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UMI
Biochemical Characterization of the Major DNA-binding Protein of Murine Cytomegalovirus

A thesis submitted to the College of Graduate Studies and Research in partial fulfillment of requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology University of Saskatchewan

By Qiaohua Wu December, 1998

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0-612-37924-8
UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

SUMMARY OF DISSERTATION

Submitted in partial fulfillment
of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

Qiaohua Wu

Department of Microbiology and Immunology
University of Saskatchewan

Winter 1998

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Biochemical Characterization of the Major DNA-binding Protein of Murine Cytomegalovirus

Human cytomegalovirus (HCMV) infection is common in human populations. While the infection in healthy adults is asymptomatic, it poses life-threatening diseases in immunocompromised individuals. Because of species specificity, the study of HCMV infection in humans is limited. Therefore, murine cytomegalovirus (MCMV) has been extensively used as a model for HCMV infection due to its striking biological similarities to HCMV. The MCMV major DNA-binding protein (MDBP) is a non-structural protein produced during early times of viral infection. The protein encoded by the M57 open reading frame (ORF) of the viral genome has previously been shown to share significant amino acid sequence similarity to both HCMV single-stranded DNA-binding protein (ssDBP) and herpes simplex virus type 1 (HSV-1) ICP8. Extensive studies have shown that HSV-1 ICP8 is one of seven essential proteins required for viral DNA replication. Although the ORF of the MCMV MDBP has been identified and characterized with respect to the analysis of transcript, no biochemical study has been reported. In this study, I characterized the biochemical properties of the MCMV MDBP, especially the single-stranded DNA-binding domain and nuclear localization signals (NLSs) of the protein. To do this, the M57 ORF encoding MCMV MDBP was first cloned into an expression vector. The histidine-tagged MCMV MDBP fusion protein was produced and used to develop a panel of monoclonal antibodies (MAbs) which recognized epitopes located within the N- and C-termini of the MDBP. These MAbs were used to monitor the expression of wild type (wt) and mutant MDBPs during infection and transfection experiments, and facilitated the characterization of the biochemical properties of MCMV MDBP. Single-stranded DNA-cellulose column chromatography was carried out to assess the ssDNA-binding property of the wild type
(wt) and mutant MDBPs synthesized in an \textit{in vitro} coupled transcription-translation reaction. Nuclear localization signal regions were identified by transiently expressing the mutant MDBP gene in COS-1 cells and observing the subcellular localization of the expressed protein with indirect immunofluorescence. Gel filtration chromatography was performed to define the native molecular weight of the MDBP extracted from MCMV-infected cells. The results demonstrated that wt MDBP was eluted from the column at 250 mM NaCl. Deletion of a region (amino acids 422-535) containing a zinc finger-like motif completely abolished ssDNA-binding. However, the ssDNA-binding activity of the protein was found to reside largely in the C-terminal half of the protein. Partial deletion (amino acids 788-824) of a putative ssDNA-binding motif significantly affected ssDNA-binding, suggesting that this region may contain an important determinant for the ssDNA-binding activities of MCMV MDBP. In indirect immunofluorescence experiments, the MDBP was localized to discrete areas of the nuclei of infected cells. In contrast, a more diffuse nuclear staining pattern was evident during transient expression of the protein in COS-1 cells. Although the C-terminus of the protein contains two clusters of basic residues capable of targeting a heterologous cytoplasmic protein to the nucleus, the N-terminus of the protein, which also contains such residues, appeared to constitute the major determinant for the nuclear localization of MCMV MDBP. Finally, while the protein migrated as a monomer with an apparent molecular weight of 133 K in SDS-PAGE, it was found mainly in high molecular weight complexes of in excess of 200 K in MCMV-infected cells, suggesting that it formed complexes with viral and/or cellular proteins. Collectively, these results indicate that MCMV MDBP is similar in many aspects but not identical to the previously reported HSV-1 counterpart, ICP8.
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ABSTRACT

Human cytomegalovirus (HCMV) infection is common in human populations. While the infection in healthy adults is asymptomatic, it poses life-threatening diseases in immunocompromised individuals. However, the strict species specificity exhibited by HCMV has hindered in vivo experimentation in animals. Murine cytomegalovirus (MCMV) has been extensively used as a model for HCMV infection due to its striking biological similarities to HCMV. The MCMV major DNA-binding protein (MDBP) is a non-structural protein produced during early times of viral infection. The protein encoded by the M57 open reading frame (ORF) of the viral genome has previously been shown to share significant amino acid sequence similarity to both HCMV single-stranded DNA-binding protein (ssDBP) and herpes simplex virus type 1 (HSV-1) ICP8. Extensive studies have shown that HSV-1 ICP8 is one of seven essential proteins required for viral DNA replication. Although the ORF of the MCMV MDBP has been identified and characterized with respect to the analysis of transcript, no biochemical study has been reported. In this study, I characterized the biochemical properties of the MCMV MDBP, especially the single-stranded DNA-binding domain and nuclear localization signals (NLSs) of the protein. To do this, the M57 ORF encoding MCMV MDBP was first cloned into an expression vector. The histidine-tagged MCMV MDBP fusion protein was produced and used to develop a panel of monoclonal antibodies (MAbs) which recognized epitopes located within the N- and C-termini of the MDBP. These MAbs were used to monitor the expression of wild type (wt) and mutant MDBPs during infection and transfection experiments, and facilitated the characterization of the biochemical properties of MCMV MDBP. Single-stranded DNA-cellulose column chromatography was carried out to assess the ssDNA-binding property of the wild type
(wt) and mutant MDBPs synthesized in an in vitro coupled transcription-translation reaction. Nuclear localization signal regions were identified by transiently expressing the mutant MDBP gene in COS-1 cells and observing the subcellular localization of the expressed protein with indirect immunofluorescence. Gel filtration chromatography was performed to define the native molecular weight of the MDBP extracted from MCMV-infected cells. The results demonstrated that wt MDBP was eluted from the ssDNA-cellulose column at 250 mM NaCl. Deletion of a region (amino acids 422-535) containing a zinc finger-like motif completely abolished ssDNA-binding. However, the ssDNA-binding activity of the protein was found to reside largely in the C-terminal half of the protein. Partial deletion (amino acids 788-824) of a putative ssDNA-binding motif significantly affected ssDNA-binding, suggesting that this region may contain an important determinant for the ssDNA-binding activities of MCMV MDBP. In indirect immunofluorescence experiments, the MDBP was localized to discrete areas of the nuclei of infected cells. In contrast, a more diffuse nuclear staining pattern was evident during transient expression of the protein in COS-1 cells. Although the C-terminus of the protein contains two clusters of basic residues capable of targeting a heterologous cytoplasmic protein to the nucleus, the N-terminus of the protein, which also contains such residues, appeared to constitute the major determinant for the nuclear localization of MCMV MDBP. Finally, while the protein migrated as a monomer with an apparent molecular weight of 133 K in SDS-PAGE, it was found mainly in high molecular weight complexes of in excess of 200 K in MCMV-infected cells, suggesting that it formed complexes with viral and/or cellular proteins. Collectively, these results indicate that MCMV MDBP is similar in many aspects but not identical to the previously reported HSV-1 counterpart, ICP8.
ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor, Dr. Lambert C. Loh. Without his guidance I would not have completed this project. Dr. Loh was always patient and understanding. His advice and encouragement were invaluable when the going got tough. I have enjoyed and will miss every moment when I worked with him.

