed that EFI factor is involved in formation of both complexes I and II. In addition, differential results from the two super shift protocols imply that this EFIa antibody recognizes an epitope near the EFI DNA-binding domain or the EFI dimerization domain.

VII. Interactions between EFI and ELE factors in forming DNA-protein complex II

The interaction between EFI and ELE factors was first suggested during investigation of their independent DNA-binding activities. Oligonucleotides which had only one of the two cellular factor binding sites were used as probes in standard mobility shift assays (Figure 3.8). In the beginning of these assays, EFI binding site was studied with an oligonucleotide which has an intact EFI binding site but an altered ELE site (lanes 1-4). This probe generated a single DNA-protein complex with un-infected cell extracts which is similar to complex I formed with wildtype PRE probe. This result suggested that one conclusion from mobility shift competition assays is that EFI is a primary component of complex I. Surprisingly, probe with an intact ELE site could not generate any sequence-specific DNA-protein complexes (lanes 5-8). In contrast,

oligonucleotide with both EFI and ELE binding sites were not only able to form complex I but also able to form complex II (lane 9-12), which suggests the functional importance of the ELE binding site in forming complex II. However, without the presence of EFI binding site, ELE binding site alone was not capable of forming complex II. The dependence of EFI binding site in complex II formation implys a co-operation of EFI and ELE factor in forming DNA-protein complex II.

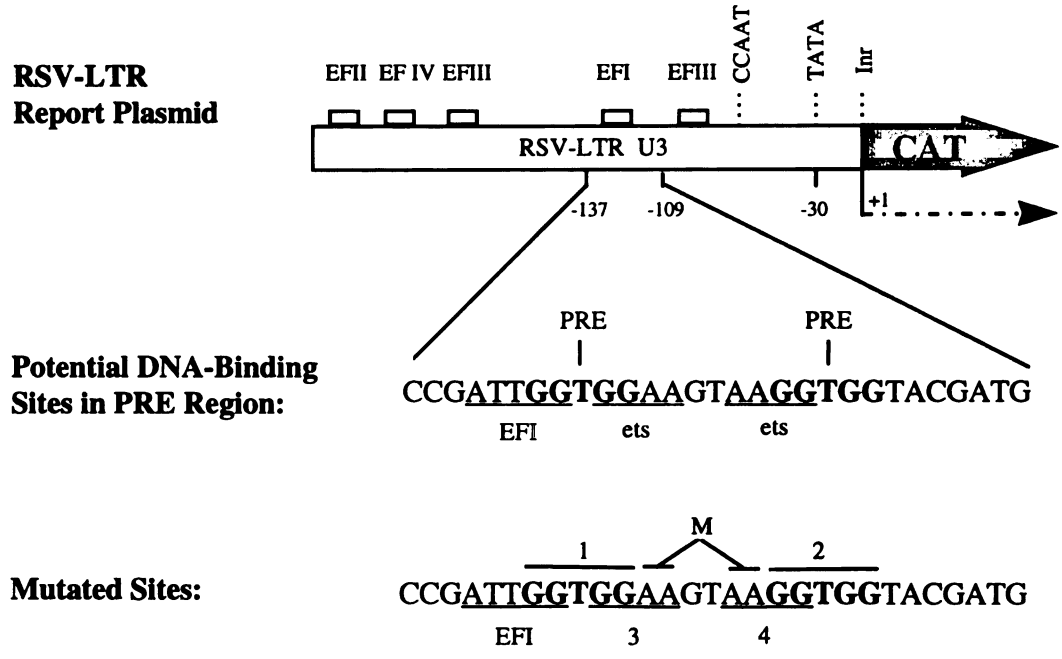
Additional evidence of EFI and ELE factor interaction was observed when we studied EFI factor binding activities. EFI is a member of the C/EBP (CCAAT element binding protein) transcription factor family and recognizes an ATTGG core sequence, an inverted CCAAT element (Faber et al., 1990; Ozer et al., 1990). To further confirm that the factor involved in formation of complexes I and II was indeed EFI, the C/EBP consensus sequence was used as an EFI specific competitor (Figure 3.9). In this experiment, wildtype PRE sequence was used as a control sequence-specific competitor. With an increasing amount of PRE competitor, both complex I and II were evenly diminished (Figure 3.9a lanes 2-5, and Figure 3.9b). However, when C/EBP consensus sequence competitors were used, an uneven pattern of competition was observed between complex I and complex II (Figure 3.9a lane 6-9, and Figure 3.9c). Complex I was eliminated more efficiently than complex II, suggesting that EFI binds to C/EBP consensus sequences efficiently, leading to a significant decrease in complex I formation. In contrast, C/EBP consensus sequence was not able to eliminate complex II formation efficiently (Figure 3.9c), perhaps due to absence of a second factor binding site, the ELE site. This result suggests that the combination of EFI and ELE binding factors (complex

II) enhances stability of EFI-DNA binding, and that the interaction between EFI and ELE factors could stabilize EFI DNA-binding.

Figure 3.1. MDV-mediated RSV-LTR transactivation with PRE promoter mutants.

a) A set of RSV-LTR promoter mutants were generated by the *site-direct in vitro mutagenesis system* (Promega Inc.). There are several potential DNA-binding protein binding sites in the PRE region, including one EFI site, two ets-like transcriptional factor binding sites, and two PRE (pentanucleotide repeat element) sites. Each single-site mutated RSV-LTR promoter correlates with a mutated DNA-binding site in PRE region.

Figure 3.1a.



