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Identification of the Marek's disease virus homolog of the herpes simplex virus type 1 (HSV-1) UL16 gene

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of the requirements for

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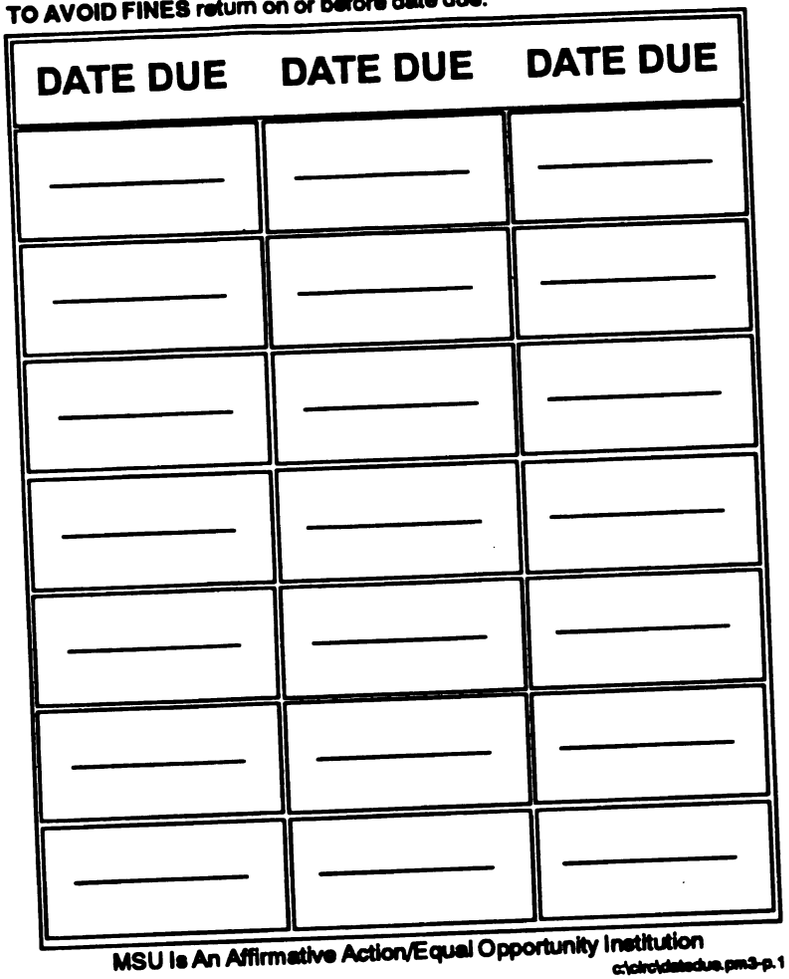

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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF THE MAREK'S DISEASE VIRUS (MDV) HOMOLOG OF THE HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) VP16 GENE

By

Mekki Boussaha

A DISSERTATION

submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

1996

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ABSTRACT

by

Mekki Boussaha

A Marek's disease virus (MDV) gene encoding a homolog to the HSV-1 VP16 protein has been identified and sequenced (Yanagida et al., 1993). The MDV VP16 coding region is 1278 nucleotides long and capable of encoding a protein with a calculated molecular weight of 48,000 daltons. Predicted amino acid sequence of MDV VP16 shows considerable homology (greater than 30 % overall in all cases examined) with VP16 homologs of other alphaherpesvirus . However, MDV VP16 is 64 amino acids shorter than HSV-1 VP16, lacking a region corresponding to the HSV-1 VP16 carboxyl-terminal acidic activation domain. In this report, we show that the MDV VP16 molecule is able to transactivate both MDV and HSV-1 immediate early gene promoters. An examination of the deduced amino acid sequence revealed two potential transactivation domains within the amino-terminal region of MDV VP16. These include a highly acidic domain (23 % acidic residues), defined by residues 1 to 60, and a proline-rich (23%) domain defined by amino acids 61 to 90. By comparing VP16 homologs using hydrophobic cluster analysis of several VP16 homologs, including MDV VP16, revealed conservation of bulky hydrophobic clusters critical for VP16 activity. Of particular interest in these studies were bulky hydrophobic residues surrounding the MDV VP16 Phe^{43} residue. Substitutions of MDV VP16 Phe^{43} with aromatic residues

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preserves MDV VP16 activity whereas substitutions of MDV VP16 Phe[43] with non-aromatic amino acids abolishes MDV VP16 transactivation function, similar to that observed with HSV-1 VP16. Furthermore, transactivation of MDV ICP4 promoters is dependent upon presence of an intact TAATGARAT element upstream of target genes. Deletion and mutational analysis within the MDV ICP4 gene promoter sequences revealed that the upstream ATGCAtATATTAT element at position 572 is important for MDV VP16 activation function. The ATGCAtATATTAT is highly homologous to the extended OCT-1 binding site ATGCAAATGARAT, which has been shown to be critical for activation functions of VP16 gene family members.

To My Parents

For

Their Love and Support

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Robert Cook, Dr.

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Acknowledgments

I am very thankful for the superb technical assistance, precious advice, and encouragment of Dr. Pau M. Coussens. I also thank Dr. Steven Triezenberg, Dr. Robert Cook, Dr. Bob silva, and Dr. Roger Maes for their technical advice. Special thanks are also due to my fellow students in Dr. Coussens lab.

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I) RNA

II) - Ger

a

b

c

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f

III) - Ba

a

I

c

IV) - Re

Table of Contents

Chapte

I) - Hist

II) - Bio

a

b

c

III) - Pat

a

b)

c)

List of Figures

Table 1

homolog

Table 2

domain

List of tables

AGP

ALV

BHV

CEF

CHX

CKC

CMV

CTD

CTF

DHFR

DPI

DR

E genes

EBV

EBNA-2

EHV

gB/C/D/E/I/K

List of Abbreviations

AGP	Agar-gel precipitin
ALV	Avian leukosis virus
BHV	Bovine herepsvirus
CEF	Chicken embryo fibroblast
CHX	Cyclohexamide
CKC	Chicken kidney cells
CMV	Cytomegalovirus
CTD	Carboxyl-terminal domain
CTF	Cellular transcription factor
DHFR	Dihydrofolate reductase
DPI	Days post-infection
DR	Direct repeat
E genes	Early genes
EBV	Epstein-Barr virus
EBNA-2	EBV nuclear antigen 2
EHV	Equine herpesvirus
gB/C/D/E/I/K	Glycoprotein B/C/D/E/I/K

GTF	General transcription factor
HCA	Hydrophobic cluster analysis
HCF	Host cell factor
HSV-1/2	Herpes simplex virus type 1 and 2
HVT	Herpesvirus of turkey
IE	Immediate early
IF	Immunofluorescence
IRL	Inverted repeat long
IRS	Inverted repeat short
Kbp	Kilobasepairs
L genes	Late genes
MD	Marek's disease
MDV	Marek's disease virus
NER	Nucleotide excision repair
NaOH	Sodium hydroxide
ORF	Open reading frame
PAA	Phosphonoacetic acid
PCR	Polymerase chain reaction
PEG	polyethelene glycol
pp14/38	Phosphoprotein 14/38
RNAPII	RNA polymerase II

SDS

SRB

TAF

TBP

TFIIA/B/D/E/

TK

TRL

TRS

UL

US

VP16

vvMDV

VZV

SDS	Sodium dodecyl-sulfate
SRB	Suppressor regulatory protein
TAF	TBP-associated factor
TBP	TATA-binding protein
TFIIA/B/D/E/F/H/K	Transcription factors II
TK	Thymodine Kinase
TRL	Terminal repeat long
TRS	Terminal repeat short
UL	Unique long
US	Unique short
VP16	Virion protein 16
vvMDV	very virulent MDV
VZV	Varicella-zoster virus

Chapter 1

Transcription regulation: Lessons from the VP16 gene family

Differentiation and development in eukaryotes is largely regulated at the level of transcription. The transcriptional control regions of eukaryotic genes often consist of networks of DNA-binding sites for factors that regulate the rate of transcription initiation at promoters. The promoter of a typical eukaryotic protein-encoding gene contains a TATA box, which is located 25 to 30 base pairs upstream of the transcription start site and an initiation sequence surrounding the start site. These elements comprise the core promoter region which is recognized by the general transcription machinery. In addition, promoter-specific factors bind to specific arrays of DNA recognition sites unique to each gene, both in the promoter proximal region and distal to the transcriptional start site. Signalling between transcriptional regulators and basal transcription factors requires co-activators that are not required for basal transcription but, rather, assist or mediate activation and perhaps repression of transcription (Pugh and Tjian, 1990).

Initiation of messenger RNA transcription by the enzyme RNA polymerase II (RNAPII) is a complex process that requires assembly of a multi-component pre-initiation complex near the transcription start site (Buratowski et al., 1989; Zawel and Reinberg, 1993). This complex is responsible for promoter recognition and response to regulatory signals (Sawadago and Sentenac, 1990; Roeder, 1991). At least six transcription factors are required, in addition to RNAPII, for accurate and

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efficient initiation of transcription. These transcription factors consist of TFIIA, -B, -D, -E, -F, and -H (Matsui et al., 1980; Samuels et al., 1982; Davison et al., 1983).

I) - RNA polymerase II (RNAPII)

Eukaryotic cells contain three nuclear DNA-dependent RNA polymerases that transcribe different classes of genes (Roeder and Rutter,1969). RNA polymerase I synthesizes ribosomal RNA precursors. RNA polymerase II (RNAPII) transcribes protein-coding genes, while RNA polymerase III transcribes genes for small stable RNAs such as 5S ribosomal RNA and transfer RNA. Each of these enzymes requires a collection of additional factors for selective promoter recognition and regulated transcription initiation. The molecular basis of transcription and its regulation are best understood for genes transcribed by RNAPII with general transcription factors (GTFs) (Roeder, 1991; Hori and Cari, 1994; Maldonado and Reinberg, 1995). Eukaryotic RNAPII enzymes share three major features. First, RNAPII is generally composed of 10 ± 2 subunits. Second, the RNAPII enzyme always contains two large subunits with molecular sizes of approximately 220 and 140 kDa. Finally, RNAPII contains three subunits of 14 to 28 kDa that are also found associated with RNA polymerases I and III in all eukaryotes. These polypeptides are called "common" or "shared" subunits.

An interesting feature of RNAPII is that its largest subunit has a unique carboxyl-terminal domain (CTD) that is absent in prokaryotic RNA polymerases, and eukaryotic RNA polymerases I and III (Corden et al., 1990). An array of tandem

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heptapeptide repeats of the sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS; single-letter amino acid code), located at the carboxyl-terminal domain (CTD) of the largest subunit of RNAPII constitute a highly conserved region essential for viability. The CTD of RNAPII is phosphorylated at different stages of the transcription cycle, and is probably involved in transcriptional regulation. For instance, transcription from the murine dihydrofolate reductase (DHFR) promoter can only be accomplished by the form of RNAPII that contains the hypophosphorylated CTD (RNAPIIA), but not by the form that lacks it (RNAPIIB). Akoulitchev et al. (1995) showed that the CTD, but not its phosphorylation, is required for initiation of transcription, and that transcription requires CTD kinase activity provided by the CDK subunit of TFIIH.

II) - General transcription factors

a - Transcription factor TFIID

The first step in assembly of initiation complexes is binding of TFIID to the TATA element (Sawadago and Roeder, 1985; Nakajima et al., 1988; Van Dyke et al., 1988; Buratowski et al., 1989). TFIID is a site-specific, DNA-binding complex required for selective initiation of transcription by eukaryotic RNAPII. Binding of TFIID at the promoter region launches sequential recruitment of transcription factors TFIIA, TFIIB, TFIIF/RNAPII, TFIIE, and TFIIH to establish a functional multi-protein complex capable of transcriptional initiation (Zawel and Reinberg, 1993).

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Transcription factor TFIID contains the TATA-binding protein, referred to as TBP (Pugh and Tjian, 1992), tightly associated with several other polypeptides referred to as TBP-associated factors or TAFs (Dynlacht et al., 1991; Pugh and Tjian, 1991; Tanese et al., 1991). The C-terminal 180 amino acid domain of TBP can replace a TFIID fraction for basal transcription *in vitro* (Hoey et al., 1990; Horikoshi et al., 1990; Peterson et al., 1990). Amino acid comparisons of the C-terminal domain of TBP from numerous species exhibit considerable conservation of structure (Hoffmann et al., 1990a, b; Peterson et al., 1990; Kao et al., 1990; Gash et al., 1990). The C-terminal domain of TBP contains two DNA-binding repeats (66-67 amino acids each). These two direct repeats are nearly 40% identical and flank a basic region. The three-dimensional and crystal structures of TBP from numerous species predicted that TBP resembled a molecular saddle in which the C-terminal direct repeats straddle the DNA (Nikolov et al., 1992; Kim et al., 1993a; Kim et al., 1993b). The inner surface of the saddle interacts with the minor groove of TATA elements, causing prominent distortion of DNA. The outer protein surface is accessible for interactions with other transcription factors.

The N-terminal regions of TBP from different species vary greatly in size, sequence, and amino acid composition. The N-terminal region of TBP has no essential role in transcription. Indeed, some strains of *S. cerevesiae* can grow when the N-terminal region of TBP is either completely missing (Cormack et al., 1991; Gill and Tjian, 1991; Poon et al., 1991; Reddy and Hahn, 1991; Zhou et al., 1991), or replaced with a very different N-terminal region of human TBP (Cormack et al.,

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1991; Gill and Tjian, 1991).

TBP is tightly associated with polypeptides called TBP-associated factors (TAFs) (Dynlacht et al., 1991; Pugh and Tjian, 1991; Tanese et al., 1991). Several studies have demonstrated direct interactions of transcriptional activators not only with TBP (Sawadago and Roeder, 1985a; Abmayr et al., 1988; Horikoshi et al., 1988a, b; Stringer et al., 1990), but also with specific TAFs. These include interactions of Sp1 with Drosophila $TAF_{II}110$ ($dTAF_{II}10$) (Hoey et al., 1993; Gill et al., 1994), interactions of USF, Tat, CTF, and E1a with human $TAF_{II}55$ ($hTAF_{II}55$) (Chiang and Roeder, 1995), interactions of VP16, p53, NF-κB p65 with $dTAF_{II}60$, $dTAF_{II}40$, $hTAF_{II}80$ and $hTAF_{II}31$, (Goodrich et al., 1993; Thut et al., 1995), and interactions of a steroid receptor with $hTAF_{II}30$ (Chen et al., 1994). The mechanism by which TAFs transduce or relay signals from activators to the basal machinery is still unclear. TAFs can also interact with other TAFs and/or with GTFs. TAF-TAF interactions include interactions of $hTAF_{II}80$ with $hTAF_{II}250$, $hTAF_{II}31$, and $hTAF_{II}20$ (Hisatake et al., 1995), and interactions of $dTAF_{II}60$ with $dTAF_{II}250$ and $dTAF_{II}40$ (Weinzierl et al., 1993; Kokubo et al., 1994; Thut et al., 1995). TAF-GTF interactions include $dTAF_{II}40$ that interacts with TFIIB (Goodrich et al., 1993), $dTAF_{II}60$ that interacts with TBP (Weinzierl et al., 1993; Kokubo et al., 1994; Thut et al., 1995), $dTAF_{II}110$ that interacts with TFIIA (Yokomori et al., 1993a), and $dTAF_{II}80$ that interacts with TBP, TFIIE and the RAP74 subunit of TFIIF (Hisatake et al., 1995), and hTAF250 interacts with RAP74 subunit of TFIIF (Ruppert and Tjian, 1995). Although the mechanism by which these interactions enhance

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transcription is unclear, they could be involved in (1) recruitment and stability of TFIID binding on the promoter (Abmayr et al., 1988; Lieberman and Berk, 1994), and (2) recruitment and functional modulation of other general transcription factors (Horikoshi et al., 1988a; Choy and Green, 1993).

b - Transcription factor TFIIA

TFIIA has been identified as a two-subunit (43 and 12 kDa) complex in yeast (Ranish and Hahn, 1991), and as a three-subunit (37, 19, and 13 kDa) complex in human and *Drosophila* (Cortes et al., 1992; Yokomori et al., 1993b; DeJong and Roeder, 1993; Coulombe et al., 1992; Aso et al., 1994). The mechanism by which TFIIA influences transcription initiation by RNAPII is not entirely understood. TFIIA can enter the transcription pre-initiation complex at any time following TBP binding. TFIIA is not required for basal transcription using TBP and highly purified factors (Ma et al., 1993). TFIIA is capable of stimulating basal transcription by altering the conformation of TBP, and thus enhancing its ability to recognize and stably associate with various TATA elements (Buratowski et al., 1989; Aso et al., 1994; Lee et al., 1992; Imbalzano et al., 1994). In this regard, TFIIA may stimulate transcription by competing for TBP binding with negative factors (NC1, Dr1/NC2, HMG-1, and ADI), which prevent formation of functional pre-initiation complexes (Meisterernst et al., 1991; Meisterernst and Roeder, 1991; Inostroza et al., 1992; Ge and Roeder, 1994; Auble and Hahn, 1993). TFIIA may also affect transcription activation by sequence-specific activators (Meistrernst et

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al., 1991; Meisterernst and Roeder, 1991; Ma et al., 1993). Indeed, TFIIA interacts directly with GAL4-AH (Wang et al., 1992), the Epstein-Barr virus activator Zta (Lieberman and Berk, 1991), and HSV-1 VP16 (Yokomori et al., 1994; Ozer et al., 1994).

c - Transcription factor TFIIB

TFIIB binds to the TBP-DNA complex, where it recruits and positions the RNAPII/TFIIF complex (Buratowski et al., 1989). TFIIB has two domains that roughly correlate with its two functions. TFIIB consists of an amino-terminal region, with a putative metal-binding site that is essential for recruitment of RNAPII and TFIIF (Ha et al., 1993; Yamashita et al., 1993; Hisatake et al., 1993), probably through interactions with RAP30 of TFIIF (Ha et al., 1993), and a protease-resistant carboxyl-terminal core (TFIIBc) composed of two long amino-acid repeats. TFIIBc contains two similar domains (each encoded by one of the two repeats) connected by a short, random-coil peptide. TFIIBc is sufficient for interactions with the TBP-promoter complex (Ha et al., 1993; Yamashita et al., 1993), and can bind RNAPII in solution (Ha et al., 1993). TFIIB functions as the "bridging" factor between TBP-DNA and RNAPII/TFIIF complexes (Buratowski and Zhou, 1993; Barberis et al., 1993; Ha et al., 1993). In combination with RNAPII, TFIIB determines the transcription start site (Pinto et al., 1992; Li et al., 1994). TFIIB is also the target of several transcriptional activators. In this regard, acidic- (Lin and Green, 1991; Roberts et al., 1993), and proline-rich (Kim and Roeder, 1994) activation domains

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can interact directly with and recruit TFIIB into the pre-initiation complex.

d - Transcription factor TFIIF

TFIIF controls activity of RNAPII at both the initiation and elongation stages of transcription (Tan et al., 1994). Mammalian TFIIF is a heterodimer that consists of two subunits of 30 and 74 kDa each (Conaway and Conaway, 1993). These are designated RAP30 and RAP74 (RAP stands for RNA polymerase II-associating protein). RAP30 binds to RNAPII and prevents it from binding non-specifically to DNA (Killeen and Greenblatt, 1992). RAP30 shares sequence similarities to the Escherichia coli σ^{70} protein, and *B. subtilis* σ^{43} (Sopta et al., 1989; McCraken and Greenblatt, 1991). The RAP30 carboxyl-terminal region shares sequence similarities with the cryptic DNA binding domain present in conserved region 4 at the carboxyl-terminal domain of a bacterial σ factor (Garrett et al., 1992). As predicted by sequence alignment, this region was found to be capable of binding DNA (Tan et al., 1994). The TFIIF RAP74 has been less extensively characterized. RAP74 contains globular amino-terminal and carboxyl-terminal domains separated by a highly charged central region. RAP74 is required for efficient transcription initiation by RNAPII (Kephart et al., 1994; Yonaha et al., 1993). However, only the RAP74 amino-terminal domain is required for transcriptional elongation (Kephart et al., 1994). Some reports suggested that RAP74 may be a helicase, although no ATP-dependent helicase activity or ATPase has been detected (Flores et al., 1990).