Many thanks to the members of my Graduate Advisory Committee, Drs. S. Hayes, S. Hemmingsen, P. Krone, and W. Xiao, for their time and helpful suggestions on this project, and Dr. Bala Chandran for serving as my external examiner.

I am extremely grateful to Dr. S. Laferté from the Department of Biochemistry, for the help in monoclonal antibody production and gel filtration chromatography.

I thank all the members and fellow graduate students of the Department of Microbiology and immunology for their help and friendship. I particularly thank Vicki Keeler and Yan Chen, for their kind and enthusiastic help and support over these years.

I gratefully acknowledge receipt of the graduate student scholarship of the University of Saskatchewan and the graduate teaching assistantship of the Department of Microbiology and Immunology, the University of Saskatchewan.

Finally, I thank my wife, Yi Fan, for her never ending support and love, and always being there when I needed her. I am also very grateful to my parents in China for their understanding and love.
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LIST OF ABBREVIATIONS

aa.............................. amino acid
AdDBP.......................... adenovirus single-stranded DNA-binding protein
αTIF.............................. a trans-inducing factor
AP................................. alkaline phosphatase
ATCC............................. American Type Culture Collection
BCIP.............................. 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
β-gal.............................. β-galactosidase
bp................................. base pairs
BrdU.............................. bromodeoxyuridine
BSA.............................. bovine serum albumin
CMV.............................. cytomegalovirus
CIP................................. calf intestinal alkaline phosphatase
CsCl.............................. cesium chloride
DAPI.............................. 4′, 6-diamidino-2-phenylindole
ddH2O............................ distilled deionized water
DMEM............................ Dulbecco's modified Eagle medium
DMSO............................. dimethyl sulfoxide
dsDNA............................ double-stranded DNA
EBV.............................. Epstein-Barr virus
E. coli.......................... *Escherichia coli*
EDTA............................ ethylenediaminetetraacetic acid
ELISA............................ enzyme-linked immunosorbent assay
FBS.............................. fetal bovine serum
FITC............................. fluorescein isothiocyanate
g................................. gravitational force
gB................................. glycoprotein B
GP5.............................. fd bacteriophage gene 5 protein
GP32............................. T4 bacteriophage gene 32 protein
HAT.............................. hypoxanthine, aminopterin, and thymidine
HCMV........................... human cytomegalovirus
<table>
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<tr>
<td>HeBS</td>
<td>N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffered saline</td>
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<td>HHV-6</td>
<td>human herpesvirus-6</td>
</tr>
<tr>
<td>hnRNP A1</td>
<td>the heterogeneous nuclear ribonucleoprotein A1</td>
</tr>
<tr>
<td>HSV-1</td>
<td>herpes simplex virus type 1</td>
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<tr>
<td>HT</td>
<td>hypoxanthine/thymidine</td>
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<tr>
<td>HveA</td>
<td>herpesvirus entry protein A</td>
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<tr>
<td>ICP8</td>
<td>infected cell polypeptide 8</td>
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<tr>
<td>IE</td>
<td>immediate-early</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IRS</td>
<td>internal repeat short</td>
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<tr>
<td>IPTG</td>
<td>isopropylthio-β-D-galactoside</td>
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<tr>
<td>kb</td>
<td>kilobase pairs</td>
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<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
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<td>MCMV</td>
<td>murine cytomegalovirus</td>
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<tr>
<td>MCS</td>
<td>multiple cloning sequence</td>
</tr>
<tr>
<td>MDPB</td>
<td>major DNA-binding protein</td>
</tr>
<tr>
<td>m.o.i</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>NBT</td>
<td>p-nitro blue tetrazolium chloride</td>
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<td>ND10</td>
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<tr>
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<td>nuclear localization signal</td>
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<td>nonidet P-40</td>
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<td>NPC</td>
<td>nuclear pore complexes</td>
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<tr>
<td>nt</td>
<td>nucleotides</td>
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<tr>
<td>OD600</td>
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<tr>
<td>OBP</td>
<td>origin binding protein</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>orILyt</td>
<td>lytic cycle DNA replication origin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>phosphonoformic acid</td>
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PFU.......................... plaque-forming units
PMSF.......................... phenylmethylsulphonylfluoride
Pol.......................... DNA polymerase
RIPA.......................... radioimmunoprecipitation assay
RPA.......................... replication protein A
RPM.......................... rotations per minutes
SDS-PAGE...................... Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRT.......................... short replicator transcripts
SSB.......................... Escherichia coli ssb gene product
ssDBP.......................... single-stranded DNA-binding protein
ssDNA.......................... single-stranded DNA
SV40.......................... simian virus 40
TAE.......................... Tris-acetate-ethylenediaminetetraacetic acid
TBS.......................... Tris-buffered saline
TBST.......................... Tris-buffered saline tween 20
TE.......................... Tris-ethylenediaminetetraacetic acid
TNF.......................... tumor necrosis factor
Tris.......................... Tris (hydroxymethyl) aminomethane
TRS.......................... terminal repeat short
UDG.......................... uracil-DNA glycosylase
UL.......................... unique long
Us.......................... unique short
1.0 INTRODUCTION

1.1 Cytomegaloviruses

1.1.1 Historical review

Prior to the isolation of cytomegaloviruses (CMVs), their characteristic cytopathology had been recognized by pathologists. Ribbert (1904) reported that in 1881 he noted for the first time large "protozoan like" cells in the sections of kidneys from a stillborn infant. Later, these cells were also found in lungs, liver, parotid glands, and thyroid of infants dying from a variety of diseases. The nature of this cytopathologic feature was not understood. Initially, the cells were thought to be protozoa, i.e., coccidia, sporozoa, or amoebas. However, over the next several decades, the similarities of these cells to those infected by varicella-zoster virus and herpes simplex virus led to the speculation that these cell lesions were caused by a virus from the herpesvirus family.