RSV-LTR Promoter Mutants:

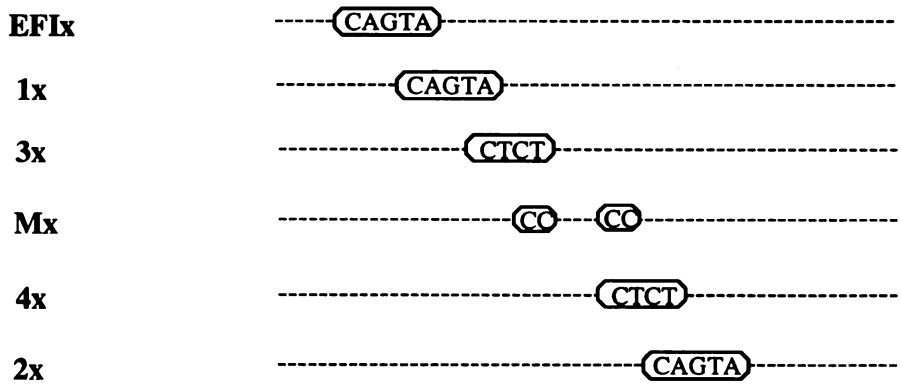


Figure 3.1.

b) Transient expression assay with single-site mutated RSV-LTR promoters were used to determine the critical binding site for LTR promoter activity. Secondary chicken embryo fibroblasts (CEF) were used for transfection. The promoter activities of these mutants were determined by CAT assay as described in materials and methods. Lane pCB presented the background CAT activities where CEFs were transfected with pCAT-basic plasmid alone either in uninfected cells or in MDV-infected cells. Lane pCS were samples transfected with pCAT-Sph plasmid, representing wildtype RSV-LTR promoter activity and MDV-mediated RSV-LTR transactivation (positive control in this assay). Lane pCS~ps were samples transfected with PRE region deleted LTR promoter mutant (negative control). In this assay, EFIx and 1x mutants, which had the EFI site completely or partially mutated, presented very low promoter activity if any, and a loss of MDV-mediated transactivation. Similar reduction of RSV-LTR promoter activities was also observed in the 4x mutant which had a mutation in the downstream *ets* binding site (ELE site). However, mutations in the sequence between EFI and ELE sites had a little or no influence on RSV-LTR basal promoter activities and MDV-mediated transactivation (lane 3x and lane Mx).

Figure 3.1b.

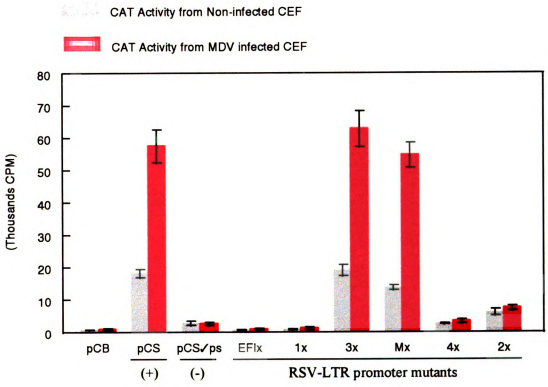

CAT Activity from Non-infected CEF
CAT Activity from MDV infected CEF
(Thousands CPM)
80
70
60
50
40
30
20
10
0
pCB
pCS
pCS✓ps
EFIx
1x
3x
Mx
4x
2x
(+)
(-)
RSV-LTR promoter mutants

Figure 3.2. Two sequence-specific cellular DNA-protein complexes are formed by PRE sequence probes. Mobility shift assay was performed as described in Materials and Methods. Briefly, 10 ug nuclear extract from secondary CEF was incubated with 1 ng of ^{32}P-labeled PRE ds DNA in the presence of 1.0 ug of poly(dI):(dC) and in the absence or presence of a 10- to 250-fold molar excess of non-radioactive labeled PRE competitor. Two sequence-specific DNA-protein complexes (complex I and complex II) and one non-specific complex are formed in this assay.

Figure 3.2.

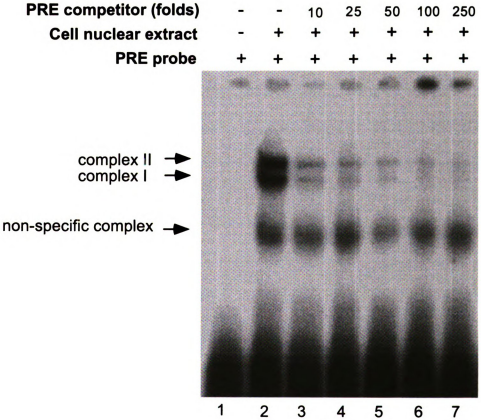

PRE competitor (folds) - - 10 25 50 100 250
Cell nuclear extract - + + + + + +
PRE probe + + + + + + +
complex II
complex I
non-specific complex
1 2 3 4 5 6 7

Figure 3.3. Study of potential DNA-binding sites involved in formation of a unique MDV DNA-protein complex.

a) Potential DNA-binding protein binding sites in the PRE region, including one EFI site, two ets-like transcription factor binding sites, and two PRE (pentanucleotide repeat element) sites. Each single-site mutated competitor correlates with a mutated DNA-binding site in the PRE region.

Figure 3.3a.

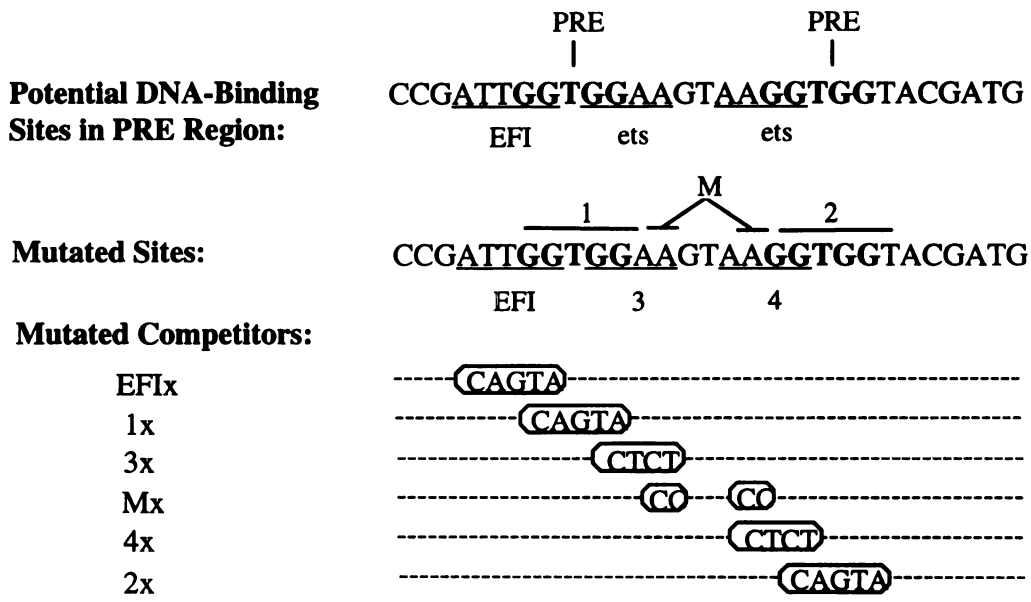

PRE
PRE
Potential DNA-Binding Sites in PRE Region:
CCGATTGGTGGAAGTAAGGTGGTACGATG
EFI
ets
ets
M
1
2
Mutated Sites:
CCGATTGGTGGAAGTAAGGTGGTACGATG
EFI
3
4
Mutated Competitors:
EFIx
1x
3x
Mx
4x
2x
CAGTA
CAGTA
CTCT
CO
CO
CTCT
CAGTA