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e - Transcription Factor TFIIE

TFIIE is a heterotetramer composed of two 56 kDa α-subunits (TFIIE-α) and two 34 kDa β-subunits (TFIIE-β) (Ohkuma et al., 1991; Inostroza et al., 1991). TFIIE enters the pre-initiation complex after recruitment of TFIIF/RNAPII (Inostroza et al., 1991). TFIIE interacts with several components of the RNAPII transcription complex. Indeed, TFIIE can bind selectively to the non-phosphorylated form of RNAPII (RNAPIIa) and to both subunits of TFIIF as well as TFIID (Maxon et al., 1994). Interactions of TFIIE with RNAPII are mediated by TFIIE-α (Maxon et al., 1994). TFIIE can also interact directly with TFIIH (Maxon et al., 1994), and thus mediate TFIIH-recruitment to the RNAPII transcription complex. TFIIH-recruitment mediates subsequent phosphorylation of the CTD of RNAPII by the TFIIH-associated kinase activity (Maxon et al., 1994). TFIIE can also interact with *ERCC-3* (Maxon et al., 1994), a DNA repair helicase of TFIIH (Schaeffer et al., 1993). These multiple interactions suggest that TFIIE is critical for both promoter clearance and transcription-coupled DNA repair (Drapkin and Reinberg, 1994).

f - Transcription factor TFIIH

TFIIH is one of the last factors to be recruited into the initiation complex. TFIIH is a multisubunit complex with several associated enzymatic activities (Gerard et al., 1991; Gileadi et al., 1992; Serizawa et al., 1992; Feaver et al., 1993). TFIIH possesses a kinase activity that is capable of phosphorylating the CTD of RNAPII, DNA-dependent ATPase and DNA helicase activities (Serizawa et

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al., 1993). TFIIH is also involved in nucleotide excision repair (NER) of DNA (Schaeffer et al., 1993). *S. cerevisiae* TFIIH exists in two forms. One form, ***HoloTFIIH***, is composed of a six subunit core/SSL2 complex tightly associated with a three subunit CTD-kinase complex designated TFIIK (Svejstrup et al., 1995). This form is active in transcription. The other form, ***repairsome***, is a multi-component complex of core/SSL2 and all other *S. cerevisiae* NER genes (RAD1, 2, 4, 10, and 14), and is active in NER (Svejstrup et al., 1995). TFIIH plays an important role in RNAPII CTD-phosphorylation, open-complex formation and promoter clearance. The CTD kinase resides with MO15/Cdk7, a cyclin-dependent kinase (Feaver et al., 1994). Cyclin H, the regulatory partner of MO15/Cdk7 is also found in the TFIIH complex (Serizawa et al., 1995). TFIIH can be targeted by several trancriptional regulatory proteins, such as HSV-1 VP16 and cellular protein p53 (Xiao et al., 1994)

III) - Basal transcription by RNAPII

a) Initiation complex formation

a-1) Template activation

DNA in eukaryotic cells is packaged into chromatin. The primary subunit of chromatin is the nucleosome core particle which consists of 146 bp of DNA wrapped around core histone octamer (Kornberg, 1974). The core histone octamer contains two (H2A-H2B) heterodimers and one $(H3\text{-}H4)_2$ tetramer (Eickbush and Moudrianakis, 1978). Chromatin structure can affect the process of RNAPII transcription by **(1)** hindering access of the general transcription machinery

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to promoter sequences, and **(2)** blocking the binding of sequence-specific transcription activator proteins to their promoter-binding sites (Felsenfeld et al., 1992). The mechanism by which RNA polymerases can unwind DNA to form a "transcription bubble" and elongate past a nucleosome without major dislocations is unclear. Recent biochemical and genetic studies imply that cellular factors - the SWI/SNF complex - are required to alter chromatin structure to allow transcription factors access to their binding sites (Kruger and Herskowitz, 1991; Hirschhorn et al., 1992; Peterson and Herskowitz., 1992; Winston and Carlson, 1992; Imbalzano et al., 1994). Interactions of SWI/SNF complex with nucleosomal DNA is dependent on an ATPase activity which is intrinsic to the complex (Côté et al., 1994). In yeast, SWI/SNF complex is composed of five subunits encoded by SWI1 (ADR6), SWI2 (SNF2), SWI3, SNF5, and SNF6 (Winston and Carlson, 1992). SWI/SNF complex can assist binding of basal transcription factors (Kwon et al., 1994; Imbalzano et al., 1994) and transcription activator proteins (Côté et al., 1994; Peterson and Herkowitz, 1992; Yoshinaga et al., 1992; Laurent and Carlson, 1992) to nucleosomal DNA, *in vitro*.

a-2) Pre-intiation complex formation

a-2-1) Stepwise model

Pre-initiation complexes are elaborate structures composed of general transcription factors and RNAPII (Buratowski et al., 1989; Zawel and Reinberg, 1993). In the stepwise model, pre-initiation complexes

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assemble in a cascade fashion starting with binding of TFIID to the promoter (Buratowski et al., 1989). TBP and TAF subunits of TFIID participate in DNA binding and promoter recognition (Verrijzer et al., 1995). No other transcription factor stably interacts with the promoter in the absence of TFIID binding (Van Dyke et al., 1988, 1989; Buratowski et al., 1989). After binding of TFIID to DNA templates, the basal factors TFIIA and TFIIB are recruited to the promoter (Buratowski et al., 1989). TFIIB can associate with the TFIID-promoter complex whether or not TFIIA is present (Buratowski et al., 1989; Peterson et al., 1990). TFIIB acts as a molecular bridge, allowing subsequent recruitment of the TFIIF/RNAPII complex to TFIID-DNA complexes (Buratowski et al., 1989). The resulting assembled complex (composed of TFIID, TFIIA, TFIIB, TFIIF, and RNAPII), is sufficient to initiate transcription. Recruitment of additional factors, TFIIE and TFIIH, appears to direct later steps such as promoter clearance, elongation, and transcription-coupled DNA repair (Goodrich and Tjian, 1994).

a-2-2) Holoenzyme model

Genetic and biochemical studies in yeast have recently identified a large pre-assembled complex, termed RNA polymerase II holoenzyme, that consists of RNAPII, a subset of GTFs, nine suppressor regulatory proteins of RNA polymerase B (SRB 2-11), GAL11, SUG1, and additional unidentified proteins. The feature that distinguishes the RNA polymerase II holoenzyme model from the stepwise model is stable interactions of RNAPII with the SRB proteins and GTFs

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independently of DNA template, *in vitro* (Kim et al., 1994; Koleske and Young, 1994). In the holoenzyme model, the two major regulatory steps in transcription initiation are formation of a TFIID-bound promoter and association of the RNA polymerase II holoenzyme with TFIID-DNA complex. Several forms of RNA polymerase II holoenzyme have been described. The larger form of RNA polymerase II holoenzyme contains RNAPII, TFIIB, TFIIF, TFIIH, and SRB regulatory proteins (Koleske and Young, 1994). The second form of RNA polymerase II holoenzyme contains RNAPII, TFIIF, and SRB proteins. These two forms of RNA polymerase II holoenzyme may exist simultaneously *in vivo*, or the smaller RNA polymerase II holoenzyme may, in fact, be a subcomplex of the larger form and appears to be a consequence of instability of the latter one during purification. RNA polymerase II holoenzymes are capable of site-specific initiation of transcription when supplemented with the missing GTFs, and are responsive to transcriptional activators (Kim et al., 1994; Koleske and Young, 1994). These features, not observed with purified RNAPII and GTFs alone (Flanagan et al., 1991; 1992), suggest a model in which transcriptional activators may function through direct interactions with the RNA polymerase II holoenzyme. Indeed, a subcomplex, called the mediator of activation, can be dissociated from the RNA polymerase II holoenzyme by using monoclonal anti-CTD antibodies (Kim et al., 1994). The purified mediator contains SRB proteins, SUG1, GAL11, and TFIIF. Genetic studies suggested previously that GAL11, SUG1, and SRB proteins are involved in transcriptional regulation (Fassler and Winston, 1989; Himmelfarb et al., 1990;

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Nishizawa et al., 1990; Vallier and Carlson, 1991; Yu and Fassler, 1993). Genetic and biochemical interactions have described physical and functional interactions between the RNAPII CTD and SRB regulatory proteins, and thus may have important implications for the function(s) of the RNAPII CTD in transcription initiation. *SRB10* and *SRB11* encode kinase and cyclin-like polypeptides that are tightly associated with the holoenzyme and appear to have roles in CTD phosphorylation (Liao et al., 1995). The role of CTD phosphorylation in transcription is not yet clear (Li and Kornberg, 1994; Serizawa et al., 1993), but it may disrupt interactions between RNAPII and GTFs to stimulate promoter clearance (Zawel and Reinberg, 1992; Dahmus and Dynan, 1992; O'Brien et al., 1994).

a-3) Open complex formation

After RNAPII and the GTFs assemble on promoter DNA (closed complex assembly), a short stretch of DNA around the transcription start site becomes unwound and serves for abortive transcription. This process is referred to as the open complex formation. Minimal initiation complexes containing TBP, TFIIB, TFIIF, and RNAPII are efficient of cycling short abortive transcripts specific to the transcription start site, indicating that the DNA around the start site is in the open or melted configuration. Transition to a fully open complex requires ATP β-γ bond hydrolysis, TFIIE and TFIIH (Laybourn and Dahmus, 1990; Wang et al., 1992). ATP may be used by DNA helicases to melt the start site and/or by CTD kinases to phosphorylate the CTD of RNAPII. TFIIH has both ATP-dependent helicase and

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CTD-kinase activities (Drapkin and Reinberg, 1994). Open complex formation may be targeted by transcriptional activators. Indeed, GAL4-VP16 derivatives were reported to facilitate the process of open complex formation (Wang et al., 1992; Jiang et al., 1994).

b) Promoter escape by RNAPII

Promoter clearance by RNAPII is the event during which RNAPII escapes the promoter to elongate primary transcripts (Goodrich and Tjian, 1994). Minimal initiation complexes containing TBP, TFIIB, TFIIF, and RNAPII are efficient in cycling short abortive transcripts specific to the transcription start site. Addition of TFIIE and TFIIH results in formation of active transcription complexes that are competent to advance through the promoter clearance stage and proceed into an elongation complex, upon addition of nucleoside triphosphates (Goodrich and Tjian, 1994). Production of extended transcripts requires hydrolyzable ATP (Goodrich and Tjian, 1994). TFIIH-associated helicase and ATP hydrolysis stretch the melted region of DNA, probably in the direction of transcription, prior to transcript formation. The RNAPII system requires a pair of GTFs (TFIIE/TFIIH) following polymerase entry because this pair somehow modifies polymerase and/or the initiation complex. Such modifications could involve the CTD of RNAPII. Phosphorylation of RNAPII CTD generates at least two RNAPII isoforms, one with CTD hyperphophorylated (RNAPIIO), the other with CTD non-phosphorylated (RNAPIIA) (Dahmus, 1981; Cadena and Dahmus, 1987; Baskaran et al., 1993).

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TFIIH contains a kinase activity capable of phosphorylating the CTD of RNAPII (Drapkin and Reinberg, 1994). RNAPIIA interacts with TBP (Usheva et al., 1992) and TFIIE (Maxon et al., 1994), and participates in complex assembly and transcription of sequences proximal to the promoter (Lu et al., 1991; Chestnut et al., 1992; O'Brien et al., 1994). RNAPIIO, however, catalyzes RNA chain elongation (Cadena and Dahmus, 1987; O'Brien et al., 1994; Weeks et al., 1993). Negative supercoiling can, however, functionally replace the requirements for TFIIE, TFIIH, and ATP hydrolysis, indicating that negative supercoiling expedites promoter clearance (Goodrich and Tjian, 1994). These findings suggested a role of TFIIH-associated kinase activity and supercoiling in promoter escape by RNAPII, but not open complex formation as previously suggested (Schaeffer et al., 1993).

c) Elongation and termination

The mechanism of transcriptional elongation of eukaryotic messenger RNA synthesis is not completely understood. Recently, however, several developments have offered new insights into possible transcriptional elongation mechanisms. Biochemical studies have defined several factors involved in the elongation process by RNAPII. These elongation factors include TFIIS (SII), TFIIF, and the elongin/SIII complex. Mechanistic studies indicate that SII is capable of increasing the overall rate of duplex DNA transcription by RNAPII (Chen et al., 1992). SII does not increase the catalytic rate of nucleotide addition to a growing transcript. Rather, SII appears to facilitate passage of RNAPII through a variety of

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transcriptional blocks (nucleoprotein complexes, intrinsic arrest sites, and pausing sites), which can lead RNAPII to pause, terminate, or resume transcription and pass through terminators (Rudd et al., 1994). Upon encountering a block to transcription, RNAPII concludes elongation, but can be reactivated by SII (Kerpolla and Kane, 1991; Kane, 1994; Reines, 1994). SII-dependent read-through is proceeded by endonucleolytic cleavage and re-extension of nascent transcripts held in the polymerase active site (Reines, 1992; Izban and Luse, 1992; Wang and Hawley, 1993). Cleavage of nascent transcripts by SII requires: (i) divalent cations (Mg^{2+}or Mn^{2+}), and is inhibited by low concentrations of α-amanitin; and (ii) physical interactions between RNAPII and the cleaved RNA.

TFIIF and elongin/SIII increase the overall rate of RNA chain elongation by RNAPII (Bengal et al., 1991; Garrett et al., 1994). TFIIF is unique among other GTFs because of its ability to support both transcription initiation and elongation . Mechanistic studies indicated that TFIIF increases the rate of RNA chain elongation by RNAPII (Bengal et al., 1991; Garrett et al., 1994). The RAP74 subunit of TFIIF stabilizes binding of TFIIF to RNAPII and increases TFIIF elongation activity (Kitajima et al., 1994). TFIIF is not capable of releasing RNAPII from arrest at transcription blocks. Rather, TFIIF decreases the likelihood that RNAPII will suffer arrest at these sites (Cu and Reines, 1995). The mechanism by which TFIIF and elongin/SIII increase the elongation rate is not completely understood.

In addition to transcription blocks that can halt elongation, RNAPII elongation complex is also the target of transcription-coupled nucleotide excision repair (NER)

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(Mellon et al., 1987). TFIIH is closely involved in NER (Schaeffer et al, 1993). The largest subunit of mammalian TFIIH is the product of the NER gene *XPB/ERCC3* (Schaeffer et al., 1993). The products of the known NER genes *RAD3/XPB, TFB1/p62,* and *SSL1/p44* are also among the subunits of S. cerevisiae and mammalian TFIIH (Feaver et al, 1993; Schaeffer et al., 1994; Humbert et al., 1994; Drapkin et al., 1995).

VI) - Regulation of RNAPII transcription

Transcriptional activation of eukaryotic protein-coding genes during development or in response to extracellular signals can be accounted by the combinatorial effect of upstream activator proteins targeted to enhancer and promoter regions by sequence-specific DNA-binding (Maniatis et al.,1987; Mitchell and Tjian, 1989). Key players in this process are sequence-specific transcription factors that select genes to be activated and assist assembly of a stable transcription pre-initiation complex at the start site of mRNA synthesis by RNA polymerase II (Ptashne and Gann,1990). GTFs and RNAPII bind in the surroundings of initiation start sites to support a basal level of transcription. To achieve an induced level of transcription, factors of the regulatory family bind to one or more of a variety of DNA sequence elements located farther away. How these regulatory factors operate to boost the frequency of initiation by RNAPII remains unclear.

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a) Activators and their targets

How transcriptional activators act to increase the frequency of transcription initiation is unclear. Transcription by RNAPII can be divided into three major steps: **(1)** Initiation complex formation, **(2)** promoter clearance, and **(3)** elongation and termination. Transcriptional regulatory factors may target some or all of these discrete steps to increase the rate of transcription by RNA polymerase II. The mechanisms through which transcriptional activators exert their influence over RNA polymerase II machinery is the focus of the following sections.

a-1) Activators and initiation complex formation

As a first step to comprehend the mechanism of activation of transcription initiation complex assembly, it is important to identify interactions of regulatory proteins with individual transcription factors. Transcripton initiation complex formation can be subdivided into three major steps: ***(1)*** template activation - disruption of nucleosomal DNA by the SWI/SNF complex; ***(2)*** pre-initiation complex formation - recruitment of TFIID, TFIIA, TFIIB, and TFIIF/RNAPII to form the closed initiation complex; and ***(3)*** open complex formation - ATP β-γ bond hydrolysis and RNAPII CTD phosphorylation may be required for this transitition. Transcriptional activators were reported to interact directly with several of the aforementioned transcription factors which are involved in the three phases of transcription initiation complex assembly. These interactions are the focus of the following sections.

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a-1-1) ***Activators and nucleosomal DNA***

The assembly of naked template DNA into nucleosomes interferes with the ability of DNA to be transcribed, by blocking access of transcription factors to the DNA template (Tsuda et al., 1986; Knezetic and Luse, 1986; Workman and Roeder, 1987; Paranjape et al., 1994). Association of several transcriptional activators with nucleosomal DNA can induce a change in nucleosomal structures which facilitate recruitment of GTFs to the template DNA (Workman et al., 1988; Workman et al., 1990; Workman et al., 1991). The SWI/SNF complex mediates an ATP-dependent disruption of nucleosomal DNA, enabling transcriptional activator proteins, such as GAL4, *Drosophila ftz* (Peterson and Herkowitz, 1992), mammalian glucocorticoid and estrogen receptor (Yoshinaga et al., 1992), LexA-GAL4 (Laurent and Carlson, 1992), GAL4-VP16 and GAL4-AH (Kwon et al., 1994), to bind to DNA within a nucleosome core.

a-1-2) Activators and TBP

TBP was the first general transcription factor shown to interact with an activation domain (Stringer et al., 1990; Ingles et al., 1991). Several other activation domains have, subsequently, been reported to bind TBP, *in vitro*. These include ***(1)*** viral activators: E1A, Zta, Tat, Tax1, and IE2; ***(2)*** cellular proteins: E2F-1, p53, PU.1, Sp1, Oct-1, Oct-2, c-Rel, and c-Fos; and ***(3)*** yeast protein GAL4 (Lee et al., 1991; Lieberman and Berk, 1991; Kashanchi et al., 1994; Caron et al., 1993; Hagemeier et al., 1992; Emili and Ingles, 1995; Truant et al., 1993;

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Hagemeier et al., 1993; Emili et al., 1994; Zwilling et al., 1994; Metz et al., 1994; Melcher and Johnston, 1995). Interactions between transcriptional activators and TBP may be involved in *(1)* recruitment and stability binding of TBP on the promoter, and/or *(2)* conquer the inhibitory effects of certain proteins (NC1, Dr1/NC2, HMG1, and ADI) that prevent TBP from initiating assembly of the pre-initiation complex (Kraus et al., 1994).

a-1-3) Activators and TAF_{II}s

Several studies have demonstrated direct interactions of a number of transcriptional activators with TAF_{II} subunits of TFIID. These include interactions of Sp1 with Drosophila $TAF_{II}110$ ($dTAF_{II}110$) (Hoey et al., 1993; Gill et al., 1994), interactions of USF, Tat, CTF, and E1a with human $TAF_{II}55$ ($hTAF_{II}55$) (Chiang and Roeder, 1995), interactions of VP16, p53, NF-κB p65 with $dTAF_{II}60$, $dTAF_{II}40$, $hTAF_{II}80$ and $hTAF_{II}31$, (Goodrich et al., 1993; Thut et al., 1995), and interactions of a steroid receptor with $hTAF_{II}30$ (Chen et al., 1994). Although the mechanism by which these interactions enhance transcription is unclear, they could be involved in *(1)* recruitment and stability of TFIID binding on the promoter (Abmayr et al., 1988; Lieberman and Berk, 1994), and *(2)* recruitment and functional modulation of other general transcription factors (Horikoshi et al., 1988; Choy and Green, 1993).