With increased use of cell cultures from animal tissues in the early 1950s, CMVs were isolated from many tissues. Smith (1954) isolated murine cytomegalovirus (MCMV) from mouse embryo cells which showed large intranuclear inclusion bodies. This pioneer work marked the beginning of the virological stage of CMV study. In 1956 and 1957, human cytomegalovirus (HCMV) was isolated in three different laboratories in the United States. Smith (1956) isolated two HCMV strains from salivary glands and kidneys which contained cytomegalic inclusion bodies. While studying the adenoviruses, Rowe et al. (1956) accidentally recovered three HCMV strains by culturing human
adenoidal tissue from three asymptomatic children. Later, another three HCMV strains were identified by Weller et al. (1957) from liver biopsy specimens and urine of infants suffering from congenital CMV disease. When the virus was initially isolated, it was called "salivary gland virus" or "salivary gland inclusion disease virus." In 1960, Weller et al. (1960) proposed the unifying term "cytomegalovirus," which is derived from "cytomegalia," originally used to describe the remarkable enlargement of cells after viral infection (Goodpasture and Taibot in 1921).

Over the past two decades, the significance of CMV infection to individual patient care and public health has steadily increased due to the epidemic of acquired immunodeficiency syndrome (AIDS) and the increase in organ transplantation. This has propelled the most recent development of CMV studies in the area of molecular biology and immunology. For example, the complete DNA sequences for HCMV and MCMV were determined (Chee et al., 1990, European Molecular Biology Laboratory Sequence Database accession number X17403; Rawlinson et al., 1996, GenBank database accession number U68299). Many CMV genes and their transcripts have been identified. And modulations of the host immune system and cell cycle by CMVs have been reported (Beersma et al., 1993; Bonin and McDougall, 1997; Campbell and Slater, 1994; Dittmer and Mocarski, 1997). Understanding these biological mechanisms along with advances in the prevention and treatment of CMV infection, promise another exciting stage in the history of CMV study.

1.1.2 Murine cytomegalovirus: a model for human cytomegalovirus infection

Worldwide, HCMV infection is common in general populations and approximately 80% of the adults shows positive seroreactivity (Ho, 1991a). There are two modes of transmission of HCMV from a carrier to a susceptible individual: vertical and horizontal.
Vertical transmission involves the infection of infants in uterus from mothers with primary or reactivated HCMV infections. Horizontal transmission occurs during and shortly after birth, mainly through the infected cervix or breast milk of mothers. After puberty, sexual transmission of HCMV probably accounts for most cases of HCMV infection. In addition, transmission of HCMV through blood transfusion has been reported (Ho, 1991a). While HCMV infection in healthy children and adults is asymptomatic, it is a major cause of birth defects in neonates and an important pathogen in immunocompromised patients (Britt and Alford, 1996). In addition, HCMV infection is associated with the development of atherosclerosis and restenosis after coronary angioplasty (Speir et al., 1994). Due to species specificity, the study of HCMV infection in humans is restricted. Therefore, the availability of an animal model for HCMV infection is important to understand the pathogenesis and immunology of infections and to develop strategies for prevention and treatment. Murine cytomegalovirus has been extensively used for the analysis of HCMV infection because of its striking biological similarities to HCMV (Ho, 1991b). The MCMV genome is found to be essentially colinear with that of HCMV over the central part of the sequence, in which 78 open reading frames (ORFs) have striking homology at the amino acid level with those of HCMV (section 1.3). Like HCMV, MCMV acutely infects a variety of organs, such as salivary glands, adrenal glands, kidneys, spleen, and lungs. Following acute infection, both MCMV and HCMV can establish latency and cause severe interstitial pneumonia after reactivation by immunosuppressive treatment. Like HCMV, acute MCMV infection also causes immunosuppression of cell-mediated immunity. In addition, both MCMV and HCMV are sensitive to the antiviral drug ganciclovir.
1.2 The family Herpesviridae

Cytomegaloviruses are members of the family Herpesviridae which consists of more than 100 viruses. Herpesviruses are characterized by the large size of their DNA genome, viral latency, and potential oncogenicity. The family Herpesviridae can be further divided into three subfamilies based on biological features (Roizman, 1996; Table 1.1). The members of alphaherpesvirinae subfamily have broad tissue tropisms, a short replication cycle, and the ability to establish latent infections primarily in nerve root ganglia. Human herpesviruses in this subfamily are herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2, and varicella-zoster virus, which cause cold sores, genital herpes and chicken pox respectively. Human herpesvirus 8, a newly identified alphaherpesvirus, is found to be associated with Kaposi’s sarcoma. Members of the betaherpesvirinae subfamily which include HCMV, MCMV, human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7) typically have a relatively long replication cycle, a highly restricted host range, and establish latent infection in variety of tissues. Viruses of the gammaherpesvirinae subfamily specifically infect lymphocytes and lymphoblastoid cells, leading often to persistent or latent infection. Epstein-Barr virus (EBV) is in this subfamily, and causes infectious mononucleosis and is linked to certain human cancers.