Figure 3.3.

b) Mobility shift competition assay was performed with 10 ug of MDV serotype 2 SB1p31 infected CEF nuclear extract in the presence of 1ug of poly(dI):(dC), 1 ng of the ^{32}P-labeled PRE oligonucleotide, and mutated competitors: EFIx (lane 5), 1x (lane 6), 3x (lane 7), Mx (lane 8), 4x (lane 9), and 2x (lane 10). Competitors were presented in a 100-fold molar excess compared with PRE probe. Unique MDV DNA-protein complex (complex III) was formed with PRE probe (lane 2). This sequence-specific complex was eliminated with specific competitor (lane 3), but not affected with non-specific competitor (lane 4). PRE mutated competitors appear to have different effects on unique MDV DNA-protein complex (complex III) formation.

Figure 3.3b.

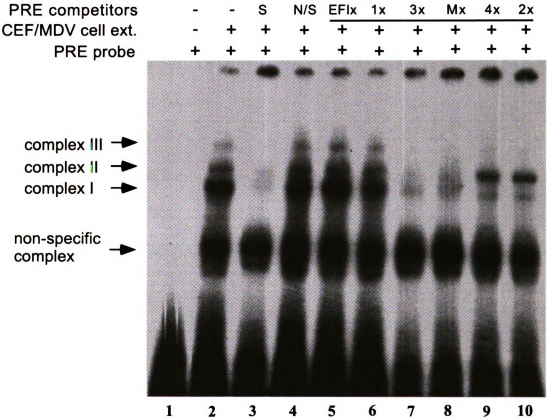

PRE competitors - - S N/S EFIx 1x 3x Mx 4x 2x
CEF/MDV cell ext. - + + + + + + + + +
PRE probe + + + + + + + + + +
complex III
complex II
complex I
non-specific complex
1 2 3 4 5 6 7 8 9 10

Figure 3.4. Requirement of both EFI and ELE DNA-binding sites in formation of unique MDV DNA-protein complex (complex III). With MDV-infected cell nuclear extract, EFI and ELE binding sites were used separately to investigate the role of each factor in forming the unique DNA-protein complex. EFI site oligonucleotide was used as probe to investigate the capacity of EFI site in formation of the unique MDV complex III (lanes 1-4). ELE site oligonucleotide was used as probe to investigate the capacity of ELE site in formation of the unique MDV complex (lanes 5-8). Oligonucleotides which had both EFI and ELE binding sites were used as positive controls in this assay, where complex III was generated as with wildtype PRE sequence (lanes 9-12).

Figure 3.4.

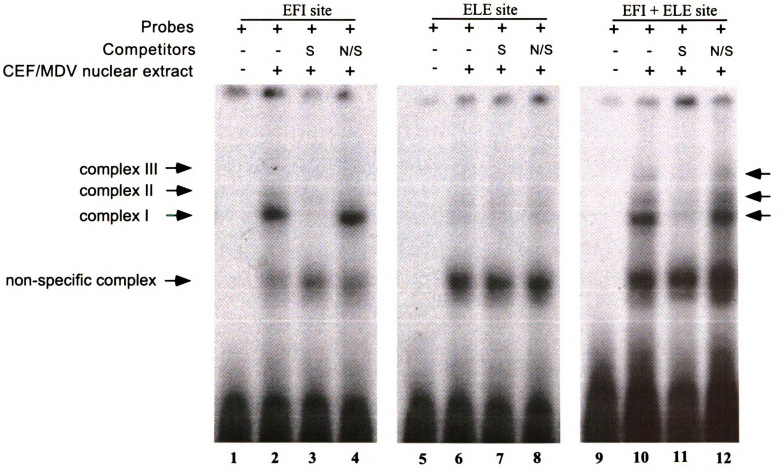

EFI site
ELE site
EFI + ELE site
Probes
Competitors
CEF/MDV nuclear extract
S
N/S
complex III
complex II
complex I
non-specific complex
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.5. Southwestern blot assay with PRE DNA sequence. Nuclear proteins from both uninfected and MDV infected cells were first separated by molecular weight under denaturing gel condition (SDS-PAGE). After transfer and fixation to a nitrocellulose membrane, denatured proteins were renatured in a buffered condition as described in materials and methods. Radioactive-labeled double-stranded (ds) PRE probes were used to detect DNA-binding proteins that recognize PRE DNA sequence. PRE probe has detected two DNA-binding proteins in MDV-infected cell nuclear extracts as well as in uninfected cell nuclear extracts. The molecular weight of these two DNA-binding proteins are 89 kDa and 49 kDa respectively.

Figure 3.5.

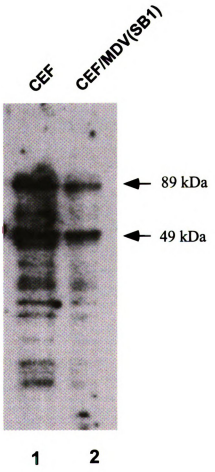


Figure 3.6. Cellular DNA-protein complex formation and related binding sites in the RSV-LTR PRE region.

a) Potential DNA-binding protein binding sites in the PRE region.

Figure 3.6a.