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a-1-4) Activators and TFIIB

TFIIB-association with TFIID-promoter DNA complex is a rate limiting step in transcription pre-initiation complex formation (Lin and Green, 1991). Acidic- (Choy and Green, 1994; Kim et al., 1994) and proline-rich (Kim and Roeder, 1994) activation domains can interact directly with TFIIB. How activators increase transcription rates by interacting with TFIIB may be a bipartate process. First, the amino- and carboxyl-terminal domains of TFIIB interact with one another, creating a closed structure which prevents TFIIF/RNAPII recruitment. Binding of VP16 to TFIIB appears to induce a conformational change in the structure of TFIIB that opens up the molecule and exposes surfaces within TFIIB which are critical for binding of TFIIF/RNAPII complexes (Roberts and Green, 1994). Second, TFIIB release during transcriptional elongation interferes with recruitment of another molecule of RNAPII for a subsequent round of transcription initiation (Reines, 1991). Interactions of activators with TFIIB may increase the rate of TFIIB-reassociation, following promoter clearance, facilitating RNAPII-recruitment and subsequent rounds of transcription initiation.

a-1-5) Activators and TFIIA

Several lines of evidence indicated that TFIIA stimulates activator-mediated transcription (Ma et al., 1993; Ozer et al., 1994; Yokomori et al., 1994). Transcriptional activators that interact with TFIIA include VP16, Sp1, NTF-1, and Zta. The role of TFIIA in activated transcription may be two fold. *(1)* TFIIA may

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act as an anti-repressor of TAF subunits of TFIID and in conjunction with activators, such Zta, overcome a slow step in pre-initiation complex formation (Chi and Carey, 1993). ***(2)*** TFIIA may increase stability of the TFIID-TFIIB-promoter complex. Interactions of TFIIA with transcriptional activators may facilitate formation of activator-TFIID-TFIIA-promoter complexes (Wang et al., 1992; Chi and Carey, 1993; Lieberman and Berk, 1994). Transcriptional activators stabilize the activator-TFIID-TFIIA-promoter complex relative to the TFIID-TFIIA-promoter complex. The increased stability of the activator-TFIID-TFIIA-promoter complex is likely to be the result of DNA-protein and protein-protein interactions. DNA-protein interactions include interactions between the activator DNA-binding domain and its binding sites and the activation domain-induced downstream TFIID-DNA interactions. Protein-protein interactions include interactions between the activator activation domain and the TBP subunit of TFIID (Lieberman and Berk, 1991). Because TAFs and TFIIA are required for formation of stable activator-TFIID-TFIIA complexes, additional contacts may occur between the activator activation domain, TFIIA, and TAFs. Interactions of TFIIA with TAF110 of *Drosophila* TFIID (Yokomori et al., 1993a), and the human co-activator PC4 (Ge and Roeder, 1994, Kretzschmar et al., 1994) are consistent with a specialized role of TFIIA in activation.

a-2) Activators and promoter escape by RNAPII

a-2-1) Activators and TFIIH

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activities involved in transcription initiation, transcriptional elongation, and DNA repair (Drapkin and Reinberg, 1994). Several reports indicate that TFIIH interacts with transcription activator proteins. These include interactions of TFIIH with HSV-1 VP16 and cellular protein p53 (Xiao et al., 1994), and with the EBV EBNA2 (Tong et al., 1995). These interactions may influence the role(s) TFIIH plays during transcription, although no stimulation of TFIIH-associated enzymatic activities by transcriptional regulatory proteins has yet been reported.

a-3) Activators and elongation and termination

Activators can stimulate transcription by increasing not only the rate of transcription initiation but also the efficiency of transcriptional elongation. Several activators have been implicated in regulation of the transcriptional elongation phase. These include GAL4-VP16, GAL4-AH, and GAL4-E1a (Yankulov et al., 1994). The mechanism by which transcriptional regulatory proteins increase the efficiency of elongation is unclear. Activators can, however, stimulate RNAPII transcriptional elongation by sequestering factors that are required during this step. These factors include SII, TFIIF, and SIII/elongin. Transcriptional activators can also stimulate transcription-coupled nucleotide excision repair (NER) by interacting with TFIIH.

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between protein factors and cis-regulatory elements within gene promoters and enhancers. Often, viruses exploit cellular mechanisms of gene regulation to control and promote expression of their own genes. In this regard, viruses offer a unique insight into eukaryotic transcriptional control mechanisms. In the case of herpes simplex virus type 1 (HSV-1), immediate-early (IE) genes are transactivated by VP16 (also called Vmw65, ICP25, α-TIF) (Campbell et al., 1984). HSV-1 VP16 is comprised of 490 amino acids and encodes a gene product of 54 kDa. HSV-1 VP16 is synthesized during the late phase of gene expresion. During virion assembly, HSV-1 VP16 is incorporated into the tegument between the capsid and virion envelope. HSV-1 VP16 is subsequently released during infection, whereupon it specifically induces transcription of viral IE genes (Post et al., 1981, Campbell et al., 1984). Specific induction of IE genes by VP16 requires at least one cis-acting DNA sequence motif, TAATGARAT (R = purine) (McKnight et al., 1987; O'Hare and Goding, 1988). HSV-1 VP16, which has no substantial affinity for double-stranded DNA (Marsden et al., 1987), functions by forming a multi-component complex on TAATGARAT sites in IE gene promoters (Triezenberg et al., 1988; Werstuck and Capone, 1989), together with the cellular POU domain protein Oct-1 and at least one other cellular factor called CFF, VCAF, or HCF (Katan et al., 1990; Xiao and Capone, 1990; Kristie et al., 1989). Mutational analysis of HSV-1 VP16 has identified a highly acidic domain (defined by 80 amino acids at the carboxyl terminus) as a potent transcriptional activating region (Triezenberg et al., 1988; Greaves and O'Hare, 1989). Activation domains have been typically classified into

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acidic (HSV-1 VP16, yeast GAL4 and GCN4), glutamine-rich (Sp1, Oct-1, Oct-2 and Jun), and proline-rich (CTF/NF1) (Mitchell and Tjian, 1989;). However, classification based upon the most conspicuous attributes of these domains may be deceptive. Recent studies on VP16 (Regier et al., 1993; Cress and Triezenberg, 1991) and the artificial domain AH (Ruden, 1994) uncovered that most activation domains are more sensitive to alterations in specific patterns of hydrophobic and aromatic amino acids than to mutations of acidic amino acids. The importance of hydrophobic and aromatic residues for activation is supported by studies of the mammalian cell acidic-rich protein p53 (Lin et al., 1994), the foamyvirus acidic-rich activator Bel-1 (Blair et al., 1994), as well as the prototypical glutamine-rich activators Sp1 (Gill et al., 1994) and Oct-1 (Tanaka and Herr, 1994). Thus, it appears that a specific pattern of bulky hydrophobic and aromatic amino acids may be more critical than were the more conspicuous and abundant acidic amino acids. In this regard, mutational studies of the HSV-1 VP16 acidic activation domain uncovered that a phenylalanine residue at position 442 (Phe^{442}) is critical for HSV-1 VP16 transcriptional activity (Cress and Triezenberg, 1991). Substitutions of HSV-1 Phe^{442} with hydrophobic and aromatic amino acids restored HSV-1 VP16 activity, whereas substitutions of HSV-1 Phe^{442} with non-aromatic amino acids abrogated HSV-1 VP16 activity (Cress and Triezenberg, 1991).

Several VP16 homologs from numerous herpesviruses have been identified. These include VP16 from several strains of HSV-1 (Pellet et al., 1985; Dalrymple et al., 1985), HSV-2 (Cress and Triezenberg, 1990), Varicalla-zoster virus (VZV)

(Davison and Scott, 1986; McKee et al., 1990), equid herpesvirus type 1 (EHV-1) (Purewal et al., 1992), equid herpes virus type 4 (EHV-4) (Purewal et al., 1994), BHV-1 (Carpenter and Misra, 1992), and MDV VP16 (Yanagida et al., 1993). VZV open reading frame 10 (ORF10) is the most thoroughly studied among these VP16 homologs. VZV encodes a protein of 410 amino acids that is homologous to HSV-1 VP16 (Davison and Scott, 1986). While VZV ORF10 and HSV-1 VP16 show considerable amino acid homology within their amino-terminal regions, VZV ORF10 is 80 amino acids shorter than VP16, lacking sequences similar to that of the HSV-1 VP16 acidic activation domain (McKee et al., 1990). A recent study (Moriuchi et al., 1993) shows that the VZV ORF10 protein acts as a transactivator for both VZV and HSV-1 IE promoters in transient-expression assays. VZV ORF10 can also substitute for the transactivation function of HSV-1 VP16. Cell lines expressing VZV ORF10 are able to complement an HSV-1 VP16 mutant which lacks the transactivation function of VP16 (Moriuchi et al., 1993). Interestingly, the transcriptional activation domain of VZV ORF10 maps to the amino-terminal region of the protein (Moriuchi et al., 1995). Hydrophobic cluster analysis (HCA) and sequence alignment of HSV-1 VP16 and VZV ORF10, using the HSV-1 VP16 Phe^{442} as a guide, revealed conservation of a pattern of bulky hydrophobic residues. Of particular interest were bulky hydrophobic clusters surrounding the VZV ORF10 Phe^{28} residue (Moriuchi et al., 1995). Similar to Phe^{442} of HSV-1 VP16, substitutions of VZV ORF10 Phe^{28} with hydrophobic and aromatic amino acids preserved VZV ORF10 activity whereas substitutions of VZV ORF10 Phe^{28} with non-aromatic amino

acids abolished VZV ORF10 transactivation function, similar to that observed with HSV-1 VP16 (Moriuchi et al., 1995). Thus it appears that a common transactivation mechanism may exist between VZV and HSV-1 VP16 gene family members, despite significant differences in protein sequences and spatial arrangements of activator domains.

BHV-1 VP16 is also capable of transactivating IE gene promoters (Misra et al., 1994). Unlike the activation domain of the HSV-1 VP16, the carboxyl-terminus of BHV-1 VP16 is largely hydrophobic in character (Misra et al., 1994). When fused to the DNA-binding domain of GAL4, the BHV-1 VP16 transactivation domain displays a poor stimulation of promoters containing GAL4 binding sites (Misra et al., 1994). Deletions of the entire C-terminus of BHV-1 VP16 does not entirely destroy its transactivation functions (Misra et al., 1994), indicating that other sequences contribute to gene activation.

EHV-1 gene12, another homolog of the HSV-1 VP16, is 479 amino acids in length. Like VZV ORF10, EHV-1 gene12 lacks sequences similar to those of the HSV-1 VP16 acidic transactivation domain (Telford et al., 1992). EHV-1 gene12 is, however, capable of transactivating both homologous and heterologous (HSV-1) IE gene promoters. Mutational analysis of the EHV-1 gene12 have identified the C-terminal seven amino acids, that are similar to the extreme C-terminal region of HSV-1 VP16, as a potent transactivation subdomain.

MDV VP16 is another homolog of the HSV-1 VP16 gene (Yanagida et al., 1993; Boussaha and Coussens, unpublished data). Identification and functional

characterization of MDV VP16 are the focus of this thesis project.

Chapter 2

Marek's disease virus

1) - Historical perspective

Marek's disease (MD) is a naturally occurring lymphoproliferative and peripheral nerve demyelinating disease of chickens (Marek, 1907). Lesions in the nervous system, described as a "polyneuritis", were first reported in 1907 by Joseph Marek (Marek, 1907). Lymphomas involving the gonads, muscle, skin, and various visceral organs are attributes of MD (Payne, 1982). During the early 1960's, it was discovered that the etiologic agent of MD is a highly cell-associated avian herpesvirus, Marek's disease virus (MDV) (Churchill and Biggs, 1967; Nazerian and Burmester, 1968). MDV is transmitted horizontally in dust and dander, and has high mortality which led to condemnation of infected birds, until development of successful live-virus vaccines in the early 1970's (Churchill et al., 1969; Okazaki et al., 1970). Prior to widespread vaccination, MD was a disease of devastating economic proportions in the poultry industry. MDV remains as an important pathogen in intensive poultry operations, particularly with the emergence of very virulent strains of MDV that are resistant to current vaccines (Witter, 1985).

For several decades, there was a great deal of confusion regarding the nature of MD. Based on similarities between many of the resulting gross lesions, MD lymphoma (caused by a herpesvirus) and lymphoid leukosis (caused by a retrovirus), were once classified under the same heading, "Avian Leukosis

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Complex" (Anonymous, 1951; Cottral, 1952; Chubb and Gordon, 1957; Biggs, 1961; Campbell, 1954, 1961). It was not until the late 1960's that visceral lymphomas were shown to include two pathologically discrete lymphoid neoplasmas. MD lymphoma is now known to be caused by a herpesvirus and is primarily restricted to T-cells, while lymphoid leukosis is caused by a retrovirus, Avian Leukosis virus (ALV), and is largely restricted to B-cells (Churchill and Biggs, 1967; Solomon et al, 1968; Nazerian and Burmester, 1968). Confusion also existed as to whether MD lesions were inflammatory or neoplastic. Both inflammatory and neoplastic lesions occur in affected nerves of infected birds and are sometimes intermixed. Finally, there was confusion over transmissibility of MD. Transmission experiments using tumor or blood cells from affected birds gave variable and generally controversial results (Olson, 1940). In spite of all of these difficulties, some significant research, in the 1960's, contributed to a greater understanding of the nature of MD, and led to virtual control of the disease by vaccination. Three significant events were accomplished during this period: (1) The experimental transmission of MD by inoculation of susceptible young chicks with blood or lymphoma cells from infected birds (Biggs and Payne, 1963); (2) Recognition of the etiologic agent of MD as a highly cell-associated herpesvirus, Marek's disease virus (MDV) (Churchill and Biggs, 1967; Solomon et al., 1968; Nazerian and Burmester, 1968); and (3) production of live virus vaccines containing either tissue-culture-attenuated MDV (Churchill et al., 1969) or the antigenically related herpesvirus of turkeys (Okazaki et al., 1970).

In addition to its enormous economic relevance to the commercial poultry industry, MD has served as an exquisite model for herpesvirus oncology. MDV is one of numerous herpesviruses that are involved in the induction of tumors in their natural hosts. These herpesviruses include several human herpesviruses (Epstein-Barr virus, human cytomegalovirus, and human herpesvirus type 6), as well as representatives from a long list of animal herpesviruses (Zur Hausen, 1980; Nahmias and Norild, 1980; Naegle and Granoff, 1980, Falk, 1980, Hinze and Chipman, 1971). Unfortunately, many of these oncogenic herpesviruses cannot be studied experimentally in their natural host system. This is especially true for human herpesviruses where there is no appropriate experimental model. MD, however, has captured attention as a model for herpesvirus oncology for several major reasons. Well-characterized genetic strains of chickens have been produced that range from extremely susceptible to remarkably resistant to the disease. Specific pathogen-free experimental animals are readily available. Third, a large spectrum of virus strains ranging from very virulent to non-oncogenic and non-pathogenic have been isolated. Finally, MD is a naturally occurring disease that can be reproduced experimentally using natural methods of exposure in the natural host. Various virological and immunological aspects of MDV infection can serve as a model of herpesvirus infections in human and in other animals. The development of herpes virus of turkey (HVT), or attenuated MDV as effective vaccines against the disease, provides the first case of a means to control a naturally occurring malignant lymphomatous disease by vaccination in any species (Churchill et al., 1969;

Okazaki et al., 1970).

2) - Biology of Marek's disease virus

A) - Virus morphology and morphogenesis

MDV is classified as a *Gallid herpesvirus 1* (Matthews, 1979). The MDV genome is a linear double-stranded DNA molecule with a molecular weight of 1.2×10^8 (Lee et al., 1971), which is slightly larger than that of the prototype herpes simplex virus (HSV). In sucrose gradients, the sedimentation coefficient of MDV DNA is 56S when compared to that of T4 phage DNA and HSV DNA (Lee et al., 1971). However, in alkaline gradients, the sedimentation coefficient of MDV DNA was determined to be 70S with a molecular weight of 6×10^7. The differences in S values implied the presence of gaps and/or nicks in the MDV DNA molecule (Lee et al., 1971). The buoyant density of MDV DNA in CsCl, is 1.705 g/cm^3, corresponding to an overall guanine + cytosine (G+C) content of approximately 46% (Lee et al., 1971), similar to the G+C content of chicken genomes. Similarity of G+C contents prevents effective separation of viral DNA from cellular DNA by buoyant density centrifugation.

The MDV capsid consists of 162 capsomeres arranged in icosahedral symmetry (Nazerian and Burmester, 1968). MDV nucleocapsids consist of a core of intact viral DNA, approximately 85-100 nm in diameter, and a protein shell, the capsid, assembled in the nucli of infected cells. Few or no enveloped virions are produced in most cell types, but rather, naked intranuclear particles are present in

infected cells (Nazerian and Burmester, 1968; Hamdy et al., 1974). The feather-follicle epithelium is unique in that it is the only tissue in which MDV infection is fully productive (Calnek et al., 1970; Witter et al., 1972). Enveloped virions are produced in cells undergoing keratinization, and these virions are spread when feathers are molted or when dead cells are lost in the form of dander. These cell-free particles contain infectious virions which contaminate the environment and therefore are important epizootiologically.

B) - Marek's disease virus serotypes

MDV has been classified into three serotypes based on agar-gel precipitin (AGP) tests and indirect immunofluorescence (IF) antibody assays (Bulow and Biggs, 1975; Schat and Calnek, 1978). MDV serotype 1 (MDV1), includes all oncogenic viruses and their attenuated derivatives. These include very virulent (vvMDV), virulent, and attenuated strains of MDV. vvMDV strains, such as RB1B and Md/5, are responsible for many outbreaks of MDV in vaccinated chickens. Only vaccinated genetically resistant lines of chickens are resistive to vvMDV infection. MDV strains GA and JM are classified as virulent strains of MDV and can result in a high incidence of MD in genetically susceptible, unvaccinated chickens. Continued *in vitro* passage of vvMDV and virulent MDV isolates attenuates their oncogenic potential, modifies their growth *in vitro* and their antigenic features. Following attenuation, these viruses are immunogenic, and appear to be protective when used as vaccines against oncogenic MDV strains (Churchill et al., 1969).

MDV serotype 2 (MDV2), consists of non-oncogenic, naturally occurring chicken herpesviruses. A cell-associated lymphotropic herpesvirus of turkey (HVT), comprises a third serologic type (MDV3) related to, but different from, both MDV serotypes 1 and 2. HVT is apathogenic for both chickens and turkeys and is used as an effective vaccine against MD in chickens. MDV2, MDV3, and attenuated MDV1 have been successfully used individually and in combination to produce monovalent, bivalent, or trivalent vaccines against MDV-induced tumors (Churchill et al., 1969; Okazaki et al., 1970; Witter, 1985).

C) - Source of Marek's disease virus

MDV is present in a cell-associated form in many tissues of MDV-infected chickens (Philips and Biggs, 1972). Non-enveloped MDV can be isolated from viable whole cells, buffy coat cells, kidney cells, or lymphoma cells (Witter et al., 1969). The feather-follicle epithelium is the only tissue from which cell-free MDV can be produced. Cell-associated and cell-free MDV can be propagated in primary fibroblast cells obtained from various avian embryos. Cell-associated and cell-free MDV produce cytopathic plaques characteristic of herpesviruses within a few days when inoculated onto tissue-culture monolayers of chick kidney cells (CKC) (Churchill, 1968), duck embryo fibroblasts (Solomon et al., 1968), and chick embryo fibroblasts (CEF) (Nazerian, 1970). Plaques consist of foci of refractile rounded or fusiform cells (Churchill, 1968). Initial absorption of both cell-associated and cell-free MDV to cultured cells is rapid. Approximately, 50% of virus is absorbed within

30 minutes at 37^{0}C (Churchill and Biggs, 1967; Sharma et al., 1969).

3) - Pathology of Marek's disease virus

The understanding of MDV pathology is aided by knowledge of the types of virus-cell interactions that occur. These interactions consist of (1) productive infections, in which virions are completely or partially formed, resulting in cell death; and (2) non-productive infections, in which there is either none or very limited expression of viral genomes, resulting in viability of infected cells and, in some instances, neoplastic transformation.

A) - Productive infection

Productive infections are characterized by synthesis of viral DNA, viral proteins, and virions. Productive infections can be subdivided into fully-productive and semi-productive infections. Fully-productive infections by MDV only occur in the feather-follicle epithelium, from which cell-free infectious virions can be produced (Calnek et al., 1970a; Nazerian and Witter, 1970). Production of cell-free virions from feather-follicle epithelium is associated with envelopment of virions in cytoplasmic inclusion bodies. Semi-productive MDV infections (restrictive or abortive infection) are seen in essentially all other tissues, including spleen, bursa, thymus and peripheral blood lymphocytes (Calnek et al., 1970; Schat et al., 1978). Infected tissues contain cells that express viral antigens, naked virions in the nucleus, and limited enveloped virions, mainly associated with nuclear membranes.

Virus is not released in an infectious form, rather, it spreads from cell to cell. Infection of *in vitro*-cultured cells by MDV is also of the semi-productive type and is closely cell-associated.

B) - Non-productive infection

Non-productive infections are usually characterized as latent infections and occur primarily in transformed T-cells. Latent infections are first detected at the end of the early cytolytic infection cycle. Latent infections are usually restricted to T-lymphocytes and are characterized by the presence of intact virus genomes, with little or no active viral gene expression. In MDV and HVT, apparently latent infections of splenic and peripheral blood cells persist, probably, for the lifetime of the bird. Virus particles are not observed in these cells *in vivo*. Yet virus can be rescued *in vitro* (Adlinger and Calnek, 1973; Payne and Rennie, 1973; Calnek et al., 1970), and by inoculation of susceptible birds. The ultimate response in serotype 1 MDV-induced MD is transformation. Transformation by MDV appears to be restricted to T-lymphocytes (Calnek et al., 1970; Nazerian, 1973).