1.3 Virion and genome structures of herpesviruses

Herpesviruses all have a similar virion structure which consists of four components: (i) an outer lipid bilayer envelope carrying viral glycoprotein spikes on its surface, (ii) an amorphous structure called the tegument which is asymmetrically distributed beneath the envelope, (iii) an icosahedral nucleocapsid which is 110 nm in diameter and composed of 162 capsomers, and (iv) within the nucleocapsid, a core
<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Representative Virus</th>
<th>Biological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha</td>
<td>Herpes simplex virus 1</td>
<td>Wide host ranges, rapid replication cycle, and latent infection mainly in ganglia.</td>
</tr>
<tr>
<td></td>
<td>Herpes simplex virus 1</td>
<td></td>
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<tr>
<td></td>
<td>Varicella-zoster virus</td>
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<tr>
<td></td>
<td>Human herpesvirus 8</td>
<td></td>
</tr>
<tr>
<td>Beta</td>
<td>Human cytomegalovirus</td>
<td>Narrow host ranges, slow replication cycle, and latent infection in various tissues</td>
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<td></td>
<td>Murine cytomegalovirus</td>
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<td></td>
<td>Human herpesvirus 6</td>
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<td></td>
<td>Human herpesvirus 7</td>
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<tr>
<td>Gamma</td>
<td>Epstein-Barr virus</td>
<td>Highly restricted host ranges, variable rate of replication, latent infection in lymphoblastoid cells, and oncogenicity</td>
</tr>
</tbody>
</table>
wrapped with a linear, double-stranded DNA (dsDNA) genome (Roizman, 1996). The size of herpesvirus virions varies from 120 to nearly 300 nm dependent upon the thickness of tegument. Hudson et al. (1976) have documented that when MCMV was propagated in mouse fibroblast cells, the majority of infectious progeny viruses are released as multicapsid virions containing a cluster of typical herpeslike capsids enclosed within a single envelope. This virion structure appears unique to MCMV. Like other enveloped viruses, herpesviruses are sensitive to fat solvents as well as extreme physical conditions and are inactivated at low pH (Hamparian et al., 1963).

Herpesviruses have extremely large genomes. For example, HSV-1 genome is approximately 150 kilobase pairs (kb; Roizman, 1996). Human cytomegalovirus (AD169 strain) has genome size of 230 kb which contains 208 predicted ORFs of greater than 100 amino acids in length (Chee et al., 1990). Herpesvirus genomes may contain both terminal and internal repeat sequences. Based on these repeat sequences, the herpesvirus DNA genomes are subdivided into six groups (Roizman, 1996; Figure 1.1). The genome arrangement of HSV-1 is classified as group E (Figure 1.1). The DNA sequence is divided into two segments termed the unique long (UL) and the unique short (Us) components flanked by large terminal and internal repeat sequences. Both UL and Us components can invert relative to each other, resulting in the generation of four isomers differing in their relative orientation. The HCMV genome structure is similar to HSV-1 (Figure 1.1), but a direct repeat called the a sequence at both ends of the viral genome in different HCMV strains is less conserved compared to different HSV-1 strains. The genome of MCMV belongs to group F, which is arranged as one long unique sequence containing several short internal repeats and a 31-bp direct repeat at each end. The MCMV genome is found to be essentially colinear with that of HCMV over the central part of the sequence, in which 78 open reading frames (ORFs) have striking homology at the amino
Figure 1.1 A Schematic depiction of the sequence arrangements in the six classes of genomes of the family herpesviridae. The genomes A, B, C, D, E and F are represented by the channel catfish herpesvirus, herpesvirus saimiri, Epstein-Barr virus, varicella-zoster virus, herpes simplex viruses, and cytomegaloviruses. In the diagram, the horizontal lines represent unique regions. The large direct repeat sequences are illustrated as rectangles while short direct repeats are depicted as vertical lines. The genome of HSV-1 contains $n$ copies of sequence $a$ next to a large sequence designated as $b$. The other terminus has one directly repeated $a$ sequence next to a sequence designated as $c$. Both termini sequences are repeated in an inverted orientation and juxtaposed internally ($ab$ and $ca$ sequences), dividing the unique sequence into long (UL) and short (Us) segments. The letters above the human cytomegalovirus genome depict the following features. The L-terminal $a$ sequence ($a_L$), zero to several ($n$) additional copies of the $a$ sequence ($a_n$), L-terminal $b$ sequence, the unique sequence of the L component (UL), the L-S junction $b$ sequence inverted repeat ($b'$), one to several ($m$) additional copies of the $a$ sequence inverted repeats at the L-S junction ($a'_m$), the L-S junction $c$ sequence inverted repeats ($c'$), the unique sequences of the S component (Us), S-terminal $c$ sequence and the S-terminal $a$ sequence ($a_r$) with a variable number of additional copies of the $a$ sequence ($a_n$). In murine cytomegalovirus genome, one unique region is flanked by short repeat sequences. The genome of Epstein-Barr virus consists of terminal repeats (TR) and several internal repeats (IR1-4) which subdivide the unique sequence into well-defined segments (U1-U5).

[Adapted from Mocarski (1996) and Roizman (1996)]
Class | Genome Arrangement | Virus
--- | --- | ---
A | LTR | The channel catfish herpesvirus
B | | Herpesvirus saimiri
C | TR U1 IR1 U2 IR2 U3 IR3 U4 IR4 U5 TR | Epstein-Barr virus
D | UL IR US TR | Varicella-zoster virus
E | a_n b UL b'a_n c' US c a | Herpes simplex viruses
F | TR TR | Murine cytomegalovirus
acid level with those of HCMV. This may contribute to extensive biological similarities between HCMV and MCMV. In the EBV genome (Figure 1.1), the unique sequence flanked by direct terminal repeats is subdivided into several well-defined segments by internal repeats.

1.4 The lytic viral replication cycle of herpesviruses

After entry into susceptible cells, herpesviruses undergo two choices, either lytic infection and the production of infectious viruses, or they remain latent in the infected host and no infectious progenies are produced. Upon appropriate stimulation, the latent viruses can be reactivated to produce infectious virus particles.

1.4.1 Virus entry

Herpesviruses initiate infection by attachment and penetration of the host cell surface membrane. For HSV-1, this process is mediated by the concerted actions between several viral membrane glycoproteins and cell surface components. The virus attaches to the cell surface through the binding of viral glycoprotein C (gC) or gB to heparan sulfate chains on cell surface proteoglycans (Herold et al., 1991; Shieh et al., 1992). This is followed by viral penetration, which involves the pH-independent fusion of the viral envelope with the surface membrane of the host cell. The viral components of this process are glycoproteins gB, gD, gH, and gL (Spear, 1993). Removing these glycoproteins from the virion disrupts penetration, but not virus attachment. Other cell surface receptors besides heparan sulfate can also mediate HSV-1 entry (Montgomery et al., 1996; Nicola et al., 1998; Terry-Allison et al., 1998; Warner et al., 1998; Willis et al., 1998). The herpesvirus entry protein A (HveA) was the first cell surface protein shown to mediate herpesvirus entry. Herpesvirus entry protein A binds to isolated glycoprotein gD of HSV-
1 and to gD in virions. The protein is a type I membrane glycoprotein and is a receptor for two members of the human tumor necrosis factor (TNF) family, lymphotoxin α and LIGHT (Mauri et al., 1998). While HveA is present in many tissues, lymphoid organs and cells appear to be the major sites for its expression. Other cell surface components that were identified as HSV-1 entry proteins including HveB, HveC, and Pvr-HveD. They are all members of the human immunoglobulin superfamily. These receptors are expressed in neuronal, fibroblastic, and epithelial cells. The diversity of herpesvirus entry proteins may account for its wide range of tissue tropisms. In EBV, the cell surface protein required for virus entry is B-lymphocyte receptor CR2 of the C3d component of complement system (Fingeroth et al., 1984). The major EBV envelope glycoprotein gp350/220 binds to the receptor to initiate penetration (Nemerow et al., 1989).