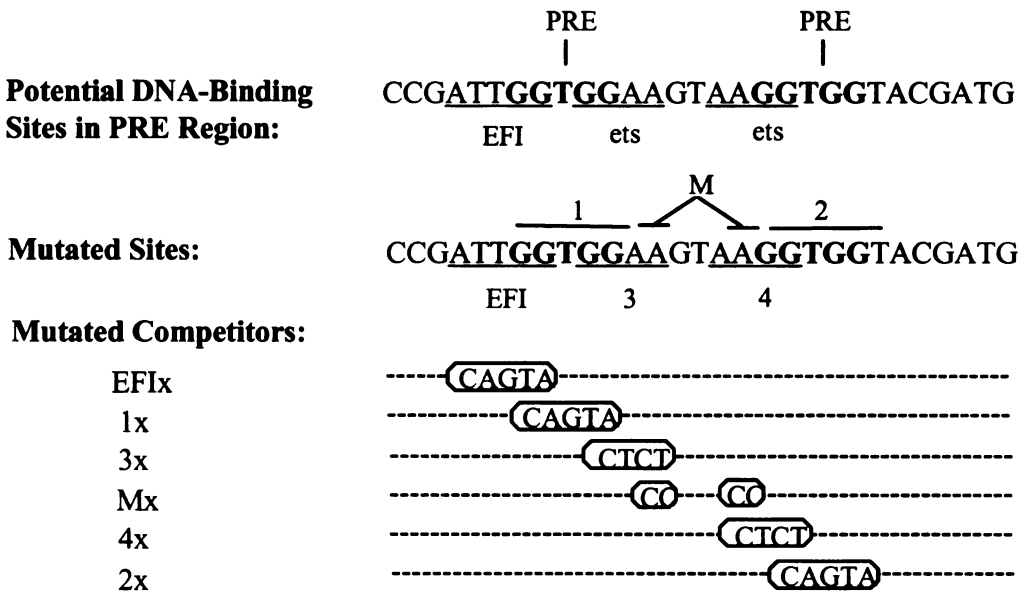

PRE
PRE
Potential DNA-Binding Sites in PRE Region:
CCGATTGGTGGAAGTAAGGTGGTACGATG
EFI
ets
ets
M
1
2
Mutated Sites:
CCGATTGGTGGAAGTAAGGTGGTACGATG
EFI
3
4
Mutated Competitors:
EFIx
1x
3x
Mx
4x
2x
CAGTA
CAGTA
CTCT
CC
CTCT
CAGTA

Figure 3.6.

b) Mobility shift competition assay was performed with a series of single-site mutated PRE competitors which were presented in a 100-fold molar excess compared to labeled PRE probe. Competitor S (lane 3) and N/S (lane 4) stand for specific and non-specific competitors respectively. Generally, non-radioactive labeled probe sequence was used as specific competitor in our studies. In the present case, PRE sequences served as specific competitor.

Figure 3.6b.

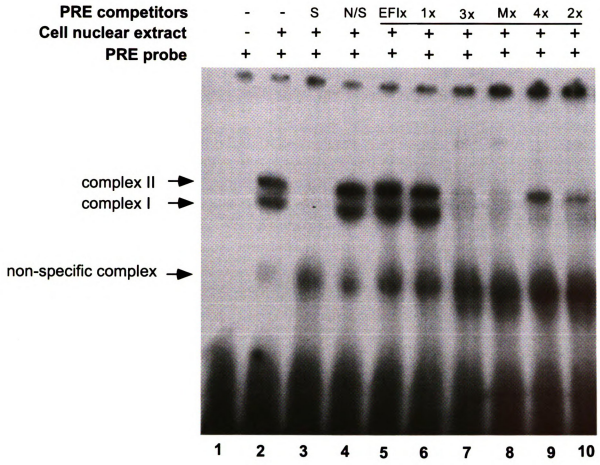

PRE competitors - - S N/S EFIx 1x 3x Mx 4x 2x
Cell nuclear extract - + + + + + + + + +
PRE probe + + + + + + + + + +
complex II
complex I
non-specific complex
1 2 3 4 5 6 7 8 9 10

Figure 3.7. Involvement of EFI factor in formation of two cellular DNA-protein complexes. **a)** Detection of EFI protein by EFIa polyclonal antibody. A 52 KDa protein was found both in un-infected and MDV-infected cell extracts. As an internal control for equal amount of sample loading, the same amount of actin was found between samples. **b)** Mobility super shift assay was used to investigate protein components of the two cellular complexes with EFIa polyclonal antibody. Two kinds of super shift assay procedures were applied in this study. In the **blocking procedure** in which antibody was added before DNA-protein complex formation, cell nuclear extract was first incubated with EFIa antibody for 15 minutes, then PRE probe was added (lane 4 and lane 5). While, in the **super shift** procedure, EFIa antibody was added after DNA-protein complex formation (lane 6 and lane 7). A new, very slowly migrating DNA-protein complex was observed in all samples with rabbit anti-serum. Even pre-immune serum caused this complex formation, which suggested that this new DNA-protein complex was not generated by specific reaction with EFIa antibody. On the other hand, both cellular DNA-protein complex I and II were eliminated with EFIa anti-serum in the blocking procedure where antibody was added before PRE probe (lane 4 and lane 5).

Figure 3.7.

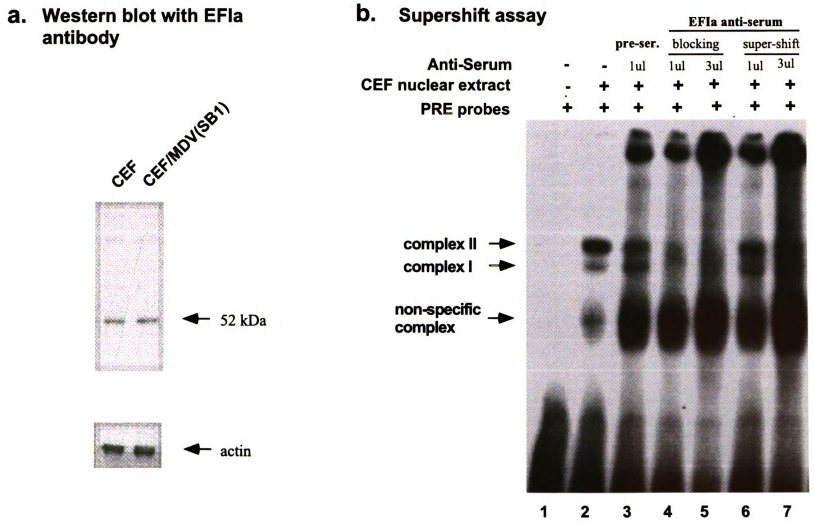

a. Western blot with EFla antibody
CEF
CEF/MDV(SB1)
52 kDa
actin
b. Supershift assay
EFla anti-serum
pre-ser.
blocking
super-shift
Anti-Serum
CEF nuclear extract
PRE probes
1ul
3ul
complex II
complex I
non-specific complex
1 2 3 4 5 6 7

Figure 3.8. Independent binding activities of EFI and ELE factors. EFI and ELE factor binding sites were used separately to study DNA-binding activities of these two factors by mobility shift assays. EFI site oligonucleotide was used as probe to investigate EFI factor DNA-binding activity (lanes 1-4), while a separate ELE site oligonucleotide was used as probe to investigate ELE factor DNA-binding activity (lanes 5-8). Oligonucleotide which had both EFI binding site and ELE binding site was used as a positive control in this assay, where two sequence-specific complexes were generated as with wildtype PRE sequence (lanes 9-12).