C) - Pathogenesis

The pattern of events which occurs sequentially in antibody-free, genetically susceptible chickens which ultimately die from tumors after infection with an oncogenic strain of MDV can be generally divided into four stages: (1) early cytolytic infection; (2) latent infection; (3) permanent immunosuppression and late

cytolytic infection ; and (4) transformation.

MDV infection usually occurs via the respiratory tract. As early as 1 to 2 days post-infection (DPI), virus can be detected in spleen, thymus and bursa of Fabricius. By 3 days post-infection, a productive-restrictive infection is detected. Few or no enveloped virions are produced. Rather, naked intranuclear particles are present in infected cells. The necrotizing effects of early infection lead to acute inflammatory changes, termed "acute reticulitis" (Payne et al., 1976). Histologic features in thymus and bursa include cytolysis of lymphocytes, loss of cortical cells (thymus) or follicular structure (bursa), infiltration by macrophages and granulocytes, and reticular cell hyperplasia. The result is atrophy of bursa and thymus, and consequently immunosuppression. Early cytolytic infection reaches a peak by 4 to 5 DPI and declines by 6 to 7 DPI (Shek et al., 1983). B-cells are found to be a primary early target for cytolytic MDV infection. Five to seven days post-infection, there is a switch in the type of infection seen in lymphocyte populations. During the second week of infection when virus activity subsides in lymphoid organs, a variety of other tissues, notably those of epithelial origin, become infected. Focal necrosis and intranuclear inclusion bodies may be seen in many organs, including the kidney, pancreas, adrenal gland, and proventriculus (Calnek and Hitchmer, 1969). These focal sites typically exhibit productive-restrictive infections. At the same time that these areas are developing focal infections, there is a reappearance of infection in the central lymphoid organs. Lymphocytes may become cytolytically infected at these sites. At about 2 to 3 weeks post-infection, a permanent immunosuppression

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involving both humoral and cell-mediated immune responses develops. Permanent immunosuppression could result from loss of responsive lymphocytes due to cytolytic infections. The ultimate response in MD is neoplastic transformation of lymphocytes. Gross lymphomas involving almost any of the visceral organs, skin, muscle, and nerves develop in most cases after 3 weeks post-infection (Payne et al., 1976; Calnek, 1980). There are two types of lesions (Payne and Biggs, 1967): (1) Type A (neoplastic) lesions consist of both infiltrative and proliferative lymphoblastic cells, reticulum cells, small and medium cells, and sometimes Schwann cells; and (2) type B (inflammatory) lesions which are characterized by scattered infiltrative lymphocytes, plasma cells, and macrophages, and Schwann cell proliferation. Other sites of infiltrative and/or proliferative lesions in MD are the iris (leading to blindness), muscle and skin (resulting in grayish-white lymphoid tumors) (Payne et al., 1976; Calnek and Witter, 1978). Blood changes include development of lymphomatosis due to increased numbers of T cells (Payne and Rennie, 1967; Evans and Patterson, 1971).

D) - Gross lesions

Clinical signs of MD usually appear at 3 to 4 weeks post-infection, and are classified as either classical or acute. Classical MD is characterized by enlargement of the celiac, autonomic, and other peripheral nerves. Affected nerves are usually 2 to 3 times their normal thickness, lose their cross-striations, and show a gray or yellow discoloration (Goodchild, 1969; Sugiyama et al., 1973). Nerve

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enlargement is caused by lymphomatous and/or inflammatory infiltrations. The acute form of MD is usually characterized by lymphoma formation. Various organs including the ovary, testis, liver, spleen, kidney, heart, lung, skin, and skeletal muscle are common targets for acute infection. Tumors may become visible in these organs by 3 to 4 weeks post-infection. By 5 weeks post-infection, reduction in bursal and thymus weight may occur, due to lymphoid atrophy (Payne and Rennie, 1973). Very virulent strains of MDV (vvMDV), cause even more severe atrophy of the bursa and thymus, and may result in cell death by 8 to 10 days post-infection (Witter et al., 1980).

4) - The molecular biology of MDV

A) - MDV genome structure

The MDV genomic DNA is a linear double-stranded DNA molecule of approximately 165 to 180 kilo-basepairs (kbp) in length and contains nicks and gaps (Lee et al., 1971; Hirai et al., 1979; Cebrian et al., 1982; Fukuchi et al., 1984; Wilson and Coussens, 1991).

Originally, MDV was classified as a gamma-herpesvirus based on its lymphotropic nature, similar to Epstein-Barr virus (EBV). Recently, however, MDV has been reclassified as an alpha-herpesvirus based on genome structure and gene collinearity with other alpha-herpesviruses, including herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) (Buckmaster et al., 1988; Roizman 1992; Brunovskis and Velicer, 1992; Velicer and Brunovskis, 1992). Similar to HSV-1 and

VZV, the genome structure of MDV belongs to the *Herpesviridae* group E genome family (Cebrian et al., 1982). MDV genomes consist of covalently linked unique long (U_L) and unique short (U_S) regions, each flanked by internal inverted repeats (IR_L and IR_S) and terminal repeats (TR_L and TR_S) (Cebrian et al., 1982; Fukuchi et al., 1984). The MDV DNA molecule also contains several direct repeats (DR1-5 sequences). These DR sequences are located within the internal or terminal repeat regions (Hirai, 1988). 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. For example vvMDV strain Md5 has only two 132 bp repeat units. MDV1 DNA in different lymphoblastoid cell lines contain either two or three repeat units. These findings suggested that two copies of the 132 bp repeats may be necessary or sufficient for induction and maintenance of oncogenic transformation by MDV1. DR2 is located within the *BamHI F* fragment of the U_L region and consists of a direct repeat of approximately 1.4 Kbp (Fukuchi et al., 1984). DR2 does not appear to differ in size and number of repeats between oncogenic and attenuated forms of MDV1 strain DNAs. DR3 is located within IR_S and TR_S adjacent to the U-S junction and terminal repeats. DR3 consists of a direct 178 bp repeat. The 178 bp sequence of DR3 does not have any homology to the 132 bp sequence of DR1 and is amplified as much as 50 fold during viral replication of both oncogenic and non-oncogenic MDV1 strain DNAs. DR4 is located within IR_S and TR_S adjacent to the

U_S and consists of a direct 200 bp repeat (Hirai et al., 1984). DR3 and DR4 may not be associated with a loss of 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.

B) - Physical map of Marek's disease virus

Physical maps of the three MDV serotypes have been reported (Fukuchi et al., 1984; Ono et al., 1992). Restriction endonuclease patterns are very different between the three serotypes, despite their antigenic similarities (Hirai et al., 1979; Ross et al., 1983). Initially MDV serotype 1 and HVT viral DNAs were shown to share less than 5% homology by DNA-DNA reassociation kenitics and Southern blot hybridization (Hirai et al., 1979). Gibbs et al. (1984), however, estimated that the homology between DNAs of MDV serotype 1 and HVT under less-stringent conditions ranged from 70 to 80% at the nucleotide level. Restriction enzyme profiles of the MDV serotype 2 DNA were also shown to differ greatly from those of the MDV serotype 1 and HVT DNAs (Hirai et al., 1984).

Gene identification and mapping indicated that genes encoded in the unique long and unique short regions of the MDV genome are collinear with those of HSV-1 and VZV (Buckmaster et al., 1988; Brunovskis and Velicer, 1992). To date, two MDV glycoproteins, A and B antigen, have been characterized as HSV-1 gC and gB homologs, respectively (Coussens and Velicer, 1988; Isfort et al., 1987; Ross et al., 1989; Chen and Velicer, 1992). Thirty-five MDV genes were also identified

by comparison to Varicella-zoster virus (VZV) (Buckmaster et al., 1988). Recently, homologs of HSV ICP4, ICP27, VP16 and gK have been identified and sequenced from serotype 1 MDV (Anderson et al., 1992; Yanagida et al., 1993; Ren et al., 1994). Genes located in the repeat regions are highly unique and specific to MDV. In this regard, a 38 kDa phosphoprotein (pp38) and a *fos/jun* oncogene homolog (*meq*), both expressed in MDV transformed cell lines, were identified within the *BamHI* H and *BamHI* I_2 fragments of the IR_L region of MDV genomes (Chen et al., 1992; Cui et al., 1991). A 14 kDa MDV protein encoded by a cDNA spanning *BamHI* H and I_2 regions has also been identified (Hong and Coussens, 1994). The potential relationship between unique genes in MDV and MDV tumorigenicity has attracted much interest in further exploring MDV repeat regions.

5) - MDV gene expression

As with other herpesviruses, MDV gene expression is regulated in a cascade fashion (Maray et al., 1988). Three major kenetic classes of MDV genes are expressed: (1) immediate-early (IE or α), (2) early (E or β), and (3) late genes (L or γ).

A) - MDV Immediate-early genes

Immediate-early genes are expressed immediately after infection. IE gene expression does not require *de novo* viral protein synthesis and hence IE mRNAs are synthesized in the presence of metabolic inhibitors such as

cyclohexamide (CHX). IE gene products are required for subsequent activation of early and late virus gene expression, and feedback regulation of their own expression.

Two MDV IE genes homologous to HSV-1 ICP4 and ICP27 were identified within the MDV genome (Anderson et al., 1992; Ren et al., 1994). The MDV ICP4 genes are located within the IR_S and TR_S regions of the MDV genome. The MDV ICP4 is 4,245 nucleotides in size (Anderson et al., 1992). The predicted structure of the MDV ICP4 gene product is similar to its counterparts in HSV-1 and VZV. In fact, the MDV ICP4 gene product contains five regions in which regions 2 and 4 are the most conserved, and an amino-terminal serine-rich domain (Anderson et al., 1992). The serine-rich domain of MDV ICP4 is unique in that it is flanked on both sides by proline- and basic-rich stretches. Whereas the serine-rich region of the HSV-1 and VZV ICP4 products are preceded by proline- and basic-rich amino acid residues, but followed by acidic rich regions. A number of potential regulatory sites were identified within or adjacent to the MDV ICP4 sequence. These include an ICP4 binding site, Oct-1 site, and TAATn3A sequence similar to the HSV-1 VP16/Oct1 recognizing motif, TAATGARAT (Anderson et al., 1992). Although the MDV ICP4 gene product is capable of homologous promoter activation, the precise function of MDV ICP4 in MDV infected cells is still unclear.

The MDV homolog of the HSV-1 ICP27 gene is located within the EcoRI B fragment of the MDV genome (Ren et al., 1994). The MDV ICP27 gene is 1,419 nucleotides in size and could potentially produce a product with a molecular weight

of 54.5 kDa (Ren et al., 1994). The carboxyl-terminal region of MDV ICP27, which likely contains its functional domain, is highly conserved when compared to other herpesviruses, including HSV-1 and VZV. The carboxyl-terminal domain of MDV ICP27 contains a zinc-finger motif highly similar to that of the HSV-1 ICP27. The zing-finger motif of the HSV-1 ICP27 gene product is involved in DNA, RNA, and protein-protein interactions (Smith et al., 1991). Intensive research is now in progress to investigate the functional properties of MDV ICP27.

A MDV unique IE gene - pp14 - has been recently identified within the *BamHI* I2 fragment of the MDV genome (Hong et al., 1994). The pp14 gene encodes a product with a molecular weight of 14 kDa and is expressed in lytically-infected cells with oncogenic strains (GA and Md11), or their attenuated derivatives, as well as in latently MDV-infected and transformed MSB-1 cell lines.

B) - MDV Early genes

Early genes are the next class of genes expressed in lytic MDV infection and their synthesis requires the activity of at least one IE protein. Early genes encode proteins required for nucleotide precursor metabolism, and viral DNA replication. Expression of E genes is enhanced in the presence of drugs that inhibit viral DNA synthesis, such as phosphonoacetic acid (PAA). Several MDV early genes homolgous to those of HSV-1 have been identified. These include thymidine kinase (TK), DNA polymerase, DNA-binding protein, etc. (Buckmaster et al., 1988). A MDV unique gene, phosphoprotein 38 (pp38), has also been identified within the

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BamHI H fragment and spans the junction of MDV U_L and IR_L regions. MDV pp38 has been identified as an MDV serotype 1-specific antigen (Silva and Lee, 1984; Nakajima et al., 1987), and is one of the viral-specific antigens consistently expressed in MDV-transformed lymphoblastoid cell lines as well as in MDV tumor cells (Ikuta et al., 1985; Naito et al., 1986). MDV pp38 is 290 amino acids in length and is relatively rich in acidic residues (15 % glutamic and aspartic acid residues) (Nakajima et al., 1987; Cui et al., 1991; Chen et al., 1992). Recently, it was reported that MDV serotypes 2 and 3 contain MDV serotype 1 pp38-related polypeptides that have epitopes or polypeptides homologous to MDV serotype 1 pp38 (Cui et al., 1992).

C) - MDV Late genes

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 VP16, an IE gene transactivator (Roizman et al., 1991). Late genes can be further subdivided into γ1 and γ2 genes. Expression of γ1 genes occurs prior to initiation of viral DNA synthesis. As viral DNA synthesis begins, high level expression of γ1 genes occurs and γ2 gene expression begins. Six MDV late genes homologous to genes of HSV-1 (gB, gC, gD, gE, gI, and gK) have been identified (Silva et al., 1984; Ikuta et al., 1983a; 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). MDV gB and gC (A and B antigen,

respectively), are the most thoroughly studied in MDV. MDV gB is located within the *BamHI* K3 and I3 fragments. MDV gB is processed into a family of proteins known as gp100, gp60, and gp49 (Yanagida et al., 1992). MDV1 gB was shown to be a major protective immunogen. Homologs of the MDV serotype 1 gB gene were identfied in serotypes 2 and 3 homologs of the MDV serotype 1 gB gene were identified (Yoshiba et al., 1994). Alignmemt of MDV serotypes 2 and 3 gB with MDV1 gB revealed predicted amino acid identities of 83 and 82 % for MDV2 and MDV3 gB, respectively (Yoshiba et al., 1994). Proteolytic cleavage sites for processing of precursors (gp100 to gp60 and gp49) are also conserved among the three gB homologs (Yoshida et al., 1994).

MDV gC is located within the BamHI B fragment of the MDV genome (Isfort et al., 1986, Coussens and Velicer, 1988). The MDV gC gene product encodes an N-linked glycoprotein of 57-65 kDa with a precursor of 44 kDa (Isfort et al., 1986). Expression of MDV gC is significantly reduced in attenuated MDV strains (Churchill et al., 1969; Bulow and Biggs, 1975; Ikuta et al., 1983; Silva et al., 1984; Isfort et al., 1986). The mechanisms leading to reduced expression of MDV gC and its relationship with MDV oncogenicity or attenuation are still unclear. It has been postulated that reduced expression of MDV gC in MDV attenuated strains may be due to alteration of viral or cellular regulatory protein(s) involved in regulation of the MDV gC gene promoter.

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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF THE MAREK'S DISEASE VIRUS (MDV) HOMOLOG OF THE HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) VP16 GENE

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ABSTRACT

We are investigating regulatory protein genes in Marek's disease virus (MDV) based, in part, on homolgy with herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV). Since the MDV genome structure is colinear with that of HSV-1 and VZV, map positions of most MDV genes may be inferred from positions of similar genes in HSV-1 and VZV. In this report, a MDV gene encoding a homolog to the HSV-1 VP16 protein has been mapped to the MDV *BamHI B* fragment. The complete nucleotide sequence of the MDV VP16 gene has been determined and used to predict the amino acid sequence of MDV VP16. The MDV VP16 coding region is 426 amino acids long with a molecular weight of 48,000 daltons. Predicted amino acid sequence of MDV VP16 shows considerable homology (greater than 30 % overall in all cases examined) with VP16 homologs of other alphaherpesvirus . Like VZV ORF10, MDV VP16 is 64 amino acids shorter than HSV-1 VP16, lacking sequences similar to that of the HSV-1 VP16 carboxyl-terminal acidic activation domain. Nevertheless, MDV VP16 is able to transactivate both homologous (MDV) and heterologous (HSV-1) immediate-early gene promoters. An examination of the deduced amino acid sequence revealed two potential transactivation domains within the amino-terminal region of MDV VP16. These include a highly acidic domain (23 % acidic residues), defined by residues 1 to 58, and a proline-rich (57%) domain defined by amino acids 59 to 80. Hydrophobic cluster analysis of several VP16

homologs, including MDV VP16, revealed conservation of bulky hydrophobic clusters critical for VP16 activity. Of particular interest in these studies were bulky hydrophobic residues surrounding the MDV VP16 Phe^{43} residue. Substitutions of MDV VP16 Phe^{43} with aromatic residues preserves MDV VP16 activity whereas substitutions of MDV VP16 Phe^{43} with non-aromatic amino acids abolishes MDV VP16 transactivation function, similar to that observed with HSV-1 VP16 and VZV ORF10. Furthermore, transactivation of MDV ICP4 promoters is dependent upon presence of an intact TAATGARAT element upstream of target genes. Deletion and mutational analysis within the MDV ICP4 gene promoter sequences revealed that the upstream ATGCAtATATTAT element at position 572 is important for MDV VP16 activation function. The ATGCAtATATTAT is highly homologous to the extended OCT-1 binding site ATGCAAATGARAT, which has been shown to be critical for activation functions of VP16 gene family members.

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INTRODUCTION

MDV, a member of the herpesviridae family, causes lymphoproliferation and nerve demyelination in chickens (Marek, 1907; Payne, 1982). The three serotypes of MDV include oncogenic MDV strains and their derivatives (serotype 1), the nononcogenic chicken herpesviruses (serotype 2), and turkey herpesviruses (serotype 3) (Payne, 1982). Originally, MDV was classified as a gammaherpesvirus, based primarily on its lymphotropic nature, similar to Epstein-Barr Virus (EBV). Recently, however, MDV has been reclassified as an alphaherpesvirus based on MDV genomic structure and gene colinearity with other alphaherpesviruses (Buckmaster et al., 1988; Roizman, 1992; Brunowski and Velicer, 1992). Recent progress in MDV research includes development of correlative maps between MDV, herpes simplex virus type 1 (HSV-1), and varicella zoster virus (VZV) (Buckmaster et al., 1988; Ross et al., 1989; Brunovskis and Velicer, 1992). These studies revealed that genes encoded in the unique long and unique short regions of MDV are highly similar to those of other alphaherpesviruses, while genes in repeat regions are highly variable and more specific to a particular virus.

As with other herpesviruses, MDV gene expression is regulated in a cascade fashion (Maray et al., 1988). Immediate-early (IE) and early (E) genes are expressed immediately after infection while late (L) genes are not expressed until onset of viral DNA replication. In the case of HSV-1, IE genes are transactivated by the virion tegument protein VP16 (Campbell et al., 1984). Specific induction of IE

genes by VP16 requires a cis-acting DNA sequence motif, TAATGARAT (R = purine), which is present in promoters of HSV-1 IE genes. Mutational analyses of the HSV-1 VP16 gene have identified a highly acidic domain (defined by 80 amino acids at the carboxyl terminus) as a potent transcriptional activating region (Triezenberg et al., 1988; Greaves and O'Hare, 1989). VP16 has no substantial affinity for double-stranded DNA (Marsden et al., 1987). Rather, VP16 interacts with Oct-1and at least another cellular factor called CFF, VCAF, or HCF (Katan et al., 1990; Xiao and Capone, 1990; Kristie et al., 1989), to form an activator complex that binds the TAATGARAT motif on IE viral promoters. Recent studies on HSV-1 VP16 have identified patterns of aromatic and hydrophobic amino acids which are important for VP16 activity. Specifically, the pattern of bulky hydrophobic residues surrounding Phe^{442} was critical in preserving VP16 activity. Substitution of Phe^{442} and neighboring leucines with non-hydrophobic amino acids destroyed VP16 activity while substitutions with other hydrophobic or aromatic amino acids preserved activity.

VZV open reading frame 10 (VZV ORF 10) encodes a protein of 410 amino acids that is homologous to HSV-1 VP16 (Davison et al., 1986). While VZV ORF 10 and HSV-1 VP16 show considerable amino acid homology at the amino-terminal region, VZV ORF 10 is 80 amino acids shorter, lacking sequences similar to that of the VP16 transactivation domain (McKee et al., 1990). A recent study (Moriuchi et al., 1993) shows that the VZV ORF 10 protein acts as a transactivator for both VZV and HSV-1 IE promoters in transient-expression assays. VZV ORF 10 can also

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substitute for the transactivation function of HSV-1 VP16. Cell lines expressing VZV ORF 10 are able to complement an HSV-1 VP16 mutant which lacks the transactivation function of HSV-1 VP16 (Moriuchi et al., 1993). The transcriptional activation domain of VZV ORF 10 maps to the amino-terminal region of the protein (Moriuchi et al., 1995).