1.4.2 Nuclear import of viral DNA and gene transcription

After the fusion of the HSV-1 envelope with the cellular plasma membrane, the capsid is released into the cytosol and quickly associates with microtubules by binding to dynein, a minus-end-directed motor protein (Sodeik et al., 1997). The capsid is then transported along microtubules to the microtubule organizing center located close to the nucleus. So far the viral protein serving as the receptor for dynein or for adapter proteins such as dynactin has not been identified. At the nuclear envelope, the capsid attaches to nuclear pore complexes (NPCs) through fibrils that protrude from the pore into the cytosol. The actual process of importing viral DNA into the nucleus is not well understood. It is speculated that transient alterations of capsid pentons at the vertices may be required for releasing viral DNA into the nucleus after the capsid binds the NPCs (Whittaker and Helenius, 1998).
The expression of HSV-1 genes is temporally regulated in a cascade fashion which can be divided into three major phases: immediate early (IE), early, and late (Honess and Roizman, 1974). The IE genes are transcribed in the absence of de novo viral protein synthesis. Their expression requires a virion tegument protein, a trans-inducing factor (αTIF), together with the cellular octamer DNA-binding protein, oct-1 (Gerster and Roeder, 1988; Pellett et al., 1985). The expression of early genes is induced by proteins encoded by IE genes and peaks prior to viral DNA synthesis. Finally, the late genes are transcribed after DNA synthesis, which generally produce the structural proteins of the virus. Herpes simplex virus 1 transcripts are processed by conventional means with relatively little splicing. Viral proteins made in the cytoplasm are extensively modified through processes that include cleavage, phosphorylation, glycosylation, sulfation, and poly(ADP)-ribosylation.

1.4.3 Viral DNA replication and packaging

Herpes simplex virus 1 DNA synthesis begins shortly after the expression of early genes that encode a large number of enzymes required for the replication of the viral genome or involved in nucleotide metabolism. DNA replication initiates within the origin of lytic replication, but the actual mechanism by which DNA replication proceeds is not clear. Several lines of evidence suggest a rolling circle model. Soon after infection, the HSV-1 linear viral genome undergoes a loss of terminal fragments and the appearance of novel fragments created by fusion of terminal fragments (Friedman and Becker, 1977), suggesting genome circularization. A concatemeric head-to-tail replicative intermediate were detected in nuclei of infected cells (Jacob et al., 1979).

For packaging the viral genome, the concatemeric DNA replication intermediate is translocated into the preformed capsid until a signal for cleavage and packaging is reached.
The DNA is then cleaved, releasing the concatemer and leaving a unit-length genome within the nucleocapsid (Ladin et al., 1982). In HSV-1, the signals for cleavage and packaging are located within the $a$ sequence of the genome (Figure 1.1). Two sequence motifs referred to as pac1 and pac2 were initially identified within the HSV-1 $a$ sequence and subsequently found at the termini of all herpesvirus genomes for which sequence data are available (Deiss et al., 1986). The molecular mechanism of the cleavage-packaging is unknown. Several proteins which participate in this process have been identified which include virus-encoded proteins that specifically recognize the pac2 site, a virus-induced DNA endonuclease that introduces double-strand cuts in the $a$ sequence, and several nonstructural and capsid proteins. A model for the cleavage-packaging reaction has been proposed by Deiss et al. (1986). During cleavage and packaging, concatemeric viral DNA binds the capsid via the pac1 site by a protein complex that includes a component of the capsid. The cleavage-packaging apparatus then spools the DNA into the capsid while it scans the DNA for the pac2 site. The juxtaposition of pac1 and pac2 sites results in generation of linear genomic DNA by deleting the DNA between the direct repeats of the $a$ sequence. Subsequently, an amplification event restores the continuity of the DNA concatemer for the next cycle of cleavage and packaging.

1.4.4 Synthesis of viral membrane proteins, virion envelopment and egress

It has been found that late in HSV-1 infection, concave or convex patches appears in nuclear membranes (Schwartz and Roizman, 1969). These structures likely are accumulations of viral membrane proteins, presumably including the viral glycoproteins on the outside surface and anchorage and tegument proteins on the inside surface (Roizman and Sears, 1996). Newly assembled herpesvirus capsids exit the nucleus through binding to the patches of nuclear membrane and budding into the lumen. In HSV-1, the UL11
gene product may play an important role in initiating this process (Baines et al., 1995). The pathway for further transport of herpesvirus through the cytoplasm is not clear. Morphological analysis by electron microscopy suggests that capsids may transit directly into the endoplasmic reticulum, follow a pathway through the Golgi apparatus that is similar to that taken by secreted soluble proteins, and finally reach the cell surface (Hanson and Grose, 1995). Alternatively, capsids may become de-enveloped at the outer nuclear membrane and a naked capsid is released into the cytoplasm. The capsids then somehow become wrapped up by membranes of the Golgi apparatus where final glycosylation takes place before transport into extracellular space (Gershon et al., 1994). The entire lytic HSV-1 replication cycle takes about 18 to 20 hours in fully permissive cells.