Figure 3.8.

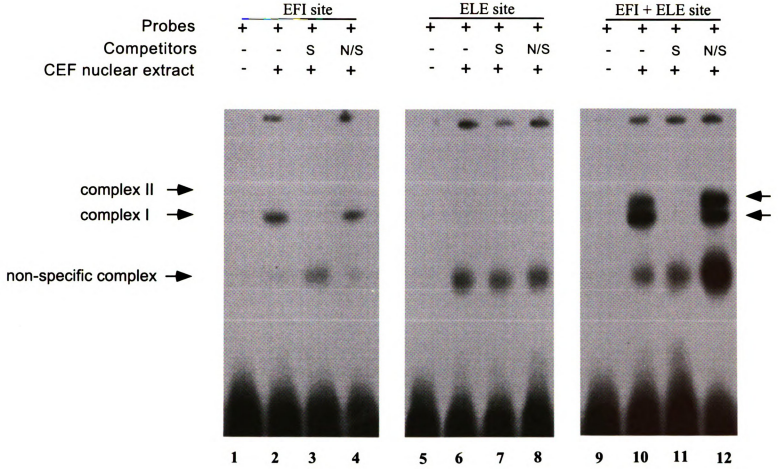
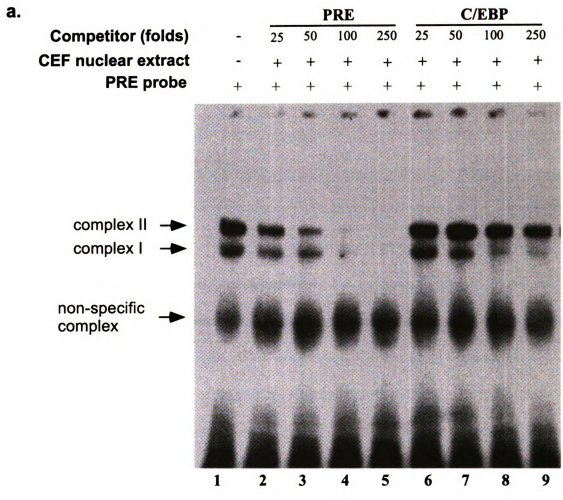
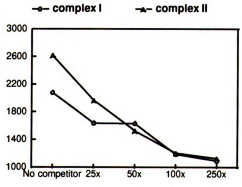
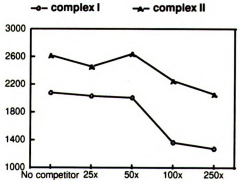

EFI site
ELE site
EFI + ELE site
Probes + + + + + + + + + + + +
Competitors - - S N/S - - S N/S - - S N/S
CEF nuclear extract - + + + - + + + - + + +
complex II
complex I
non-specific complex
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.9. The role of EFI factor in cellular DNA-protein complex formation was studied with C/EBP consensus sequence. **a)** In this mobility shift assay, C/EBP consensus sequence was used as an EFI specific competitor. As a control, non-radiolabeled PRE sequence, which contained both EFI and ELE binding sites, was also used as a competitor with 25- to 250-fold molar excess (lanes 2-5). Both complex I and complex II were eliminated with increasing amount of PRE competitor, **evenly** and **efficiently**. In comparison, the C/EBP consensus sequence which presented only an EFI binding site was capable of eliminating only complex I formation. Cellular DNA-protein complex II was only slightly affected with very high concentrations of C/EBP competitor (lanes 6-9). **b)** Analysis of complex I and II formation in the presence of PRE competitors. **c)** Analysis of complex I and II formation in the presence of C/EBP consensus sequence competitors.

Figure 3.9.


a.
PRE
C/EBP
Competitor (folds) - 25 50 100 250 25 50 100 250
CEF nuclear extract - + + + + + + + +
PRE probe + + + + + + + + +
complex II
complex I
non-specific complex
1 2 3 4 5 6 7 8 9


b. PRE competitor
complex I
complex II
3000
2600
2200
1800
1400
1000
No competitor 25x 50x 100x 250x


c. C/EBP competitor
complex I
complex II
3000
2600
2200
1800
1400
1000
No competitor 25x 50x 100x 250x

Chapter 4

Discussion and Future Research

I. Summary and Conclusion

MDV-enhanced ALV tumorigenesis presents a potential side effect of using polyvalent vaccines against vvMDV infection. During the past ten years, studies of ALV/MDV interaction have revealed that vaccine strains from MDV serotype 2 were responsible for MDV-enhanced ALV tumorigenesis and MDV-mediated ALV-LTR promoter up-regulation. Since ALV tumorigenicity largely depends on its LTR promoter activity, the up-regulated ALV-LTR promoter in MDV co-infected cells can profoundly affect ALV tumorigenesis either by increasing the number of infectious ALV virions or by further deregulating c-myc gene expression. Therefore, studies of MDV-mediated ALV-LTR up-regulation are very important in the search for putative mechanisms that cause MDV-enhanced ALV tumorigenesis. RSV-LTR promoter was successfully used as an *in vitro* model to study MDV-mediated ALV-LTR up-regulation (Tieber et al., 1990; Banders et al., 1994). Promoter deletion studies have revealed a 28 bp (PRE) region responsible for MDV-mediated RSV-LTR promoter transactivation (Banders et al., 1994). This region also formed a unique MDV DNA-protein complex (complex III) in mobility shift assays with extracts from MDV-infected cells.

The overall aims of this project have been to characterize interactions between MDV-specific factors and the 28 bp MDV-responsive DNA fragment (PREs) in the RSV-LTR promoter. Understanding these interactions will allow us to identify MDV-specific factors involved in RSV-LTR transactivation, revealing critical information on general mechanisms of herpesvirus transactivation, and even allowing the further development of new MDV

vaccines without the side effects of LL augmentation.

Interaction between MDV-specific factors and PRE region were first suggested by formation of a unique MDV DNA-protein complex (Banders et al., 1994). However, in the same experiment, two cellular DNA-protein complexes were also generated with PRE region DNA sequences, suggesting existence of cellular factor binding sites in addition to the potential target of MDV-specific factors. Since cellular factors also bind to this 28 bp region, the target of MDV-specific factors could be DNA sequence independent from cellular factor binding, overlaped with cellular factor binding, or even the surface of cellular DNA-binding proteins. Based on these possibilities, three interaction models have been proposed. They are the independent-binding model, the compete-binding model and the indirect-binding model (Figure 1.9).