Like all other VP16 homologs, MDV VP16 is of interest to study for several reasons. IE gene expression is an essential step for viral replication, and thus defines an early point at which control can be exerted during the entire replication cycle. In addition, MDV VP16 provides a well-defined model for gene expression and tools to further clarify our understanding of how eukaryotic transcriptional activators work. Of particular interest in these studies are comparisons of VP16 homologs of diverse herpesviruses. Since the MDV genome structure is colinear with that of HSV-1 and VZV, the gene encoding the tegument protein, VP16, is predicted to be located within the unique long region near the MDV glycoprotein C (gC) homolog gene (Coussens and Velicer, 1988). In this study, we report the identification, molecular cloning, complete nucleotide sequence, and functional characterization of the MDV VP16 gene. The MDV VP16 coding region is 426 amino acids. Another report of the MDV VP16 gene sequences (Yanagida et al., 1993), is consistent with our data. To examine the function of MDV VP16, we expressed MDV VP16 in transient-expression assays. MDV VP16 is able to transactivate both homologous (MDV) and heterologous (HSV-1) herpesvirus immediate-early gene promoters. An extended Oct-1 motif (ATGCAtATATTAT)

within the MDV ICP4 gene promoter (Anderson et al., 1992) is critical for MDV VP16 transactivation function. Deduced amino acid analyses of MDV VP16 revealed two potential transactivation domains located within the amino terminal region of MDV VP16. These include a highly acidic domain (23%) defined by amino acids 1 to 58 and a proline-rich sub-domain (57%) bounded by amino acids 59 to 80. Comparison of several VP16 homologs using hydrophobic cluster analysis (HCA), revealed conservation of potential hydrophobic clusters which may play a critical role for MDV VP16 transactivation function. This hydrophobic motif is centered at Phe^{43} and its predicted spatial arrangement was similar to residues surrounding Phe^{442} and Phe^{28}, a residue known to be critical for transactivating HSV-1 VP16 and VZV ORF10, respectively. *In vitro* site-specific mutagenesis revealed that substitutions of MDV VP16 Phe^{43} with aromatic amino acids preserved MDV VP16 activity whereas substitutions of MDV VP16 Phe^{43} with non-aromatic residues reduced MDV VP16 activity, similar to results obtained with HSV-1 VP16 Phe^{442} and VZV ORF10. Our studies suggest that a common transactivation mechanism may exist between MDV, VZV, and HSV-1 VP16 despite significant differences in protein sequences and arrangement of these sequences within the polypeptide. Our results and studies reported previously (Greaves and O'Hare, 1989; Moriuchi et al., 1995) indicate that transactivation domains of VP16 gene family members are highly similar, and may have evolved from a common ancestral domain.

Materials and methods

Cells, viruses and transient-expression assays

Preparation, maintenance and infection of chick embryo fibroblasts (CEF) were performed as described (Glaubiger et al., 1983). MDV strains GA and Md11 have also been described (Payne, 1982; Schat and Calnek; 1978). Transfection of plasmid DNA (5 ug of activator and 3 ug of reporter DNA were added to each group of three 60-mm tissue culture dishes) for transient-expression assays was carried out in 60-mm tissue culture dishes. Primary cells were trypsinized and plated at 1.2 x 10^6 cells per plate, 24 hours prior to transfection. Cells were transfected using the calcium phosphate procedure as described by Graham and Van der Eb (Graham et al., 1973). Transfectants were harvested following incubation at 37°C in 95% air and 5% CO_2 for 48 hours post-transfection. Cell lysates were prepared as described by Gorman et al. (Gorman et al., 1982). Chloramphenical acteyl transferase (CAT) assays were performed essentially as described by Neumann et al. (Neumann et al., 1987).

Southern blot hybridization

DNA in EtBr-stained agarose gels was nicked by irradiation with UV light for 3-5 minutes, transferred to nylon membranes (Zeta-probe, Bio-Rad, Inc., Richmond, CA) under alkaline conditions (0.4 N NaOH), and hybridized to radiolabeled DNA

probes as described (Budowle and Baechtel, 1990). HSV-1 VP16 probes were derived from pMSVP16.

Nucleotide sequence and data analysis

Localization and subcloning of DNA encoding the MDV homolog of the HSV-1 VP16 gene is represented schematically in Figure 1. Following preliminary localization of the MDV VP16 gene by southern blot hybridization (see Results), a 2.122 kbp *SmaI-SalI* subfragment of the MDV *BamHI B* fragment was cloned into pUC18 to generate pMDVP16. Random *Sau3AI* and *TaqI* pieces of this subclone were sequenced and potential open reading frames (ORF's) analyzed for homology to predicted VP16 sequences of HSV-1 and other herpesviruses. Homologous segments were aligned with HSV-1 VP16 sequences to establish gene limits and orientation. DNA sequencing was performed using the dideoxy chain-termination method (Sanger et al., 1977). In all sequencing reactions, [^{35}S]-dATP (NEN, Boston, Ma) and the sequenase enzyme (United States Biochemical Corp., Cleveland, OH) were used as recommended by the manufacturer. Amino acid sequences of potential ORFs were compared against entries in the Swiss-protein data base. Final alignment of amino acid sequences was performed using the GAP program of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

Computer analysis of DNA sequences and GenBank accession number.

MDV VP16 nucleotide sequences have been determined in our laboratory

(GenBank accession number I26485), and by others (Yanagida et al., 1993).

Hydrophobic cluster analysis (HCA) was used to compare and align several protein sequences (Gaboriaud et al., 1987; Moriuchi et al., 1995). HCA relies upon a two-dimensional representation of the sequences, as if the sequences were folded on an alpha-helical pattern. Clusters of hydrophobic residues were highlighted and the pattern was compared and analyzed by eye. The classifications of hydrophobic residues that we have used in these studies were based on previous proposals (Gaboriaud et al., 1987).

Northern blot hybridization

Total RNA from uninfected CEF cells and CEF cells infected with GA and Md11 strains of MDV was isolated using the guanidinium-phenol:chloroform isolation method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated using the polyATrack mRNA kit (Promega, Madison, Wisconsin), as recommended by the manufacturer. Poly(A)$^+$ RNA (10 ug), was resolved on 1.2 % formaldehyde gels and transferred to nylon membranes (Amersham Corp., Arlington Heights, IL) using 2X saline sodium citrate (SSC). MDV VP16 DNA was labelled with α-[^{32}P]-dCTP using a random primed labelling kit (BRL Life Technologies, Inc., Gaithersberg, MD), as specified by the manufacturer. Northern blots were hybridized in 10% PEG, 1.5 x SSPE, 7% SDS, 100 ug/ml of sonicated salmon sperm DNA and the denatured, labelled probe in a 50°C shaking water bath overnight. Membranes were washed twice in 2 x SSPE, 0.1% SDS at room

temperature, and twice in 1 x SSPE, 0.1% SDS at 55°C for ten minutes each. Filters were removed and subjected to autoradiography.

Three DNA probes have been used for Northern blot hybridization (Figure 5). Probe P1 included a 520 base-pair fragment located at the amino-terminal region of MDV VP16. P2 is encoded within the MDV VP16 coding region. Finally, P3 encoded a 320 base-pair DNA fragment spanning the carboxyl-terminal domain of the MDV VP16 gene.

Construction of MDV ICP4 gene promoter plasmids and derivatives

A schematic representation of the MDV ICP4 gene promoter as described previously (Anderson et al., 1992) is presented (Figure 9A). The MDV ICP4 gene promoter was PCR amplified from the MDV GA genome. The upstream primer used for PCR amplification is located 1110 bp upstream of the transcription start site and contains a *HindIII* site, whereas the downstream primer overlaps the transcription start site. A new *XbaI* restriction site was introduced near the transcription start site. The 1.1 Kbp PCR product was digested with *HindIII* and *XbaI* and cloned into pCAT Basic vector (Promega, Madison, Wisconsin), to generate pICP4cat.

Several deletion mutants of the MDV ICP4 gene promoter linked to CAT as in MDV ICPcat were engineered (Figure 9A). pSH3cat was constructed by deleting a *AvaI* fragment from the MDV ICP4 gene promoter thus removing a potential AP-1 binding site, but leaving intact Oct-1, and TATA and CAAT elements. In pAH3cat,

which contains only the TATA box, all potential transcriptional activator binding sites were deleted. pSXbcat contains only the AP-1 binding site and lacks binding sites for Oct-1 and CAAT factors, as well as the TATA element.

Construction of MDV VP16 plasmids

The MDV VP16 gene is encoded by a 1.651 Kbp *SpeI-SalI* region within the MDV *BamHI B* fragment of the GA strain. The MDV *BamHI B* fragment was digested with *SpeI* and *SalI* restriction enzymes. The 1.651 Kbp *SpeI-SalI* sub-fragment was electro-eluted and cloned into the pBK-CMV plasmid (Stratagene), which contains the CMV IE gene promoter. The cloning junctions of pBKCMVP16 construct was sequenced to verify the identity and orientation of the insert. Cells transfected with pBKCMVP16 expressed a protein of the expected size, as detected in western blots using HSV-1 VP16 antisera.

***In vitro* site-specific mutagenesis**

Site-specific mutagenesis was performed using the pAlter system (Promega, Madison, Wisconsin), essentially as detailed by the manufacturer. The MDV VP16 gene was cloned as an *SpeI* to *SalI* insert into the pAlter-1 plasmid, and the resulting construct was used to generate mutations within the Phe^{43} residue of MDV VP16. Mutated sites were sequenced to ensure proper insertion of the appropriate mutation. Appropriate MDV VP16 mutant versions were recloned into the pBK-CMV system as described above, and western blot analysis was used to determine

whether MDV VP16 mutant constructs encode a polypeptide when transfected into cultured cells . MDV VP16 mutagenesis primers used were:

Original sequence: AACAATTGAAGAA**TTT**GATGAAACGCTTCT

F43A primer: AACAATTGAAGAA**GCA**GATGAAACGCTTCT

F43P primer: AACAATTGAAGAA**CCA**GATGAAACGCTTCT

F43Y primer: AACAATTGAAGAA**TAC**GATGAAACGCTTCT

The MDV ICP4 promoter was also cloned as a *HindIII* to *XbaI* insert into the pAlter-1 plasmid. The resulting pAICP4 construct was used to generate multiple base pair mutations within the Oct-1 site within the MDV ICP4 gene promoter sequences. Mutated sites were sequenced to ensure the proper insertion of the appropriate mutation. MDV ICP4 promoter mutants were recloned into the pCAT Basic vector, as described above. MDV ICP4 promoter mutagenesis primers were:

Original Oct-1 sequence: TTCCCAAGT**ATGCATATATTATAT**CAGCTT

Oct1A$^-$ primer: TTCCCAAGT**ATGCATGCCTTATAT**CAGCTT

Oct1B$^-$ primer : TTCCCAAGT**CGACATATATTATAT**CAGCTT

Western blot analysis

Cultured cells were prepared as described previously (Sambrook et al., 1989). Proteins (10 ug) were separated on 12.5% polyacrylamide/1% SDS gels. Separated proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk and probed with antibodies raised against HSV-1 VP16 protein. Donkey anti-rabbit immunoglobulin

conjugated with horseradish peroxidase was used as a secondary antibody. Detection was performed using an ECL Western blot kit (Amersham Corp., Arlington Heights, Il), and subsequent fluorography. Protein sizes were estimated by comparison to pre-stained protein molecular weight standards (Bio-Rad, Richmond, Ca), electrophoresed on the same gel.

RESULTS

Localization of the MDV VP16 gene

The HSV-1 VP16 ORF maps to a 2.6 Kbp fragment located in the unique long region of the HSV-1 genome, between map coordinates 0.688 and 0.679. HSV-1 VP16 is predicted to contain a maximum of 490 amino acids with a molecular weight of 54,000 (Dalrymple et al., 1985). Since the MDV genome structure is collinear with that of HSV-1 and VZV, the MDV homolog of HSV-1 VP16 was predicted to be located within the unique long region of the MDV genome between the gC (Figure 1A) (Coussens and Velicer, 1988) and UL52 (Boussaha and Coussens, unpublished data) gene homologs. This region is entirely contained within the MDV B*amHI B* fragment (Figure 1B).

To examine this region for homology to HSV-1 VP16 coding sequences, DNA from purified MDV strain GA *BamHI B*, and *EcoRI C* fragments, was electrophoresed, transferred to nylon membranes and hybridized with radiolabelled DNA probes containing the HSV-1 VP16 gene (see Materials and Methods). HSV-1 VP16 probes hybridized to both the *BamHI B* and *EcoRI C* fragments (Figure 2). These results suggested that VP16-related sequences were indeed located within the 3'-one half of the MDV *BamH1 B* fragment, downstream of the MDV gC homolog gene. Restriction mapping and random sequencing further located the putative MDV VP16 gene within a 2.122 Kbp *SmaI-SalI* segment of the *BamHI B*

fragment (Figure 1C). These results were consistent with predictions based on gene colinearity and with results reported previously (Yanagida et al., 1993). The 2.122 Kbp *SalI-SmaI* fragment was subsequently cloned into pUC18, generating plasmid pMDVP16 for further analysis and complete nucleotide sequencing.

Nucleotide sequence of MDV VP16

The entire 2.122 Kbp *SalI-SmaI* fragment was sequenced in both directions as described in Materials and Methods. Sequences were examined for potential open reading frames and for homology to HSV-1 VP16. The complete nucleotide sequence of the MDV VP16 gene, including 5' and 3' flanking regions, is shown in Figure 3. The MDV VP16 structural gene is 426 amino acids long (Figure 3). Based on nucleotide sequence similarity with HSV-1 VP16 and VZV ORF 10, the MDV VP16 translation start site is predicted to be at nucleotide 259 (Figure 3). Though the second ATG codon beginning with nucleotide 271 is more favorable on the basis of other eukaryotic translation initiation sites (Kozak, 1983), the predicted amino acid sequence encoded between nucleotides 259 and 271 is highly similar to the predicted amino-terminus of VZV ORF 10 (3 of 4 identical amino acids and one conservative substitution). There are at least three potential TATA sequences located upstream of the predicted translation start site. (nucleotides 224 to 229, 196 to 199, and 148 to 153). In addition, there are at least two potential CAAT box homologies located at nucleotides 6 to 10 and nucleotides 45 to 49 of the sequence presented in Figure 3. A putative polyadenylation signal, (AATAAA), is located 83

bp downstream of the termination codon. Also of interest is a potential polyadenylation signal in the region immediately upstream of the MDV VP16 coding sequences (nucleotides 208 to 213, Figure 3). This polyadenylation signal may function in processing of a transcript derived from sequences immediately upstream of the MDV VP16 gene.

Deduced amino acid sequences were compared with sequences from the GenBank database. The MDV VP16 gene product was found to have the highest identity with EHV-1 gene 12 (42 %), EHV-4 UL48 (43 %), BHV-1 UL48 (38 %), VZV ORF 10 (38 %), HSV-1 VP16 (35 %), and HSV-2 VP16 (36 %) (Table 1). Amino acids 327 to 403 of MDV VP16 are highly similar with other alphaherpesvirus VP16 homologs (Figure 4), while the carboxyl-terminus (amino acids 403 to 426) is not well conserved. Furthermore, MDV VP16 amino acids 382 to 403 are highly similar to HSV-1 VP16 amino acids 360 to 391, which contains the sequence R-E-H-A-Y-S-R-A (Figure 4), involved in transcription complex formation (Hayes and O'Hare, 1993). Like VZV ORF10, the MDV VP16 protein is 64 amino acids shorter than HSV-1 VP16, lacking sequences similar to the transactivation domain.

Detection of MDV VP16 transcripts

Based on nucleotide sequence analysis, the MDV VP16 structural gene is predicted to be 1278 nucleotides long. Assuming MDV VP16 is not translated from a bi- or polycistronic message, the major MDV VP16 mRNA was predicted to be about 1.4 Kb in size. To determine if sequences from the proposed MDV VP16

gene were transcribed in infected cells, poly (A)$^+$mRNA was prepared from CEF cells and CEF cells infected with MDV strain GA, electrophoresed and hybridized as described in Materials and Methods. Probes P1 and P2 hybridized to 7.8 and 2.5 Kb transcripts in all infected cell RNA samples but not in uninfected cell RNA (Figure 5). P3, however, hybridized to three major transcripts of 7.8, 4.6, and 2.5 kb. Based on sequences reported previously, transcription of the 7.8 kb mRNA may start upstream of the MDV VP16 gene and contain sequences from the UL49, VP16, UL48, and UL47 homologs (Figure 5) (Yanagida et al., 1993). The polyadenylation signals located upstream and downstream of MDV VP16 were not recognized. The 4.6 kb transcript may start immediately downstream of MDV VP16 and may encode information from the UL47 and UL46 genes. The 2.5 transcript starts upstream and terminates immediately downstream of MDV VP16. Like HSV-1 VP16, transcription of the MDV VP16 gene may be through a bicistronic message which includes information from the UL49, UL47, and UL46 genes.

Detection of MDV VP16 gene product

Detecting the VP16 gene product will help in understanding the function of MDV VP16 and guide future research directions. To accomplish this goal, antibodies (antibody C8-5) raised against the HSV-1 VP16, were used to detect the MDV VP16 translation product in extracts of mock-infected CEF cells, and MDV-infected CEF cells. A 48 kDa protein was specifically detected in cell extracts from CEF infected with MDV strain Md11 (Figure 6). A similar protein was not, however,

detected in mock-infected CEF cells.

Transactivation of IE promoters by MDV VP16

Previous studies have shown that the HSV-1 VP16 and VZV ORF 10 act as strong transactivators of viral IE gene promoters. Like VZV ORF10, MDV VP16 lacks the carboxyl-terminal acidic activation domain present in HSV-1 VP16, but contains two potential activation domains within the amino-terminal 80 amino acids. Transient-expression assays were performed to determine whether MDV VP16 is capable of transactivating expression from MDV IE gene promoters.

Transient-expression studies were performed using pBKCMVP16, which contains the entire MDV VP16 structural gene and 5'-flanking sequences, under the control of CMV IE gene promoter (Figure 7). Reporter constructs consisted of the promoter for immediate-early genes (ICP4 from Md11 low passage, ICP4 from Md11 high passage, and HSV-1 ICP4), MDV early gene (pp38), and MDV late gene (gB), linked to the gene for chloramphenicol acetyl transferase (CAT). In transient co-transfection experiments, cells containing pBKCMVP16 and MDV ICP4cat expressed five- to six- fold more CAT activity than cells containing a similar amount of pBK-CMV and ICP4cat (Figure 8). pBKCMVP16 did not, however, activate CAT expression directed by the MDV pp38 and gB gene promoters. These results suggested that MDV VP16 could transactivate MDV immediate-early gene promoters. We also assessed the effects of MDV VP16 on an HSV-1 ICP4 gene promoter. pBKCMVP16 activated CAT expression directed by the HSV-1 ICP4

gene promoter by up to thirty five-fold (Figure 8). Thus, like and VZV ORF10, the MDV VP16 gene transactivates both homologous and heterologous IE gene promoters. These results suggest that a common transactivation mechanism may exist between MDV, VZV, and HSV-1 VP16, despite significant differences in protein sequences.

Identification of MDV ICP4 promoter sequences critical for MDV VP16 activation function

A number of potential transcriptional regulatory sites have been identified within and adjacent to the MDV ICP4 sequence (figure 9A). Of particular interest, a sequence (ATGCAtATATTAT) resembling the octamer motif (ATGCAAAT) often extended to ATGCtAATGARAT, lies 5' to the predicted MDV ICP4 coding region (Anderson et al., 1992). HSV-1 VP16 functions by forming a multi-component complex on TAATGARAT sites in the IE gene promoters together with the cellular POU domain protein Oct-1 and at least one other cellular factor called CFF, VCAF, or HCF (Gerster and Roeder, 1988; Kristie et al., 1989; O'Hare and Goding, 1988; Wilson et al., 1993; Xiao and Capone, 1990). The role of the upstream ATGCAtATATTAT element in MDV ICP4 promoter responsiveness to MDV VP16 was determined, using MDV ICP4 promoter deletion mutants.

Transient co-transfection assays were performed using pBKCMVP16 as effector plasmid. Target constructs consisted of MDV ICP4cat and several deletion versions of the MDV ICP4 gene promoter linked to CAT as in MDV ICP4cat.