1.4.5 The lytic viral replication cycle of cytomegaloviruses

Similar to HSV-1, the CMV lytic replication cycle starts with the attachment of the virus to the surface of susceptible cells. This process is dependent upon the presence of cell surface heparan sulfate proteoglycans (Compton et al., 1993; Kimpton et al., 1989; Neyts et al., 1992) binding the HCMV envelope glycoprotein complex II (gC-II) and gB (Boyle and Compton, 1998; Kari and Gehrz, 1992). This heparin-dissociable binding state is rapidly converted to a stable attachment followed by the penetration of host cells by pH-independent fusion of the viral envelope glycoproteins gB and gH with the plasma membrane (Compton et al., 1992; Keay and Baldwin, 1991; Navarro et al., 1993). Although cellular receptor responsible for stable binding or fusion is not known, several cell surface proteins are suggested to be important for this process. Grundy et al. (1987) proposed that HCMV binds to class I human leukocyte antigen molecules via virus-bound β2-microglobulin. Cellular annexin II, a member of the annexin-lipocortin family whose functions are related to calcium-dependent interactions with phospholipid membranes
(Crompton et al., 1988), has also been implicated as a receptor for HCMV on endothelial cells (Wright et al., 1994). Pietropaolo and Compton (1997) have demonstrated that HCMV gB can physically interact with annexin II. Human cytomegalovirus was found to bind to 30 K and 92 K proteins on a wide range of cell types (Adlish et al., 1990; Keay et al., 1989; Taylor and Cooper, 1990). The cell surface molecule CD13 (human aminopeptidase), a metalloprotease, was also linked to the cellular attachment or penetration of HCMV (Soderberg et al., 1993). Therefore, HCMV may use multiple host cell surface molecules for its entry.

Following the fusion of the viral envelope with the plasma membrane, the virus enters a cell. Little is known about the uncoating or delivery of the viral genome into the nucleus. However, the tegument protein pp71 and the viral DNA are transported into the nucleus about one hour post-infection (Liu and Stinski, 1992). The protein appears to function in trans-activation of specific viral genes, like the HSV-1 virion tegument protein αTIF. After entry into the nucleus, the viral DNA circularizes by fusion of the genome termini (LaFemina and Hayward, 1983; Marks and Spector, 1984, 1988; McVoy and Adler, 1994). A set of IE and early genes are then expressed, followed by the initiation of DNA synthesis from the origin of lytic replication (oriLyt). Subsequently, long head-to-tail concatamers are generated, probably by a rolling-circle mechanism. The concatamers are cleaved into unit-length genomic DNA through the herpesvirus-conserved pac1 and pac2 motifs (McVoy et al., 1998), followed by the packaging of the viral genome into preformed capsids encoded by the late genes. The viral progeny mature by budding through the nuclear membrane and are released most likely into extracellular space through pathways similar to HSV-1. In comparison to HSV-1, the lytic infection cycle of CMVs is longer, i.e., 24 to 36 hours for MCMV and 48 to 72 hours for HCMV (Stinski, 1983).
1.5 Origins of herpesvirus lytic DNA replication

The significance of studies of herpesvirus DNA replication is two fold. First, the viruses provide a useful model for studying DNA replication in eukaryotic cells because their genomes encoding many proteins that are directly required for viral DNA synthesis. Second, most human herpesviruses are important pathogens whose control relies primarily on antiviral drugs. Information learned about viral DNA replication will lead to the development of effective antiviral treatments.

The HSV-1 genome contains three origins of DNA replication: One designated as ori\textsubscript{L} is mapped near the middle of the U\textsubscript{L} component and the other two called ori\textsubscript{V} are located in the c sequence flanking the Us component. It is not clear why the HSV-1 genome carries three origins of replication. Recombinant viruses lacking ori\textsubscript{V} or one or both copies of ori\textsubscript{L} have no obvious growth defect either in cultured cells or in mouse models (Igarashi et al., 1993; Polvino-Bodnar et al., 1987). These origins of DNA replication appear to be redundant in function. The sequences of HSV-1 ori\textsubscript{V} and ori\textsubscript{L} are similar. Both contain a large inverted repeat sequences (45 and 144 bp respectively for ori\textsubscript{V} and ori\textsubscript{L}) that center around an A+T region of 18 and 20 bp (Challberg, 1996). As exemplified in Figure 1.2 A, the core region of HSV-1 ori\textsubscript{L} contains at least five domains: an AT-rich region flanked by two high-affinity binding sites (referred to as site I and site II) for the HSV-1 encoded origin-binding protein (OBP); a sequence homologous to site I, but with much lower affinity for OBP, called site III; and a binding site for a nuclear protein called OF-1. The OBP-binding affinity of site II is about 5-fold lower than that of site I. Interestingly, site III differs in sequence from site I at only one position, but its affinity for OBP is reduced to about one-thousandth of that of site I.
Figure 1.2 Functional components of the origins of herpesvirus lytic DNA replication. (A) The 70-bp core ori of herpes simplex virus type 1. The herpes simplex virus type 1 core origin sequence is composed of at least five functional domains: two high-affinity binding sites for the origin-binding protein (site I and site II); an AT-rich region; site III, a sequence homologous to site I, but with much lower affinity for the origin-binding protein; and a binding site for a nuclear protein called OF-1 (Adapted from Challberg, 1996). (B) The 1.1-kb lytic DNA replication origin of Epstein-Barr virus. The upstream and downstream core components of the origin are represented by the indicated open rectangles; the auxiliary regions are shaded. The bent arrow shows the position and direction of transcription initiation. Binding sites for the transcriptional activator Z and presumptive cellular factors (?) are indicated (Adapted from Yates, 1996).
In addition to the core components, origins of HSV-1 DNA replication contain auxiliary regions that can modulate the efficiency of replication initiated at the core. The auxiliary regions are located in the promoter-regulatory elements of divergently transcribed genes such as the UL29 gene encoding the single-stranded DNA-binding protein (ssDBP) known as infected cell polypeptide 8 (ICP8) and the UL30 gene coding for the DNA polymerase (Pol). The presence of transcriptional regulatory elements in origins of viral DNA replication suggests the possibility of interplay between factors affecting transcription and initiation of DNA replication (Nguyen and Schaffer, 1998).

The EBV genome has two essentially identical copies of a 1055-bp oriLyt located approximately opposite each other on the circularized viral genome. Like the origins of HSV-1 DNA replication, the EBV replication origin is composed of two core components separated by about 400 bp and several auxiliary components (Yates, 1996; Figure 1.2 B). The upstream core component of oriLyt is a strong early promoter with four identical binding sites for EBV transcriptional activator Z. Replication from oriLyt requires that Z interacts with these four sites. The downstream core component of oriLyt is a region less than 90 bp in length that seems to play no role in transcription. The factors that functionally interact with this critical region have not been identified, but genetic studies imply that they must be provided by host cells. The downstream auxiliary region of oriLyt is able to enhance viral DNA replication.