As a first step toward examining interaction between MDV-specific factors and PRE DNA sequence, we employed mutational studies to further map critical sequences for MDV-mediated RSV-LTR transactivation. With generation of site-directed mutants and transient expression assays, a previously reported EFI site and a newly discovered *ets*-like-element (ELE) were found essential for both RSV-LTR basal promoter activity and MDV-mediated RSV-LTR transactivation. Because EFI and ELE sites appeared to be important for RSV-LTR basal promoter activity, both sites could be recognized by cellular regulatory proteins. Since these two sites are also important for MDV-mediated RSV-LTR transactivation, the MDV-specific factor either recognizes the same binding sequence as these cellular factors, or interacts with the cellular DNA-binding factors by protein-protein interaction.

Since EFI and ELE binding sites appeared to be essential for MDV-induced RSV-

LTR promoter up-regulation, we used mobility shift competition assays to further explore the role of these cellular DNA-binding sites in formation of the unique MDV DNA-protein complex. Among competitors with different mutation sites, only competitors with mutated EFI sites lost their ability to compete for formation of unique MDV complex (complex III). This result suggests that the EFI binding site is a major cellular DNA-binding site responsible for interacting with an as yet unkown MDV transactivator.

Combined results from transient expression studies and mobility shift competition assays, we exclude the possibility that MDV-specific factors bind independently from cellular factor DNA-binding proteins ("independent-binding model"). However, we as yet could not distinguish between the "compete-binding model" and the "indirect-binding model". In other words, MDV-specific factors might directly bind to EFI site by DNA-protein interaction, or indirectly through association with EFI factor by protein-protein interaction.

Two experiments experiments were used to clarify the interaction between MDV-specific factors and EFI DNA sequence. Since EFI binding sites play an essential role in formation of the unique MDV DNA-protein complex, a probe with EFI binding site alone was used in mobility shift assays to investigate whether an EFI site alone could generate the unique MDV-associated complex III. Interestingly, no unique MDV DNA-protein complex (complex III) was formed with EFI site alone. Only a small cellular DNA-protein complex (complex I) was detected in this assay. However, in the presence of both EFI and ELE binding sites, unique MDV complex III was formed with co-existence of both cellular DNA-protein complexes (complex I and complex II). This result suggests that MDV-specific

factors would not directly bind to EFI binding sites, and would seem to exclude the compete-binding model, at least where isolated EFI sites are concerned. Instead, both EFI and ELE factors might be required for unique MDV complex formation. Consistant with these results, southwestern blot assays failed to detect unique PRE binding proteins in MDV-infected cell extracts when compared to un-infected cell extracts. Since the same two DNA-binding proteins detected in MDV-infected cells were also present in un-infected cells, MDV-specific regulatory proteins may only associate with RSV-LTR promoter PRE regions through interaction with cellular DNA-binding proteins rather than by direct binding to PRE DNA sequences.

As a realized caveat of the southwestern blot result, the procedure of SDS-PAGE during southwestern blot assays may cause the loss MDV-specific factor DNA-binding activity, particularly if this factor exists as a multi-subunit complex. However, as demonstrated in mobility shift studies, MDV-specific factors would not directly bind to an EFI binding site. Since mobility shift assays are performed under in non-denaturing

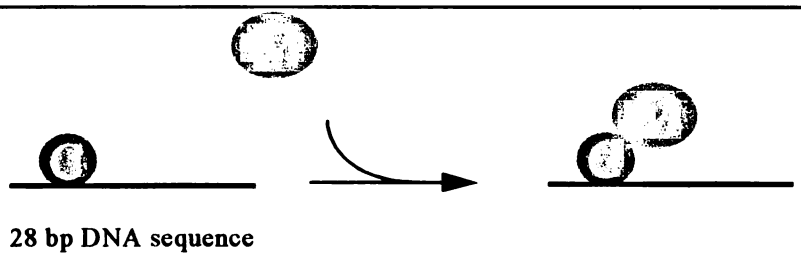


Figure 4.1. The formation of MDV unique DNA-protein complex by indirect-binding model.

conditions (which would not disrupt the DNA-binding activities of potential multiple subunit MDV factors), this result suggests that if a PRE-binding factor exists in MDV-infected cells, it can not bind to the EFI site. In total, the evidence obtained by southwestern blot assay and mobility shift assays surpport the "indirect-binding model", in which MDV-specific factors would target PRE cellular DNA-binding proteins rather than directly bind to PRE DNA sequence (Figure 4.1), while the compete-binding model is not strongly supposed.

As implied from above studies, cellular DNA-binding proteins might play an essential role in mediating MDV-enhanced RSV-LTR transactivation. Therefore, identification of these cellular proteins and understanding their interactions in formation of DNA-protein complexes are critical to further characterization and isolation of the MDV-specific factor(s).

In the RSV-LTR PREs region, there are several potential DNA-binding sites besides the previously reported EFI binding site. Among these sites, two sites are similar to recognized *ets* transcription factor core sequences (5'-GGAA-3'); and two sites are identical to Tax response elements (TRE, 5'-GGTGG-3') from the HTLV-LTR promoter. Results of mobility shift competition assays either from MDV-infected cell extracts or from un-infected cell extracts suggest that there are at least two cellular factor binding sites which are functionally important in the PRE region. One was the EFI binding site, and the other was one of the two *ets* binding sites in PRE region. This *ets*-related binding site has never been reported before, and was termed *ets*-like-element (ELE) in this study. Binding of these two cellular factors to the PRE region likely results in formation of the observed cellular protein-DNA complexes.