Although removal of an AP-1 binding site in pSH3cat reduced basal activity of the ICP4 promoter by one-half, this construct retained the ability to respond to MDV VP16 (Figure 9B). Deletions from *SmaI* to *XbaI* in pSXbcat completely abolishe CAT expression directed by the MDV ICP4 promoter (Figure 9B). Restoration of the TBP-binding site as in pAH3cat was sufficient to restore at least partial basal activity to the MDV ICP4 promoter. However, pAH3cat was not responsive to MDV VP16. These results suggest that the TATA and AP-1 sites are not sufficient for MDV VP16-mediated activation of MDV ICP4 gene promoters, although they are important for basal activity. The *AvaI* regions, which encodes recognition sites for AP-4, Sp1, CAAT, and Oct-1 factors plays a critical role in MDV VP16-mediated activation of MDV ICP4 promoter directed transcription. Further dissection of this region was complicated by a lack of suitable restriction sites for deletion mutant construction.

Given the importance of Oct-1 in HSV-1 VP16 mediated promoter activation, this site within the MDV ICP4 promoter seemed a more likely candidate than the other factor binding sites to play an important role in MDV ICP4 responsiveness to MDV VP16. To determine whether the upstream ATGCAtATATTAT element is specifically involved in MDV VP16 response, several mutations were engineered into pICP4cat using site-directed mutagenesis. In transient co-transfection experiments, mutations in either the Oct-1 site or the GARAT motif (Figure 10) completely abrogated activation of MDV ICP4 promoter directed CAT expression by MDV VP16 gene product. These results confirm that the upstream

ATGCAtATATTAT motif is important for MDV VP16 responsiveness. Furthermore, dependence on an intact TAATGARAT homology in target promoters suggests that activation mechanisms used by MDV VP16 are similar to HSV-1 VP16, despite significant differences in amino acid sequences.

Potential activation domains within MDV VP16

MDV VP16 is 64 amino acids shorter than HSV-1 VP16, lacking sequences similar to the HSV-1 VP16 carboxyl-terminal acidic activation domain. However, MDV VP16 is capable of transactivating both homologous and heterologous immediate-early gene promoters. Examination of the deduced amino acid sequence of MDV VP16 revealed at least two potential activation regions. The amino terminal amino acids 1 to 58 of MDV VP16 are highly rich in acidic residues (23 %), whereas the region defined by amino acids 59 to 80 is highly rich in proline residues (57 %) (Table 2). Acidic and proline rich domains have been implicated in promoter activation by other transcription factors. However, previous work on VP16 (Cress and Triezenberg, 1991; Regier et al., 1993) revealed that specific patterns of hydrophobic and aromatic residues were equally or more critical than were the abundant acidic amino acids. This hypothesis was also supported by studies of other activators including the mammalian cell proteins p53 (Lin et al., 1994), RelA (Blair et al., 1994), Sp1 (Gill et al., 1994), and several other classes (Ruden, 1992; Tanaka and Herr, 1994). Thus a pattern of hydrophobic clusters common to activation domains of several classes may be more important for

activation function than the more obvious features used to distinguish these classes. To be sure that these results are interpreted with caution, hydrophobic cluster analysis was used to compare and analyze several VP16 gene family members.

Hydrophobic cluster analysis (HCA) is an efficient method to analyze and compare protein sequences, especially for related proteins (Gaboiraud et al., 1987). HCA is not based primarily on maximizing amino acid identity but rather on detecting similar structural components within hydrophobic cores of proteins. HCA presents amino acid sequences in two dimensions. Clusters of hydrophobic amino acids are highlighted and the pattern is compared and analyzed by eye. HCA has been previously used to identify a motif centered at Phe^{28} of VZV ORF10 (Moriuchi et al., 1995), that strongly resembles regions surrounding Phe^{442} of HSV-1 VP16, known to be critical for its activity. When the HCA technique was used to compare HSV-1 VP16 with homologous proteins in other herpesviruses (including MDV), potential segments or clusters of homologous proteins which may function as activation domains were identified. Of particular interest were regions surrounding MDV VP16 Phe^{43}, near the amino terminus of MDV VP16. Spatial arrangements of hydrophobic residues near MDV VP16 Phe^{43} were similar to regions surrounding HSV-1 VP16 Phe^{442} and VZV ORF10 Phe^{28} (Figure 11A). The HCA plot indicates that these regions contain a horseshoe-shaped array of hydrophobic amino acids with Phe residues at their apices (Figure 11A). MDV VP16 Phe^{43} falls within the acidic domain previously identified in the MDV VP16 amino-terminal region.

Alignment of several transcription factors and activators with HSV-1 VP16 using the six hydrophobic residues surrounding Phe^{442} as a guide revealed conservation of this pattern, despite dissimilar amino acid sequences (Figure 11B). Of particular interest in these comparisons were bulky hydrophobic residues surrounding the MDV VP16 Phe^{43} (Figure 11B).

Location within the MDV VP16 amino-terminal acidic domain as well as conservation of nearby hydrophobic residues suggested the MDV VP16 Phe^{43} may play a critical role in activation of IE gene promoters. To test this hypothesis, site-directed mutagenesis of MDV VP16 Phe^{43} was initiated. Mutants were made changing Phe^{43} to tyrosine (pBKCMV-F43Y), alanine (pBBCMV-F43A), and proline (pBKCMV-F43P).

Transient expression studies were performed using pBKCMV-F43Y, pBKCMV-F43A, and pBKCMV-F43P. pBKCMV-VP16 which contains the entire MDV VP16 gene was used as a positive control. Reporter constructs consisted of the MDV ICP4 gene promoter linked to a CAT gene. In transient co-transfections experiments, cells containing wild type MDV VP16 and ICP4cat expressed three- to four-fold more CAT activity than cells containing a similar amount of pBK-CMV and ICP4cat (Figure 12A). In the same experiment, pBKCMV-F43Y in which Phe^{43} is replaced by tyrosine was capable of activating MDV ICP4 target promoters nearly as efficiently as wild type MDV VP16 (Figure 12A). Substitution of Phe^{43} with proline or alanine significantly reduced the ability of MDV VP16 to activate CAT expression directed by the MDV ICP4 gene promoter (Figure 12A). Western blot

analysis of MDV VP16 construct and derivatives was performed to demonstrate that cells transfected with MDV VP16 constructs expressed a protein of the expected size (Figure 12B). Our results suggest that substitutions of MDV VP16 Phe^{43} with aromatic amino acids can preserve MDV VP16 activity whereas substitutions of MDV VP16 Phe^{43} with non-aromatic amino acids destroy MDV VP16 transactivation function. These results demonstrate that MDV VP16 Phe^{43} is critical in activation of IE gene promoters. Furthermore, our results indicate that aromatic amino acids at position 43 can efficiently substitute for Phenylalanine. Thus it appears that a common transactivation mechanism exists between MDV and HSV-1 VP16, despite significant differences in protein sequences and spatial arrangement of activator domains.

DISCUSSION

The MDV VP16 gene has been identified, cloned, and completely sequenced, in our laboratory and by others (Yanagida et al., 1993). The predicted MDV VP16 gene product strongly resembles its counterparts among other herpesviruses. Using low stringency hybridization and random sequencing, the MDV VP16 gene was localized to a 2.122 Kbp subfragment of the MDV *BamHI B* fragment (Figure 1). MDV VP16 structural gene encodes a polypeptide of 426 amino acids with a molecular weight of approximately 48,000 daltons (Figure 6). Predicted MDV VP16 amino acid sequences have been compared with VP16 homologs from other herpesviruses. MDV VP16 has the highest similarity with EHV-1 gene 12, EHV-4 genB5, VZV ORF 10 BHV-1 UL48, HSV-1, and HSV-2 VP16 (Table 1). Similarity between MDV VP16 and VP16 homologs of other viruses adds to the evidence that MDV is more closely related, on a molecular level, to the alphaherpesvirus group than to the gammaherpesvirus such as Epstein-Barr Virus (EBV).

Like VZV (ORF 10), MDV VP16 is 64 amino acids shorter than HSV-1 VP16, lacking sequences similar to the HSV-1 VP16 acidic activation domain. However, data presented in this report suggest that MDV VP16 is able to transactivate both MDV and HSV-1 IE gene promoters (Figure 8). Transactivation of MDV ICP4 promoters is dependent upon presence of an intact TAATGARAT homology 5' of target genes. Deletion and mutational analysis within the MDV ICP4 gene promoter

sequences revealed that the upstream ATGCAtATATTAT element is important for MDV VP16 activation function (Figure 10). The ATGCAtATATTAT is highly homologous to the extended Oct1 binding site ATGCAAATGARAT, which has been shown to be critical for activation functions of both HSV-1 VP16 and VZV ORF10. Oct-1 binds directly to the TAAT region of the motif, which in many cases forms the second half of an octamer binding site. Recruitment of VP16 is dependent on the presence of the adjacent GARAT sequence (Gerster and Roeder, 1988; O'Hare et al., 1988). The overall level of conservation in the amino-terminal region of this protein family suggests that mechanisms utilized by various VP16 homologs in forming a complex on IE gene promoter DNA may be similar. With regards to the mechanism of activation, deduced amino acid sequence analysis revealed that MDV VP16 contains a region K-E-H-V-H-V-Q-K-L, homologous to the HSV-1 sequence R-E-H-A-Y-S-R-A (Figure 4). These eight amino acids, as well as other residues located between amino acids 360 and 390 (Shaw et al., 1995), have been implicated in VP16 binding to a cellular factor involved in transcriptional complex formation (Hayes and O'hare, 1993). Further investigation of this motif should reveal its role in MDV VP16-directed complex formation.

Transcriptional activation domains have been classified into four main groups , according to their structure and composition. These include the acidic group (HSV-1 VP16), the proline-rich activator group (Spl and CTF), the glutamine-rich group (Spl transcription factor), and the glutamine and proline-rich group (jun oncogene) (Triezenberg, 1995). Examination of the deduced amino acid sequence of MDV

VP16 reveals at least two potential activation regions. The amino-terminal amino acids of MDV VP16 (1 to 58) are highly acidic (23 % acidic residues), whereas the region defined by amino acids 59 to 80 is rich in proline (57 %) (Table1).

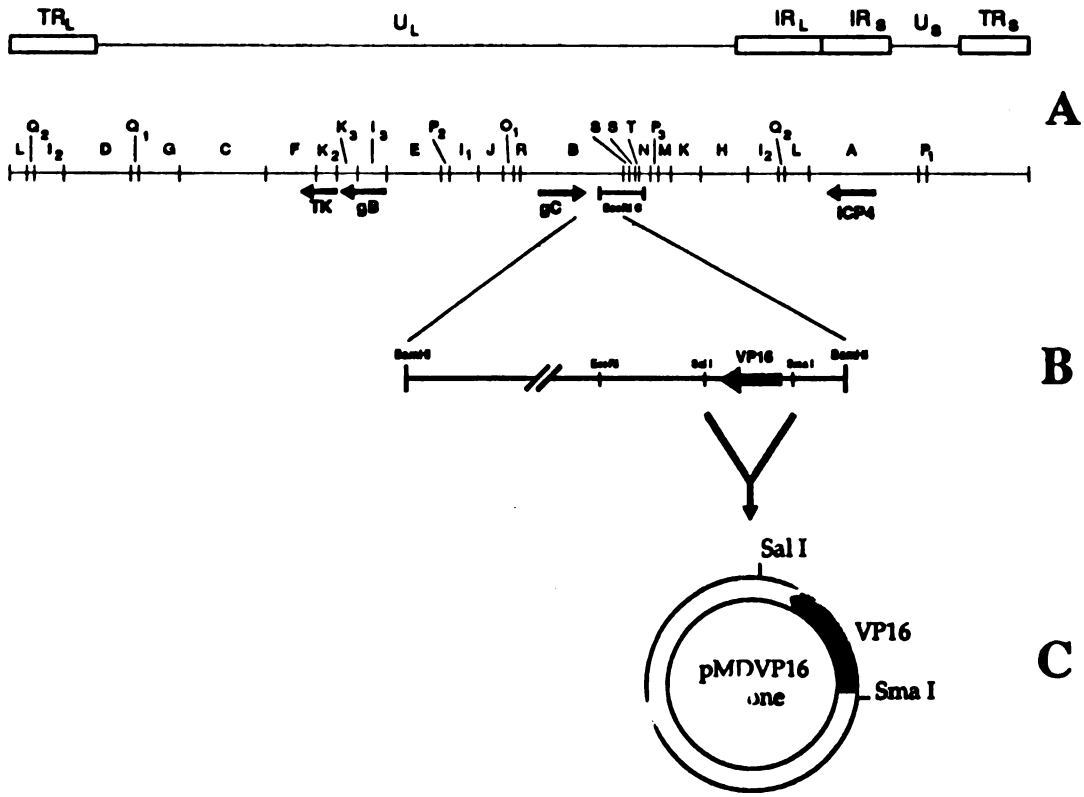
Using the six hydrophobic amino acids surrounding HSV-1 $VP16^{442}$ as a guide, alignment of bulky hydrophobic residues in a number of VP16 homologs revealed striking conservation of these units, despite significant differences in location within the VP16 molecule. These results were further supported by the hydrophobic cluster analysis. Of particular interest in these studies were bulky hydrophobic residues surrounding the MDV VP16 Phe^{43} residue (Figures 11 and 12). Both linear and two-dimensional analyses suggested that residues surrounding MDV VP16 Phe^{43} would be important for transcriptional transactivation. Indeed, substitutions of MDV VP16 Phe^{43} with non-aromatic (alanine), and helix-breaker (proline) residues destroyed MDV VP16 activity, while substitutions of MDV VP16 Phe^{43} with aromatic residues (tyrosine) preserved MDV VP16 activity. These results imply that secondary structure, related to hydrophobic interactions, may be more important to VP16 activation function than either primary sequences, net charge, or position within the protein.

The function of potential proline-rich region identified within the MDV VP16 amino-terminal domain is still unknown. However, considerable effort is being made in our laboratory to determine whether this region is able to transactivate gene promoter expression. Briefly, our strategy is to PCR amplify acidic+proline-rich domain, acidic-rich subdomain, and proline-rich subdomain. PCR products are

cloned upstream of the yeast GAL4 DNA-binding domain (GAL4 DNA-binding domain was cloned into the pBK-CMV plasmid). The cloning junctions of the generated pBK-G4VP16) constructs are sequenced to confirm proper insertion , and western blot analysis are used to determine whether GAL4-VP16 constructs encode a fusion protein of the expected size when transfected into cultured CEF cells. Antibodies raised against HSV-1 VP16 and GAL4 DNA binding domain are used for western blot analysis.

Figure 1:

Localization and cloning of the MDV VP16 gene. **Panel A:** Schematic diagram of MDV DNA and BamHI restriction map based on the results of Fukuchi et al., 1984). The approximate positions and orientation of major MDV genes are shown below the restriction map. The approximate position of the MDV EcoRI C fragment is also indicated (Silva and Witter, 1985). **Panel B**: Partial restriction map of the BamHI B fragment. The approximate position and orientation of the MDV VP16 gene, as determined by partial sequence analysis, are indicated by a solid arrow. **Panel C:** Molecular cloning of the 2.122 Kbp *SmaI-SalI* subfragment of MDV BamHI B into pUC18 to generate pMDVP16. The MDV VP16 gene and its orientation are shown by a solid arrow within the plasmid.


TR_L
U_L
IR_L
IR_S
U_S
TR_S
A
TK
gB
gC
ICP4
B
VP16
C
Sal I
VP16
pMDVP16
Sma I

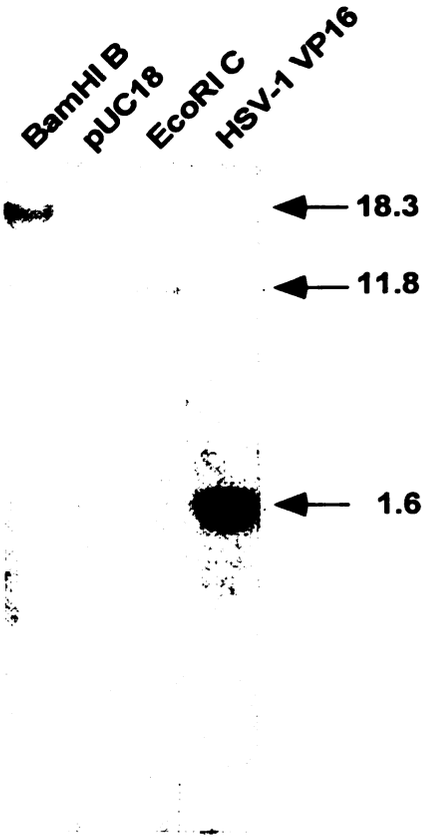


Figure 2: Confirmation of MDV VP16 gene location by Southern blot hybridizaion. DNA from MDV BamHI B (Lane 1), pUC18 (Lane 2), MDV EcoRI C (Lane 3), and HSV-1 VP16 (Lane 4), were separated by electrophoresis in agarose gels. DNA was transferred to nylon membranes and hybridized with radilabelled DNA probe containing the HSV-1 VP16 gene, as detailed in Materials and Methoids.

Sma I
CCCGGGGTCAGAGCTGTACAAAGT**AATAAA**TTCGCTTTCAGTACGGCTCCTTCATC

AGCATCTAGCACTTGGAGATCAAATACAGTGGCATTTAATCAGCGTATGTTTTGCG

GAGCGGTTGCAACTGTGGCTCAATATCACGCATACCAAGGCGCGCTCGCCCTTTGG

CGTCAAGATCCTCCGCGAACAAATGAAGAATTAGATGCATTTCTTTCCAGAGCTGT

CATTAAAATTACCATTCAAGAGGGTCCAAATTTGATGGGGGAAGCCGAAACCTGTG

CCCGCAAACTATTGGAAGAGTCTGGATTATCCCAGGGGAACGAGAACGTAAAGTCC

AAATCTGAACGTACAA**CCAAAT**CTGAACGTACAAGACGCGGCGGTGAAATTGAAAT

CAAATCGCCAGATCCGGGATCTCATCGTACACATAACCCTCGCACTCCCGCAACTT

CGCGTCGCCATCATTCATCCGCCCGCGGATATCGTAGCAGTGATAGCG**AATAAT**GT

AACATAGGAACAGCCCTACTACTGCAAGTAAGTCGTCTTT**TATA**GACATCCGAATT

AAAAACTAGTACA**TATATA**TCTTATCTACTCATTATTGTATAGTGTGA **ATG** GAG
M E

GCA	AAT	ATG	AGT	TTT	GAA	AAC	GAT	TAC	TAC	AGT	CCT	ATC	CAA
A	**N**	**M**	**S**	**F**	**E**	**N**	**D**	**Y**	**Y**	**S**	**P**	**I**	**Q**
TTA	TTC	GCT	GAA	ATT	GAG	GCG	TAT	GCG	AAT	ACT	ATG	GAT	AAG
L	**F**	**A**	**E**	**I**	**E**	**A**	**Y**	**A**	**N**	**T**	**M**	**D**	**K**
TCC	CCG	GAT	CTT	GAT	ATC	CTA	CGA	ACA	ATT	GAA	GAA	TTT	GAT
S	**P**	**D**	**L**	**D**	**I**	**L**	**R**	**T**	**I**	**E**	**E**	**F**	**D**
GAA	ACG	CTT	CTC	TCT	GAA	ATT	GAA	GTC	AGA	ACC	CAA	TCT	ATT
E	**T**	**L**	**L**	**S**	**E**	**I**	**E**	**V**	**R**	**T**	**Q**	**S**	**I**
CCA	TCG	CCT	CTG	GTT	GCT	CCG	TCT	GTT	ACT	AAA	ATG	TCA	CTT
P	**S**	**P**	**L**	**V**	**A**	**P**	**S**	**V**	**T**	**K**	**M**	**S**	**L**
CCA	TCC	CCA	TCT	CCA	GCT	CCT	CCC	AAT	TCT	CTC	TAC	ACT	AGA
P	**S**	**P**	**S**	**P**	**A**	**P**	**P**	**N**	**S**	**L**	**Y**	**T**	**R**
CTG	TTG	CAT	GAG	TTG	GAT	TTT	GTC	GAA	GGC	CCG	TCT	ATA	TTA
L	**L**	**H**	**E**	**L**	**D**	**F**	**V**	**E**	**G**	**P**	**S**	**I**	**L**
GCA	CGT	CTG	GAA	AAA	ATA	AAT	GTA	GAT	CTA	TTC	TCA	TGT	TTC
A	**R**	**L**	**E**	**K**	**I**	**N**	**V**	**D**	**L**	**F**	**S**	**C**	**F**
CCT	CAT	AAT	AAA	CAC	TTA	TAT	GAA	CAT	GCA	AAA	ATT	TTA	TCT
P	**H**	**N**	**K**	**H**	**L**	**Y**	**E**	**H**	**A**	**K**	**I**	**L**	**S**
GTA	TCT	CCT	TCG	GAA	GTA	TTG	GAA	GAG	TTG	TCG	AAG	AAT	ACA
V	**S**	**P**	**S**	**E**	**V**	**L**	**E**	**E**	**L**	**S**	**K**	**N**	**T**
TGG	ACG	TAT	ACG	GCT	TTA	AAT	TTA	AAT	GAA	CAT	GGA	GAG	ATG
W	**T**	**Y**	**T**	**A**	**L**	**N**	**L**	**N**	**E**	**H**	**G**	**E**	**M**
GCC	CTG	CCC	ATG	CCT	CCC	ACA	ACA	AAG	GCC	GAT	TTA	CCT	TCT
A	**L**	**P**	**M**	**P**	**P**	**T**	**T**	**K**	**A**	**D**	**L**	**P**	**S**
TAT	GTA	GAC	GAC	ATA	CAA	AAC	TTC	TAT	TTG	GGA	GAA	TTA	GAA
Y	**V**	**D**	**D**	**I**	**Q**	**N**	**F**	**Y**	**L**	**G**	**E**	**L**	**E**