Since EBV does not encode a protein homologous to the HSV-1 OBP which has DNA helicase activity, a cellular helicase might be recruited by EBV to act at oriLyt (Yates, 1996). Interestingly, Fixman et al. (1995) have demonstrated that an EBV oriLyt-containing plasmid is capable of amplification as long as the Z protein of EBV and a set of six core replication proteins from HSV-1 was provided. The result would suggest that the replication intermediates formed during initiation by Z and cellular proteins at the EBV
lytic origins are similar or identical to those produced during initiation by the HSV-1 OBP at the HSV-1 origins of replication.

1.6 Viral genes and proteins required for herpesvirus DNA replication

Genes and proteins required for HSV-1 DNA replication are well characterized. A set of seven viral genes that are both necessary and sufficient for origin-dependent DNA replication have been identified using a transient complementation assay in which cloned segments of HSV-1 DNA were tested for their ability to support the replication of a cotransfected plasmid containing the origin of replication (Challberg, 1986; Wu et al., 1988). The genes identified in the transfection assay were verified by detailed mapping of available temperature-sensitive mutants with defects in DNA synthesis and by the construction of viruses containing targeted null mutations (insertions or deletions) in these genes (Orberg and Schaffer, 1987).

The products of seven HSV-1 DNA replication genes consist of OBP and six core replication proteins that function at the replication fork, including Pol, the Pol accessory protein, ICP8, helicase, primase, and the primase-helicase associated factor. Investigators using a similar transfection assay for identifying HSV-1 DNA replication genes identified the essential genes required for both HCMV (Pari and Anders, 1993; Pari et al., 1993; section 1.9.1) and EBV (Fixman et al., 1992, 1995) DNA replication. The results are summarized in Table 1.2 and indicate that the six required genes encoding core proteins are conserved in herpesviruses.

The HSV-1 UL9 gene encodes an OBP of 851 amino acids with an apparent molecular weight of 83 K. Like the simian virus 40 (SV40) large T antigen and the E1 proteins of bovine and human papilloma viruses (Hassell and Brinton, 1996; Stenlund, 1996), HSV-1 OBP functions as a DNA replication initiator protein whose role is to make
the origin accessible to the DNA replication machinery. At least three functional domains have been identified within the protein. The first is the conserved ATP-binding and DNA helicase motifs in the amino-terminal two-thirds of the protein. These motifs are essential for viral DNA replication. The second mediates dimerization, presumably through a leucine-zipper motif encompassing residues 150 to 171. Finally, the carboxyl-terminal 317 amino acids contains a sequence-specific DNA binding domain which binds to sites I, II, and III of HSV-1 ori, with different affinities.

How DNA synthesis initiates following the binding of the OBP to the core origin sequence is not understood. On the basis of biochemical analysis and observations under the electron microscope, a model of OBP-mediated DNA unwinding has been proposed (Boehmer and Lehman, 1997). First, the binding of OBP dimers to the sites I and II of the origin results in bending, looping, and distortion of the AT-rich region. The DNA within the origin is then pulled through the protein complex and spooled out as ssDNA. Protein-protein interactions between the OBP and ICP8 likely stimulate this reaction and stabilize unwound regions of DNA. Finally, a primase is recruited to the origin to initiate DNA synthesis by interactions between the OBP and the primase-helicase associated factor or the cellular replication protein DNA polymerase α-primase.

A great deal of work has gone into the characterization of HSV-1 Pol because the enzyme serves as a target for antiviral drugs and as an excellent model for eukaryotic DNA polymerase (Challberg, 1996). The HSV-1 Pol, a 136 K protein encoded by the UL30 ORF, possesses 3’ to 5’ exonuclease activity which ensures high fidelity of DNA replication. The functional domains of both DNA polymerase and 3’ to 5’ exonuclease activities are mapped to the central and carboxyl-terminal portions of the protein. In HSV-1 infected cells, the polymerase exists as a heterodimer in which the protein is associated with a polymerase accessory protein. The site of interaction between these two proteins
<table>
<thead>
<tr>
<th>HSV-1</th>
<th>HCMV</th>
<th>MCMV</th>
<th>EBV</th>
<th>Protein and Function</th>
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<tr>
<td>UL30</td>
<td>UL54</td>
<td>M54</td>
<td>BALF5</td>
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<td>M57**</td>
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<td></td>
<td></td>
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<td>organizes the formation of replication compartments in HCMV infection</td>
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<td>M122/m123</td>
<td></td>
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<td>IRS1/TRS1</td>
<td>m128/m139</td>
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</tr>
<tr>
<td>UL84</td>
<td>M84</td>
<td></td>
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<td>promotes the initiation of HCMV oriLyt DNA replication</td>
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</table>

* Gene product for UL29 is ICP8.

** Gene product for M57 is MDBP.
has been located to the carboxyl-terminal 227 residues of the Pol and the amino-terminal 340 residues of the polymerase accessory protein. This latter protein of 65 K is the product of the *UL42* ORF. Similar to *E. coli* DNA polymerase III β subunit and the eukaryotic proliferating cell nuclear antigen (Baker and Bell, 1998), the HSV-1 DNA polymerase accessory protein likely acts to increase the processivity of Pol by forming a sliding clamp that prevents dissociation of Pol from the primer-template. In contrast to other DNA polymerase clamp proteins, the assembly of the HSV-1 DNA polymerase accessory protein on the DNA does not require clamp loaders. The HSV-1 DNA polymerase accessory protein itself binds DNA by an intrinsic dsDNA-binding activity (Vaughan *et al.*, 1985; Gallo *et al.*, 1988).