Primary studies of this newly discovered ELE factor have revealed a unique feature of its DNA-binding activity. Stable ELE factor binding to its own recognition sequence appears to be enhanced by protein-protein interaction with EFI factor. In support of this, ELE binding site alone was not able to generate any DNA-protein complex in electrophoretic mobility shift analyses. However, when both ELE and EFI binding sites are present and intact, a large cellular DNA-protein complex is generated (complex III), EFI binding to its site alone appears to be responsible for formation of the smaller cellular DNA-protein complex (complex I). The requirement for both EFI and ELE sites in forming a large DNA-protein complex (complex II) suggests that interaction between EFI and ELE factors might enhance ELE DNA-binding activity. In the other words, the interaction between EFI and ELE may convert a low-affinity ELE binding to a high-affinity ELE binding, or induce ELE DNA-binding activity by releasing an inhibited DNA-binding domain. In the first case, ELE alone may have a very low DNA-binding affinity. A similar situation was also found in HSV-1 VP16 where the DNA-binding affinity of HSV-1 VP16 is extremely low (Kristie et al., 1990). In order to bind to VP16 *cis*-acting sites, VP16 requires an interaction with an adjacent cellular factor, Oct-1 (Gerster et al., 1988; O'Hare et al., 1988). Protein-protein interaction between VP16 and the Oct-1 POU-homeo domain stabilizes the interaction between VP16 and its *cis*-acting promoter site (Roizman and Sears, 1996). Alternatively, ELE DNA-binding domains may be inhibited in their native form. Protein-protein interaction with EFI factor could cause a conformational change in ELE factor and release ELE DNA-binding domains. Release of the protein DNA-binding domain has been reported in studies of CBF and Ets interaction (Wotton et al., 1994; Giese et al., 1995; Wargnier et

al., 1995). For example, in the TCRα enhancer, cooperative DNA binding was found between the lymphocyte-specific proteins PEBP2α (CBFα) and Ets-1 (Giese et al., 1995). Such interactions may antagonize an inhibitory domain between amino acid 207 and 280 of Ets-1 that was found previously to impair DNA-binding activity of Ets-1 (Lim et al., 1992). A similar situation may occur here since the EFI factor is highly related to CBF (Faber et al., 1990), and the ELE factor recognizes an *ets*-like-element. This CBF/Ets interaction has been proposed in another retrovirus promoter, Murine leukosis virus (MLV) LTR promoter (Wotton et al., 1994). Furthermore, detection of a potential ELE candidate in southwestern blot assays (Figure 3.5) may have occured when the DNA-binding inhibition domain was released due to the denature/renature process, where ELE protein ternary structure only partially recovers on nitrocellulose membranes.

Besides a potential role in activating ELE factor DNA-binding activity, EFI/ELE interaction also resulted in formation of a more stable DNA-protein complex. In mobility shift competition assays, C/EBP consensus sequence was used as an EFI specific competitor to investigate the role of EFI factor in forming complex I and complex II. Consistent with the idea that EFI factor is a component of both complex I and complex II, both complexes were eliminated with an increasing amount of C/EBP competitor. However, the rate of dissociation among these two complexes was significantly different. Reduction of complex II was much slower than that of complex I, suggesting that binding of EFI factor in complex II was more stable than complex I. Under the current model, in cellular protein-DNA complex II, EFI factor not only binds to its DNA site but also interacts with ELE factor. The interaction between EFI and ELE factors may have stabilized DNA-protein interaction

between EFI factor and its cognate DNA-binding site.

Functional study of these two cellular factors has revealed that both EFI and ELE sites are critical for maintaining strong RSV-LTR promoter activity. Point mutations in either EFI or ELE sites significantly reduces RSV-LTR promoter activity. From our understanding of cellular protein-DNA complex formation, only complex II requires both EFI and ELE binding sites to be intact. Therefore, formation of cellular protein-DNA

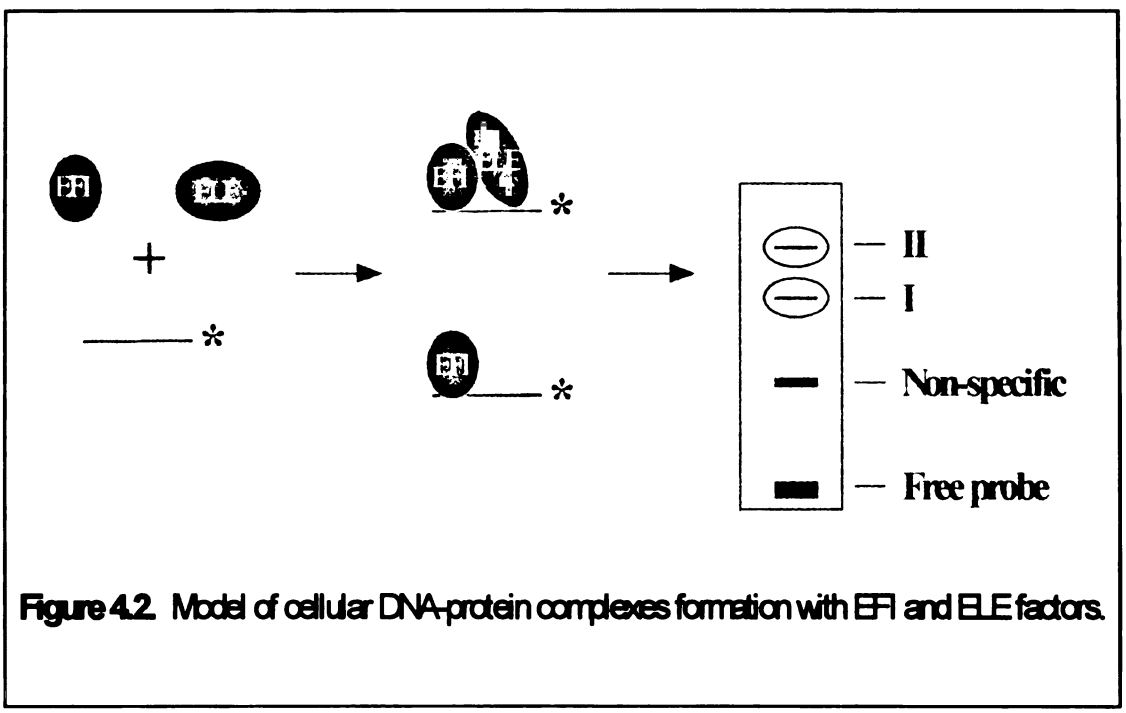


Figure 4.2 Model of cellular DNA-protein complexes formation with EFI and ELE factors.

complex II may be critical for maintaining RSV-LTR promoter activity, while cellular protein-DNA complex I may represent an intermediate form of protein-DNA interaction, before formation of functional complex II, as illustrated in Figure 4.2.