GCA AGG GAG AAA TCA TAT GCT ACT ATG TTT TAT GGA TAC TGT
A R E K S Y A T M F Y G Y C

CGT GCT CTG GCA GAG TAT ATT AGA CAG TCG GCG ATT AAA GAT
R A L A E Y I R Q S A I K D

CTT CGG GAC GCT CGA GTC GAA GAT AAA AAT ATT GGT GCG TGT
L R D A R V E D K N I G A C

AGT AAA GCA CGA CAA TAC ATC GCC GAA AGG TAT TAT CGA GAA
S K A R Q Y I A E R Y Y R E

GCG GCA AGG TTT GCG AAG CTC TTA TAT GTG CAC TTA TAC CTT
A A R F A K L L Y V H L Y L

TCT ACC ACG CGG GAT GTA TCA CAG CGA CTC GAA GCA TCT CAA
S T T R D V S Q R L E A S Q

ATG GGA CGA CAA AAT ATA TTT GTA TAT TTG AAA TGC GAA TGG
M G R Q N I F V Y L K C E W

TTA CAA GAA AGA CAC TTT CAT TGT TTA TTT CAA CCT GTA ATT
L Q E R H F H C L F Q P V I

TTT AAC CAT GGC GTA GTT ATT GTC GAA GGG CGT GTT TTG ACT
F N H G V V I V E G R V L T

GCT CCT GAA CTC CGG GCT CAG AAT TAT ATA CGT TCC GAG TTC
A P E L R A Q N Y I R S E F

GGT CTT CCT CTA ATA CGA TGT AAA TTA GTA GAA GAG CCT GAC
G L P L I R C K L V E E P D

ATG CCG TTG ATC TCG CCC CCA CCG TTT TCA GGT GAT GCT CCT
M P L I S P P P F S G D A P

CGG GCA TCA GTA TAT TTA TTG CAG TGT ATT AGA TCA AAG TTG
R A S V Y L L Q C I R S K L

GAA GTA TAT TCT CTA TCA CAC CCC CCT AAC CCG CAA TTG CAT
E V Y S L S H P P N P Q L H

GTG CAT AAG GAA CAT GTA CAT GTT CAA AAG CTA GAA TCA CCG
V H K E H V H V Q K L E S P

CCA AAC TAT GGA ACT ACT GTC GAA GCA TTG TTG ATG GAC TCT
P N Y G T T V E A L L M D S

TCA GAC AGA AAT TCA ATT TCC CCT GGT GAT CCT GTT GCC ACT
S D R N S I S P G D P V A T

ACC ATC AGT ACT TTA **TAA**GGAGGTATTTGTCTATTAGTATGATGGTGAGT

T I S T L ★

TGGCAGAACGAAACTATACTTAAATGATATTATACGTAAATGTGACGTTT**AATAA**

ACGAATTTGTCGTATTCGCTTTGTCTTTGTTTCTCACGTCCCATCAATGATGTAA

TATTATTGATGCAATTACACTTCCGCCGTTAGATCAATACATTATAAAACGGCGT

AGTTTTGATAACAGTATGCTGGTAGCACATTCCACCGAAGAATGCAAATGCCTTC

TATGCATCGGTATGGACATCCTGGTCAAAATCAACGACGGGAAAACCAATCAATC

AGAAATTACTT**GTCGAC**

Sal I

Figure 3: Nucleotide and deduced amino acid sequence of the entire *SmaI-SalI* subfragment of the *BamHI* B fragment of the MDV GA strain. MDV VP16 amino acid sequences were shown in bold letters. Potential TATA and CAAT elements, as well as poly(A) signals are underlined.

Figure 4:

Comparison of the predicted amino acid sequences of the carboxyl portion of the MDV VP16, VZV ORF 10, HSV-1 VP16, and EHV-1 gene 12. Gaps have been introduced into the sequence for the best alignment. Boxed amino acids are identical residues or conserved substitutions.

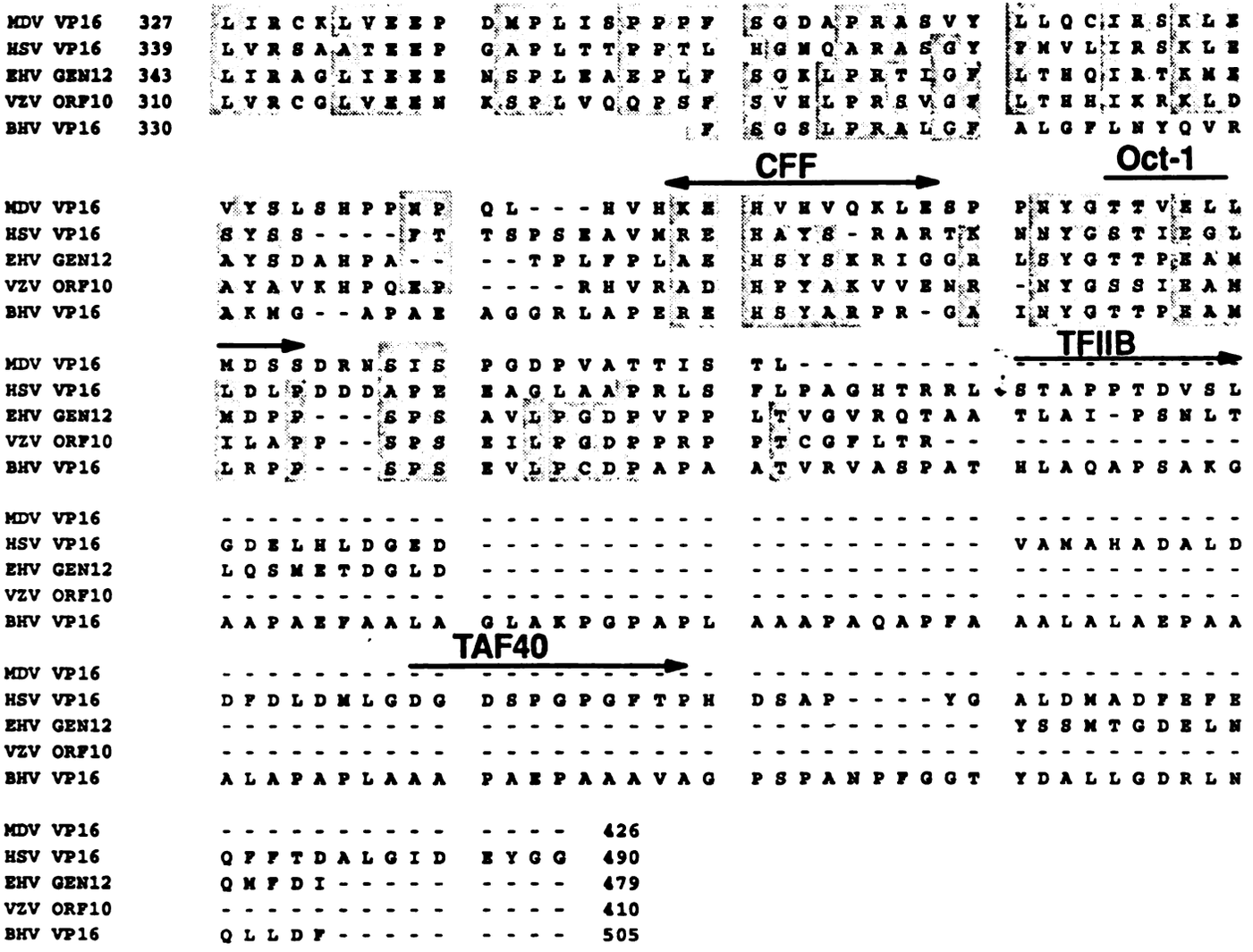
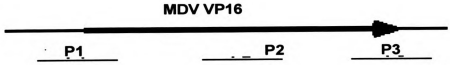

CFF
Oct-1
TFIIB
TAF40

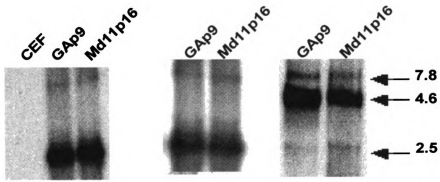
Figure 5:

Detection of the MDV VP16 transcript. Poly(A)$^+$mRNA was isolated, transferred to supported nylon membranes, and hybridized as detailed in Materials and Methods. **(A)** Map of the three MDV DNA probes used for Northern blot analysis. **(B)** RNA was hybridized with each of the DNA probes. Lane 1: uninfected CEF; Lane 2: CEF infected with MDV strain GAp9; Lane 3: CEF infected with MDV strain Md11p16. The primary (7.8, 4.6, and 2.5 Kb) MDV VP16 transcripts are indicated by an arrow to the right. Prediction of the map of the transcripts, according to our results and results published previously (Yanagida et al., 1993).

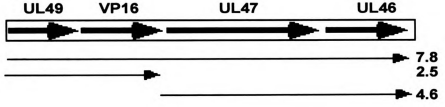
A


MDV VP16
P1
P2
P3

B


CEF
GAp9
Md11p16
GAp9
Md11p16
GAp9
Md11p16
7.8
4.6
2.5

C


UL49
VP16
UL47
UL46
7.8
2.5
4.6

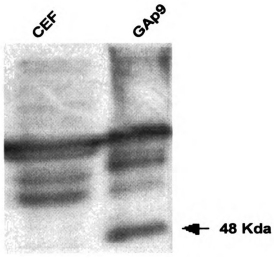


Figure 6: Detection of MDV VP16 gene product by Western blot analysis. Proteins were extracted from uninfected CEF and CEF infected with MDV GAp9 strain. 10 ug of proteins were subjected to Western blot analysis using a poly-clonal antibody raised against the HSDV-1 VP16 gene product.

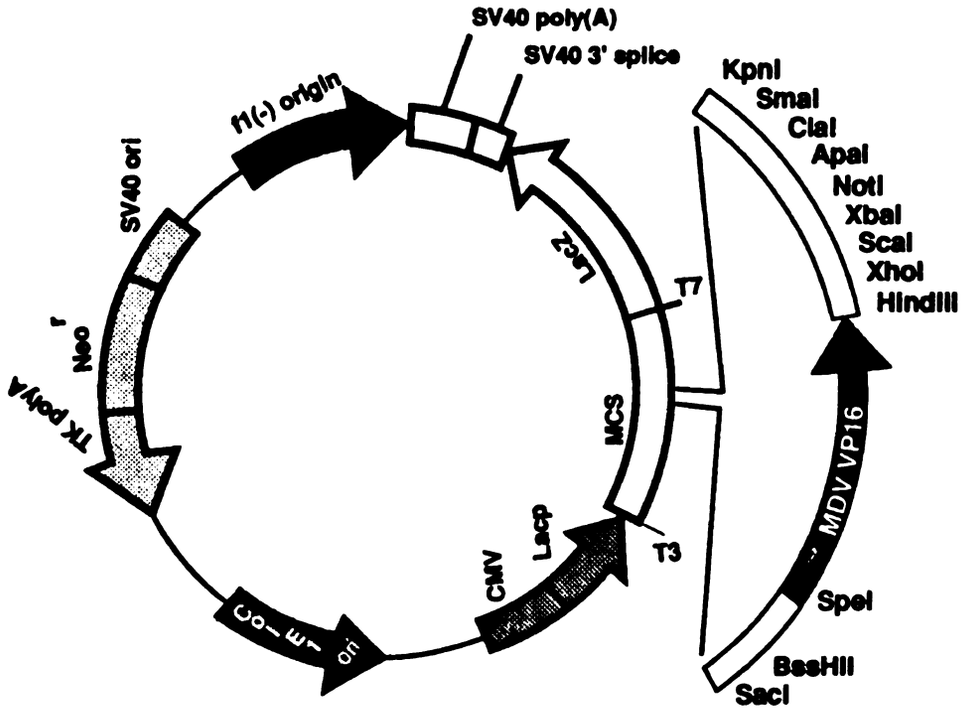


Figure 7: Map of pBKCMVP16. The 1.615 *SpeI-SalI* fragment, which contains the entire MDV VP16 coding region was cloned into pBK-CMV, as described in Materials and Methods.

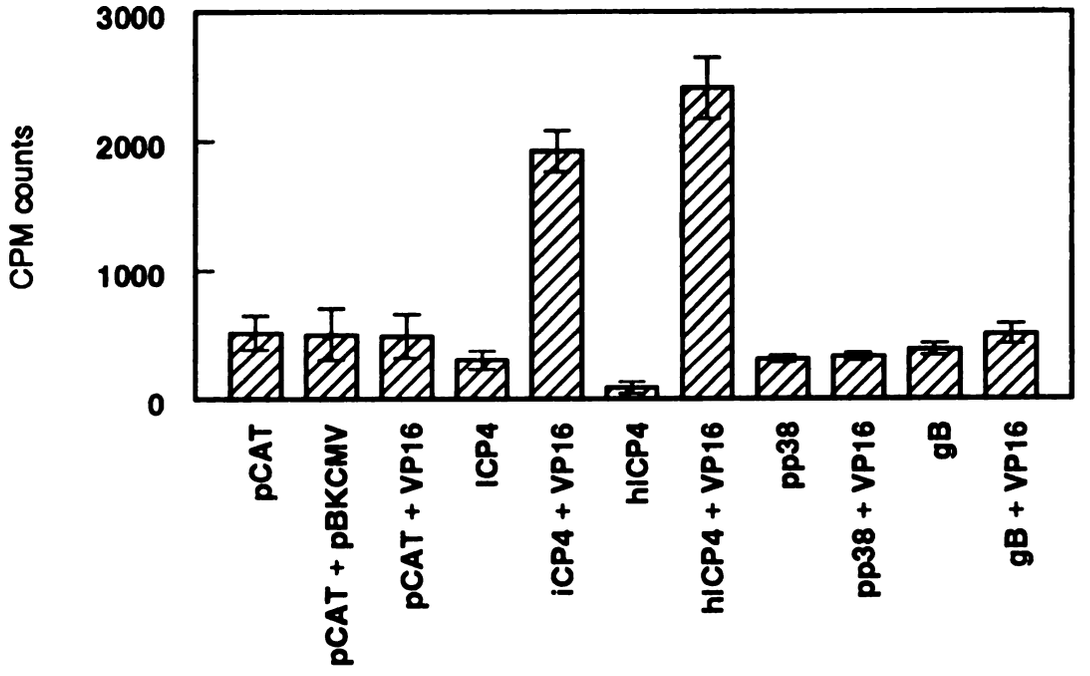


Figure 8:

Transactivation of MDV and HSV-1 IE gene promoters by MDV VP16. CEF cells were cotransfected with 3 ug of plasmid DNA containing either the MDV ICP4 (from low and high passage), pp38, and gB gene promoters or the HSV-1 ICP4 promoter linked to the CAT reporter gene, and 5 ug of pBKCMVP16. Controls were transfected with an amount of pBK-CMV DNA equivalent to that of pBKCMVP16.

Figure 9A:

Schematic diagram indicating relative positions of potential transcription factor binding sites in the MDV ICP4 promoter sequences (upper figure). Deletion versions of the ICP4 are below the wild type MDV ICP4 gene promoter.

ICP4 PROMOTER MUTANTS

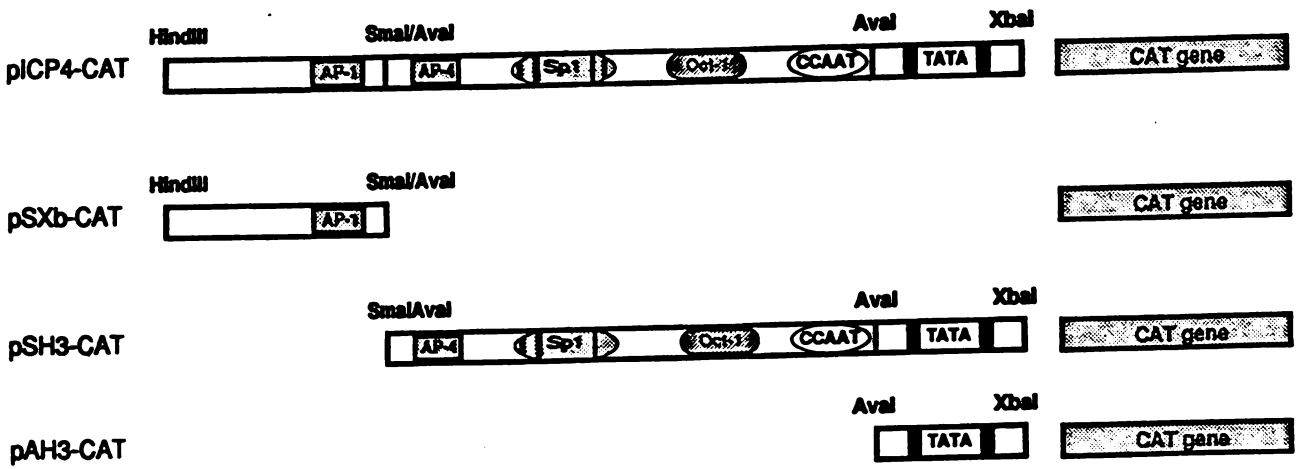


Figure 9B:

Effect of deletions within the MDV ICP4 gene promoter sequences on basal and MDV VP16 activated transcription. Lane 1: mock, lane 2: pCAT, lane 3: pCAT+VP16. Lane 4 &5: MDV ICP4 in the absence and presence of VP16, respectively. Lanes 6 & 7: pSH3CAT in the absence and presence of VP16, respectively. Lanes 8 & 9: pAH3CAT in the absence and presence of VP16. Lanes 10 & 11: PSXbCAT in the absence and presence of VP16.

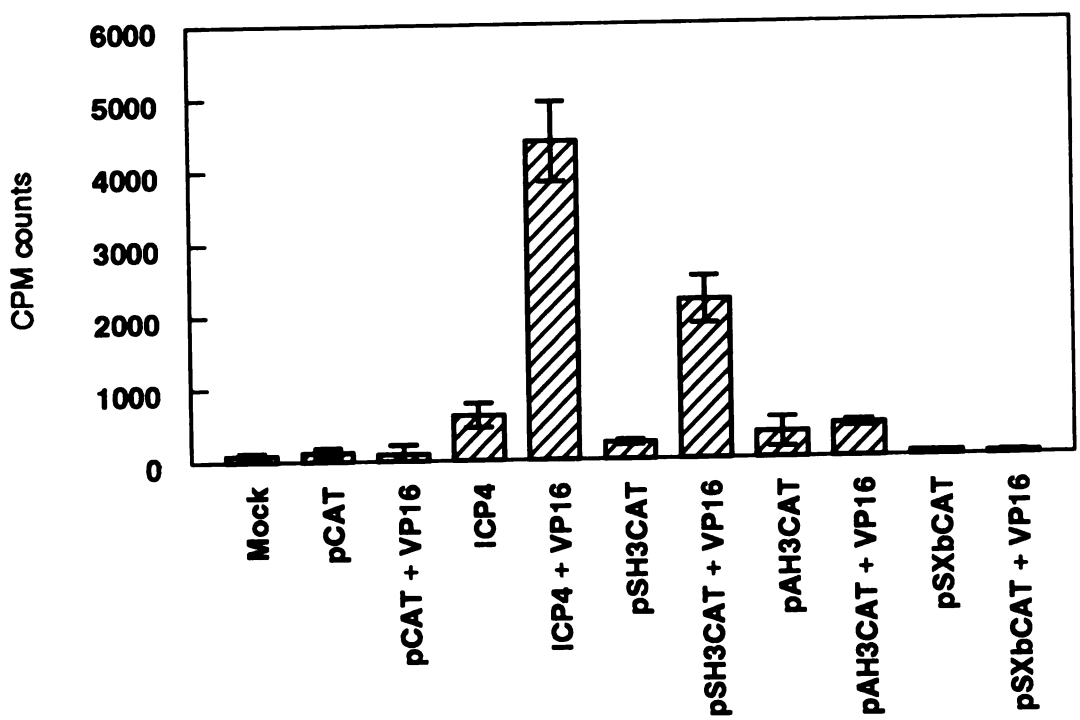

CPM counts
6000
5000
4000
3000
2000
1000
0
Mock
pCAT
pCAT + VP16
ICP4
ICP4 + VP16
pSH3CAT
pSH3CAT + VP16
pAH3CAT
pAH3CAT + VP16
pSXbCAT
pSXbCAT + VP16

Figure 10:

Effect of point mutations within the MDV ICP4 promoter Oct-1 site on MDV VP16 activated transcription. Lane 1: mock, lane 2:pCAT, lane 3: pCAT+VP16. MDV ICP4, Oct1A-, abd Oct1B- expressions were assessed in the absence (lanes 4, 6, and 8), or in the presence on MDV VP16 (lanes 5, 7, and 9).