Results from mobility shift competition assays using MDV-infected cell extracts

further confirmed findings from southwestern blot and mobility shift assays, that is, MDV-specific factors interact with the PRE region through association with cellular DNA-binding proteins rather than by direct binding to PRE DNA sequences. Although both cellular protein-DNA complexes (I and II) could have served as targets for MDV-specific factors, only one unique DNA-protein complex was found following addition of MDV-specific factors, indicating that only one of the two cellular protein-DNA complexes could be recognized by MDV-specific factors. Results from mobility shift assays suggest that neither EFI alone nor ELE alone could form this unique MDV protein-DNA complex. However, with both sites present, the unique complex could be easily detected. Only cellular protein-DNA complex II requires both EFI and ELE binding sites. This suggests that MDV specific factor(s) could only recognize cellular protein-DNA complex II, therefore, formation of the large cellular protein-DNA complex (complex II) appears to be critical for formation of the

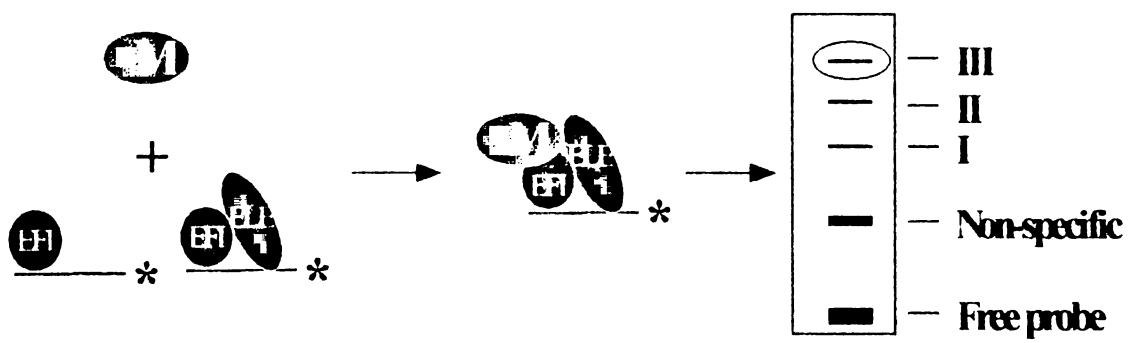


Figure 4.3. Model of the MDV-unique DNA-protein complex (complex III) formation.

MDV unique complex. Consistent with this interpretation, MDV-mediated RSV-LTR transactivation requires both EFI and ELE binding sites to be intact. Mutation in either the EFI binding site or the ELE binding site diminished basal RSV-LTR promoter activity as well as MDV-mediated RSV-LTR transactivation, illustrated in Figure 4.3.

In conclusion, systematic studies of RSV-LTR promoter activity and related DNA-protein interactions, suggest that cellular DNA-binding proteins, rather than PRE DNA sequence directly, interact with MDV-specific factor(s). two cellular transcription factor binding sites found in the PRE region were important for MDV-mediated RSV-LTR transactivation. Among the two cellular factor binding sites, the ELE site represents a newly observed ets-like element. A unique feature of ELE protein-binding activity was the dependence of ELE binding proteins on interaction with EFI factor. Protein-protein interactions between EFI and ELE factors enhanced ELE DNA-binding activity and led to the cellular protein-DNA complex II formation. Cellular protein-DNA complex II then serves as a target for MDV-specific factors and results in formation of a unique MDV DNA-protein complex (III). Loss of ability to form the unique MDV DNA-protein complex (III) coincided with loss of MDV-mediated RSV-LTR transactivation.

II. Future Research

As mentioned in the literature review, herpesvirus IE genes encode viral regulatory proteins which affect homogenous gene expression as well as heterologous promoter activities. Several MDV-encoded viral transactivators have been found in MDV infected cells. MDV ICP4 and MDV ICP27 are both capable of modifying RSV-LTR promoter activity (Banders et al., 1994; Ren et al., 1997). These two factors may partially account for MDV-mediated RSV-LTR transactivation. However, a unique feature of MDV-mediated enhancement of ALV induced LL was that only serotype 2 MDV was strongly associated with LL enhancement while the other serotypes (1 and 3) were not. This suggests that only serotype 2 MDV specific factor may be involved. This (these) unique factor(s) may be specific forms of MDV ICP4 and ICP27. Alternatively, there may be an, as get undiscovered, serotype 2 specific factor responsible for ALV and RSV promoter activation. In the future studies, our findings of DNA-protein and protein-protein interactions involved in formation of the unique complex identified to MDV infected cell lysates will facilitate isolation and identification of these MDV-specific factors. Based on protein-protein interaction between cellular DNA-binding proteins and MDV-specific factor, co-immunopercipitation assay can be used to detect and isolate the MDV-specific factors. However finding a MDV serotype 2 specific gene which encodes this(these) protein(s) will have to depend on other methods, such as the yeast two-hybrid system, or RNA differential display.

All the studies of MDV-mediated RSV-LTR transactivation and the future isolation

of MDV-specific transactivator were aimed to elucidate the role of MDV co-infection in ALV tumorigenesis. As discussed in the literature review, MDV-mediated ALV-LTR up-regulation could result in a higher level of ALV replication, and further enhance ALV tumorigenesis at the initiation stage. Many evidence including the results from this study have supported this mechanism.

As mentioned earlier, ALV-LTR promoter also involves in the second stage of ALV tumorigenesis (tumor promotion), theoretically, up-regulated ALV-LTR promoter in MDV co-infected cells could result in a further deregulation of c-myc gene expression. This increased c-myc gene expression may also contribute to LL enhancement in ALV/MDV co-infected chickens. However, several evidences from previous studies suggested that MDV was latent in the tumor promotion stage. In ALV/MDV co-infected birds, an in situ hybridization of ALV-induced preneoplastic follicles had detected MDV genome but not MDV antigens in those follicles, which strongly suggested MDV latency in ALV/MDV co-infected cells in promotion stage (Marsh et al., 1995). Generally, most viral antigen found in MDV lytic infection are no longer expressed in MDV latently in fected cells. In the case of ALV/MDV co-infection, the transition of MDV lytic infection to MDV latent infection could result in loss of MDV-specific factor which is reponsible for MDV-mediated ALV-LTR transactivation. To investigate the role of MDV in the second stage of tumor formation, a transient expression assay can be used to study ALV-LTR promoter activity in MDV latently infected cells. MDV serotype 2 latent infection has been found in tumor cells isolated from ALV/MDV co-infected birds. Therefore, cell line derived from those tumor cells can be used to exam RSV-LTR promoter activity in the transient expression assay.

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