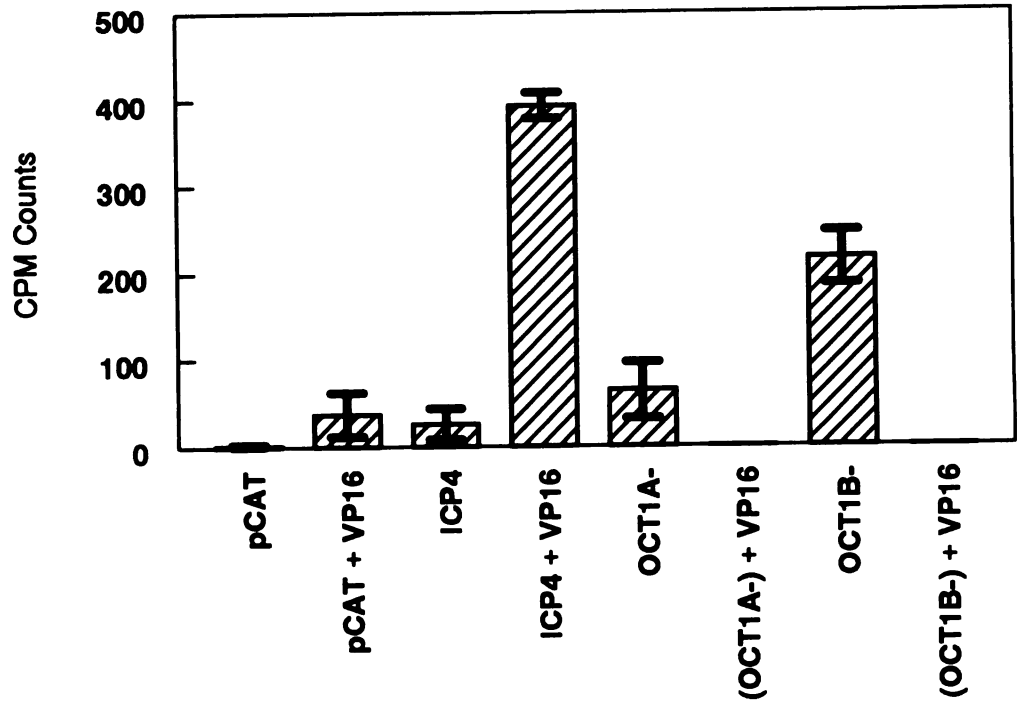
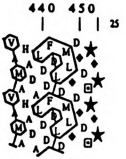
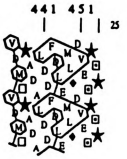

CPM Counts
500
400
300
200
100
0
pCAT
pCAT + VP16
ICP4
ICP4 + VP16
OCT1A-
(OCT1A-) + VP16
OCT1B-
(OCT1B-) + VP16

Figure 11A:

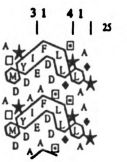
Hydrophobic Cluster analysis of several HSV-1 VP16 gene homologs. HCA profiles show the characteristic horse shoe-shaped cluster and the centrally located Phe residues.



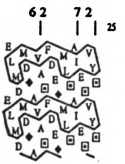
VPI6-1



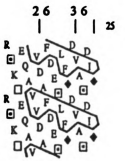
VP16-2



BHVUL48



EHV-1 GEN12



VZVGEN10

MDVUL48

Figure 11B:

Linear alignment of several HSV-1 VP16 homologs using the six hydrophobic residues surrounding HSV-1 VP16 Phe^{442} residue as a guide. Shading indicates conserved residues. Boldface letters indicate conserved bulky hydrophobic residues. Dark shading denotes conserved acidic residues. Open ovals represent residues conserved among homologs but not present in HSV-1 VP16.

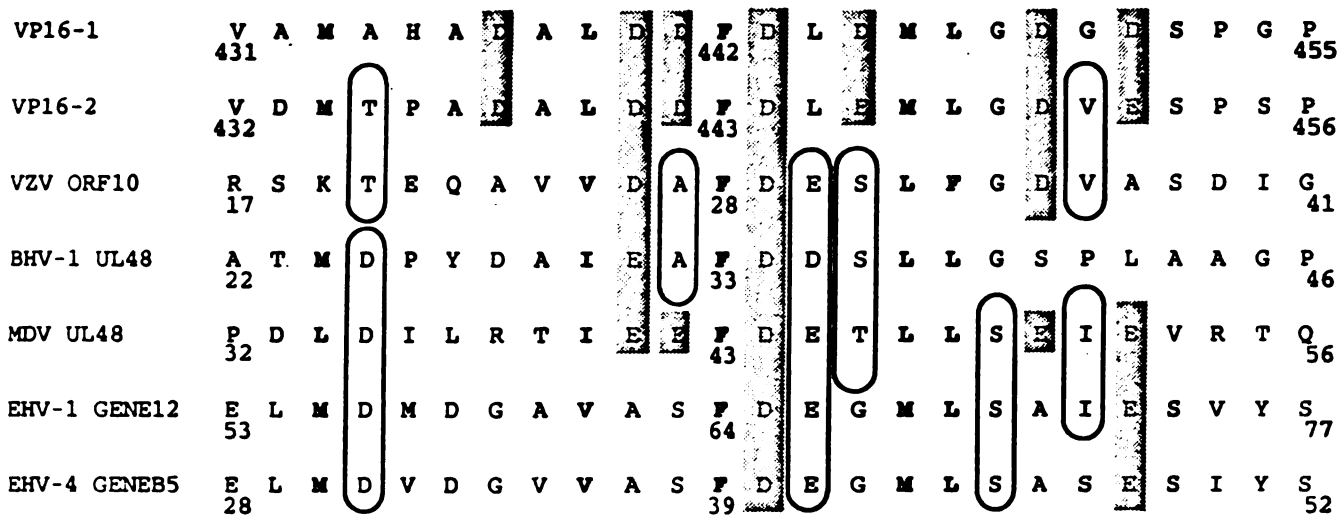
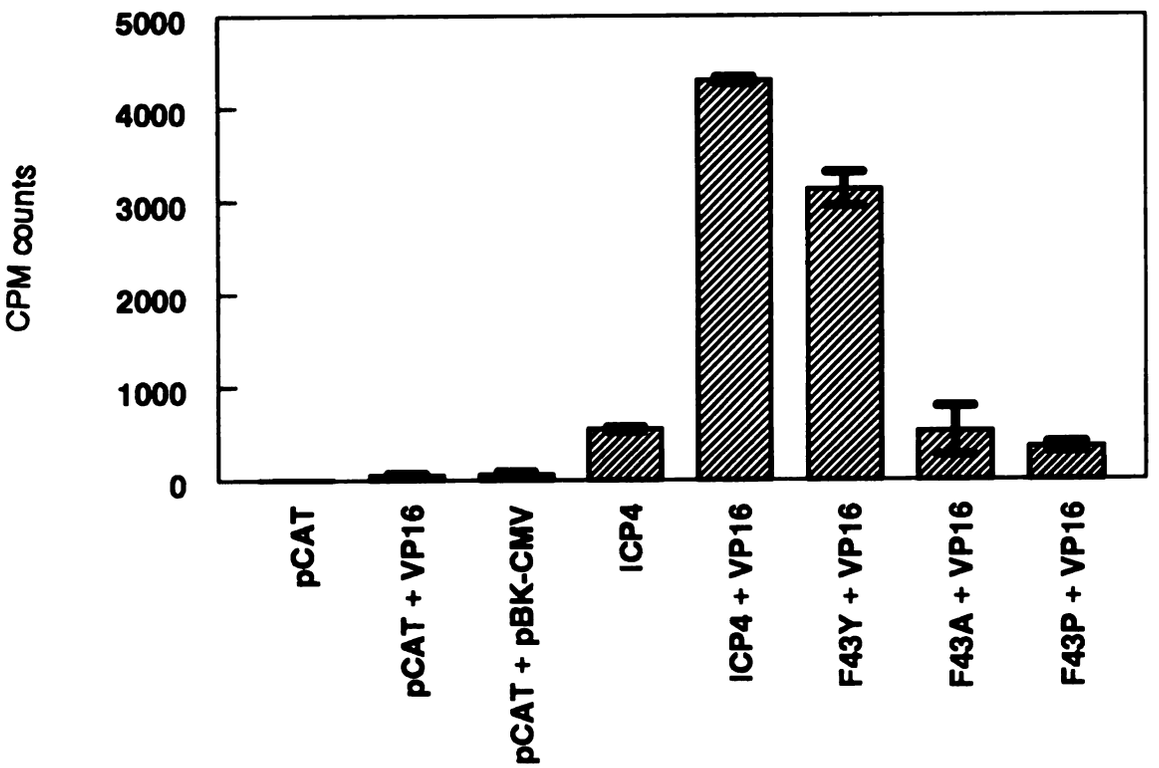

VP16-1 V 431 A M A H A D A L D D F 442 D L D M L G D G D S P G P 455
VP16-2 V 432 D M T P A D A L D D F 443 D L E M L G D V E S P S P 456
VZV ORF10 R 17 S K T E Q A V V D A F 28 D E S L F G D V A S D I G 41
BHV-1 UL48 A 22 T M D P Y D A I E A F 33 D D S L L G S P L A A G P 46
MDV UL48 P 32 D L D I L R T I E E F 43 D E T L L S E I E V R T Q 56
EHV-1 GENE12 E 53 L M D M D G A V A S F 64 D E G M L S A I E S V Y S 77
EHV-4 GENEB5 E 28 L M D V D G V V A S F 39 D E G M L S A S E S I Y S 52

Figure 12A:

Effect of aromatic amino acids at position 43 on the MDV VP16 activation function. Lane 1: mock, lane 2: pCAT, lane 3: pCAT+VP16. Expression of MDV ICP4, or MDV ICP4 mutant constructs was assessed in the absence of VP16 (Lanes 4, 6, 8, and 10) or in the presence of wild type VP16 (lane 5), F43Y (lane 7), F43A (lane 9), and F43P (lane 11).


CPM counts
5000
4000
3000
2000
1000
0
pCAT
pCAT + VP16
pCAT + pBK-CMV
ICP4
ICP4 + VP16
F43Y + VP16
F43A + VP16
F43P + VP16

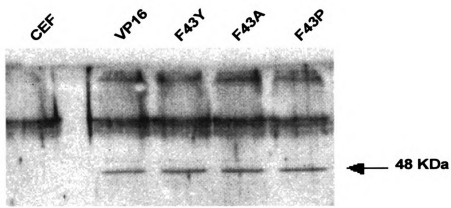


Figure 12B: Western blot analysis of MDV VP16 mutants. Proteins were extracted from mock-infected (Lane 1), and CEF infected with MDV VP16 (Lane 2), F43Y (Lane 3), F43A (Lane 4), and F43P (Lane 5). 10 ug of p[roteins were subjected to western blot analysis using a poly-clonal antibody raised against the HSV-1 VP16 gene product.

MDV VP16	EHV-1 GEN12	EHV-4 GEN12	BHV-1 UL48	VZV ORF10	HSV-1 VP16	HSV-2 VP16
% similarity	58	60	56	58	56	57
% identity	42	43	38	38	35	36

Table 1: Deduced amino acids comparisons between several MDV VP16 homologs.

Activators	Activation domains	% Acidic residues	& proline residues
MDV VP16	1 to 58	23.0	5.0
MDV VP16	59 to 80	3.0	57.0
VZV ORF10	5 to 79	20.0	7.0
HSV VP16	400 to 490	23.0	8.0

Table 2:

Comparison of putative MDV VP16 amino-terminal region activation domains with known transactivation domains from other alphaherpesvirus VP16 homologs.

Chapter 4

General conclusion and future research

General conclusion

HSV-1 VP16 is one of the most thoroughly studied transcriptional activators of gene expression. Like other regulatory proteins, HSV-1 VP16 contains a prototypical activation domain located at the carboxyl-terminus and a DNA-binding domain located at the amino-terminus. To investigate the relevance of VP16 protein to the life cycle and evolution of herpesviruses, intensive efforts were made to identify and investigate VP16 homologs from several other herpesviruses. These include Varicella-zoster virus (VZV), Equine herpesvirus type 1 and 4 (EHV-1, and EHV-4), Bovine herpesvirus type 1 (BHV-1), and more recently Marek's disease virus (MDV).

Our studies have focused primarily on identification and functional characterization of the MDV VP16 gene. MDV VP16 gene was localized within the *BamHI* B fragment of MDV strain GA. The MDV VP16 gene encodes a polypeptide of 426 amino acids and apparent size of approximately 48,000 kDa. Like VZV ORF10, MDV VP16 lacks sequences similar to the HSV-1 VP16 carboxyl-terminal acidic transactivation domain. Nevertheless, MDV VP16 is able to transactivate both homologous (MDV) and heterologous (HSV-1) IE gene promoters.

HSV-1 VP16 interacts with IE gene promoters through the TAATGARAT

motif. In the case of the MDV ICP4 gene promoter sequences, at least one clearly defined TAATGARAT element has been identified, providing a possible target during activation by the MDV VP16 gene product. Our studies demonstrated that this motif is, indeed, required for VP16 recruitment to the MDV ICP4 gene promoter region, and subsequent enhancement of ICP4 gene transcription. Mutations within the MDV ICP4 TAATGARAT region resulted in loss of MDV VP16 activity. Our results imply that MDV VP16 may function through a mechanism similar to HSV-1 VP16, despite significant amino acid sequence differences. The involvement of other promoter elements, however, can not be excluded.

Like EHV-1 gene12 and VZV ORF10, MDV VP16 lacks the highly acidic carboxyl-terminal transactivation domain present in HSV-1 VP16. Deduced amino acid sequence analysis, however, revealed that MDV VP16 possesses two potential transactivation domains located within the amino-terminal region of the protein. An acidic-region is encoded by amino acids 1 to 58, and a proline-rich region is defined by amino acids 59 to 80. Acidic and proline-rich domains have been previously identified as potent transactivation domains. The use of hydrophobic cluster analysis further helped in identification of a hydrophobic cluster surrounding MDV VP16 Phe^{43} that is highly conserved with that surrounding Phe at position 442 (HSV-1), 28 (VZV), 64 (EHV-1), and 33 (BHV-1). Site-specific *in vitro* mutagenesis revealed that substitutions of Phe^{43} of MDV VP16 with an aromatic residue perserved its activity, whereas substitutions with two non-aromatic amino acids completely destroyed MDV VP16 activity, similar to results obtained with either

HSV-1 VP16 Phe^{442} or VZV ORF10 Phe^{28}. Studies reported from our laboratory and by others indicate that transactivation domains of VP16 gene family members are highly similar, and thus may have evolved from a common ancestral domain.

The use of the GAL4 protein chimera system offers advantages in preliminary identification of activator subdomains, especially, in large regulatory proteins since it allows the analysis of relatively small segments of a complex protein independent of its spatial distribution as determined by the rest of the polypeptide in the native molecule. Intensive efforts are now being made in our laboratory in order to identify the functional characteristics of the proline-rich region identified within the amino-terminal domain of the MDV VP16 molecule. Three different constructs are made: **(1)** acidic- and proline rich domains are cloned into the GAL4 DNA-binding domain, **(2)** only the acidic-rich subdomain is cloned into the GAL4 system, and **(3)** only the proline-rich subdomain is fused to GAL4. The last two constructs will allow us to investigate the functional characteristics of acidic- and proline-rich regions independently of each other, whereas the first construct will allow us to determine whether there is any additive effect on gene activation when acidic- and proline-rich regions are both present, compared to the activation level produced by each of the domains independently..

Future research

Elucidation of the mechanism of action of VP16 gene family members is important for understanding not only the mechanism of transcriptional control, but also the interactions between activation domains of regulatory proteins and components of the basal transcription machinery.

1) - MDV VP16-target protein interactions

Previous studies have indicated that the cellular factors Oct1 and HCF are critical for both HSV-1 VP16 and VZV ORF10-mediated transactivation of IE gene expression (Katan et al., 1990; Xiao and Capone, 1990; Kristie et al., 1989; Moriuchi et al., 1995). Detailed mutational analysis of the MDV ICP4 gene promoter regulatory element has revealed the presence of a TAATGARAT element overlapping an Oct1-binding site that is critical for MDV VP16 activity. Definitive identification of this element as a specific DNA-recognition site for the cellular factor Oct1 is lacking. Mobility shift assays, using oligonucleotides containing intact or mutated Octamer/TAATGARAT-like elements of the MDV ICP4 promoter, should reveal whether MDV VP16 directs assembly of a multi-component protein-DNA complex. Monoclonal antibodies raised against MDV VP16, Oct1, and HCF are now available, and can be used to determine whether MDV VP16 transactivates IE gene promoters through direct interactions with the cellular factors Oct1 and HCF.

2) - MDV VP16 sequences involved in VP16-cellular factor complex assembly

In HSV-1, a region extending between amino acids 360 to 390 contains critical sequence elements for binding of both cellular factors Oct1 and HCF (Hayes and O'Hare, 1993; Shaw et al., 1995). This region is well conserved among other VP16 homologs including MDV VP16. Previous work identified several individual residues within this region that are important for complex formation (Hayes and O'Hare, 1993; Shaw et al., 1995). Most of these residues are conserved in MDV VP16. In addition, a critical segment consisting of three consecutive D residues (385-387) is missing in all VP16 homologs. Deletion of these residues dramatically reduced the ability of HSV-1 VP16 to form a complex with cellular factors and TAATGARAT. The lack of these D residues, however, did not interfere with complex formation (Elliot, 1994). MDV VP16 contains only one D residue. Amino acid substitutions within MDV VP16 should reveal whether these amino acids are, indeed, important for MDV VP16 interactions with Oct1 and HCF.

3) - Interactions of MDV VP16 with components of RNAPII transcription machinery

HSV-1 VP16 interacts with several components of RNAPII transcription machinery. These include interactions with TBP and TAFs, TFIIA, TFIIB, and TFIIH. The entire HSV-1 VP16 carboxyl-terminal region (amino acids 412-490) is required for its interaction with RNA polymerase II transcription factor

TFIIB, whereas only amino acids 452-490 of HSV-1 VP16 are critical for its interaction with $TAF_{II}40$. These amino acid signals are, however, missing in MDV VP16 and VZV ORF10. These differences could account for any functional differences of both MDV and HSV-1 VP16 proteins. Identification of interactions of MDV VP16 with components of the RNA polymerase II transcription machinery will provide new insights into transcription control mechanisms by VP16 gene family members.

4) - MDV VP16 and viral replication

HSV-1 VP16 is essential for viral replication (Weinheimer et al., 1992). The VZV homolog ORF10, however, is dispensable for VZV replication, in vitro (Cohen and Seidel, 1994). Unfortunately, the effect of both HSV-1 VP16 and VZV ORF10 on viral replication has never been studied in their natural host (human in this case). MDV VP16, however, is unique in that its effect on MDV replication can be investigated directly in the natural host, chickens. This experiment can be achieved by constructing MDV VP16 mutants with either stop codons in the VP16 coding region or MDV VP16 deleted. Studying the effect of MDV VP16 on viral replication is facilitated by the availability of continuous growing CHCC-OU2 cells, in our laboratory (Abujoub and Coussens, 1995). OU2 cells can be used to create VP16-expressing cell lines. To determine whether MDV VP16 is essential for viral replication, cell-free MDV mutants can be prepared by sonicating infected OU2 cells that express VP16, and removing the cell debris by centrifugation. The supernatant

which contains cell-free MDV will be used to infect CEF cells that do not express VP16. Seven days post-infection, cytopathic effects typical of MDV can be examined. Cell-free MDV VP16 mutants can also be injected into birds. This will allow us to determine the effect of VP16 on MDV replication and virulence, in vivo. Uninfected and MDV-infected birds would be used as controls.

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