Herpes simplex virus 1 produce a virus-specific DNA helicase-primase which comprises a 1:1:1 association of UL5, UL8, and UL52 proteins encoded by the *UL5*, *UL8*, and *UL52* ORFs (Boehmer and Lehman, 1997). This tripartite complex has a native molecular weight of about 270 K. The helicase activity of the complex resides in the UL5 protein which can use either ATP or GTP as a co-factor for DNA unwinding. The protein contains six sequence motifs that are typical of helicases and mutations in highly conserved residues within each of these six motifs abolish the ability of the protein to support DNA synthesis *in vivo*. However, the full helicase activity of UL5 requires the UL52 protein that is responsible for the primase activity of the DNA helicase-primase. The function of UL8 is currently not well understood. However, it has been shown the UL8 subunit is required for optimal DNA helicase, DNA-dependent nucleoside triphosphatase, and primase activity in the presence of ICP8 (Tenny *et al.*, 1995).
1.7 Single-stranded DNA-binding protein

1.7.1 Overview

Herpes simplex virus 1 ssDBP known as ICP8 is a homolog of the MCMV major DNA-binding protein (MDBP). Herpes simplex virus 1 ICP8 belongs to a class of DNA-binding proteins that have higher affinity for DNA than RNA, and for single-stranded DNA (ssDNA) than dsDNA (Chase and Williams, 1986; Kornberg and Baker, 1991b). The ssDNA-binding protein binds cooperatively to ssDNA with no sequence specificity and is required during DNA synthesis in amounts that are stoichiometric, rather than catalytic as are polymerases and helicases. The major function of ssDBP is to stabilize unwound DNA in a single-stranded conformation and to facilitate the use of these structures as templates for the DNA polymerase holoenzyme (Alberts and Sternglanz, 1977). Single-stranded DNA-binding proteins are present in both prokaryotic and eukaryotic systems. Examples are the T4 bacteriophage gene 32 protein (GP32), fd bacteriophage gene 5 protein (GP5), *Escherichia coli ssb* gene product (SSB), adenovirus ssDBP (AdDBP), HSV-1 ICP8, yeast replication protein A (RPA), and human RPA (Chase and Williams, 1986; Hubscher et al., 1996).

Herpes simplex virus 1 ICP8 has been the subject of intense study since its initial identification (Hones and Roizman, 1973). The protein is encoded by the HSV-1 UL29 ORF and consists of 1196 amino acids with a molecular weight of about 130 K. The absolute requirement for ICP8 in HSV-1 DNA replication is defined by the inability of temperature-sensitive ICP8 mutants to grow at nonpermissive temperatures (Weller et al., 1983) and by the fact that ICP8 is essential for amplifying a plasmid containing the origin of HSV-1 replication (Wu et al., 1988). Further extensive genetic and biochemical studies demonstrate that ICP8 functions at the replication fork as a classic ssDBP based on (i) its
preferential and cooperative binding to ssDNA (Lee and Knipe, 1985; Ruyechan, 1983),
(ii) its ability to stimulate the polymerase activity of HSV-1 and the helicase activity of
OBP (Boehmer et al., 1994; O'Donnell et al., 1987), and to optimize the helicase-primase
activities of the heterotrimeric helicase-primase complex (Crute et al., 1989), and (iii) its
requirement for the synthesis of long stretches of DNA molecules (Hernandez and
Lehman, 1990). As described above, ICP8 also acts in concert with other viral proteins,
namely the helicase-primase complex, OBP, Pol, and the Pol accessory factor, to form
DNA replication compartments (Liptak et al., 1996). Furthermore, ICP8 inhibits late gene
expression from parental genomes and promotes late gene expression following viral
dNA replication (Chen and Knipe, 1996; Dodowski and Knipe, 1985; Dodowski and
Knipe, 1986).

1.7.2 Functional domains of ICP8

1.7.2.1 ssDNA-binding domain

The ssDNA-binding domain of ICP8 has been mapped to a region containing
amino acid residues 300 to 849 (Gao et al., 1988; Gao and Knipe, 1989; Leinbach and
Heath, 1988; Leinbach and Heath, 1989; Wang and Hall, 1990). The identification of the
ssDNA-binding domain has provided us with an insight into the structure-function
relationships of the protein. Analysis of the amino acid sequence of the ssDNA-binding
domain reveals two important regions. One, encompassing residues 499-512, conforms to
the consensus sequence (Cys-X2.5-Cys-X2.5-Cys/His-X2.4-Cys/His, where X is any
amino acid) for a zinc-finger like motif (Berg, 1986, 1996; Gao et al., 1988). The other,
from residues 803-849, contains a putative ssDNA-binding motif (Prasad and Chiu, 1987;
Wang and Hall, 1990; Figure 1.3) that consists of eight aromatic and basic amino acids
separated by variable numbers of unrelated residues. These aromatic and basic residues are conserved in other prokaryotic and viral ssDBPs, such as GP5, GP32, SSB, and AdDBP. Based on information about the three-dimensional structures as well as analysis of these ssDBPs by site-directed mutagenesis (Bochkarev et al., 1997; Brayer and McPherson, 1983; Brayer and McPherson, 1984; Folmer et al., 1995; Kanellopoulos and van der Zandt, 1995; Shamoo et al., 1995; Tucker et al., 1994), it is suggested that conserved amino acid residues within the ICP8 ssDNA-binding motif bind to ssDNA via two general types of interactions: i) aromatic side chains of the protein stack upon the bases of the DNA chain; ii) the phosphate backbone of DNA interacts electrostatically with a series of appropriately positioned lysyl and arginyl side chains of the ssDNA-binding motif.

Although no direct evidence is available to demonstrate that a zinc atom binds to the putative zinc-finger like motif of ICP8 and that the putative ICP8 ssDNA-binding motif is actually involved in binding to ssDNA, a large body of work from different laboratories confirms the requirements of these motifs for the overall function of ICP8. Simultaneous substitution of the first two cysteines with glycines within the ICP8 zinc-finger like motif produced a mutant protein that severely reduced its ability to rescue a temperature sensitive ICP8 mutant at the nonpermissive temperature (Gao et al., 1988). Subsequently, Gupte and co-workers (1991) showed that ICP8 is a potential zinc metalloprotein that contains 1 mol of zinc/mol of protein. When they removed zinc from ICP8 by treatment of the protein with a reversible sulphydryl reagent followed by reconstitution of free sulphydryl groups, they found that the ssDNA-binding activity of the zinc-depleted ICP8 is similar to that of native ICP8. However, the modified protein gradually lost the binding activity after several days storage at 4°C. In addition, the zinc-depleted ICP8 is highly sensitive to protease. These results indicate that zinc in ICP8 appears to be essential for maintaining the
Figure 1.3 The putative single-stranded DNA-binding motif. Partial protein sequence of the single-stranded DNA-binding proteins from various organisms were aligned with the single-stranded DNA-binding region of the fd bacteriophage gene 5 protein. The analysis revealed several conserved aromatic and basic amino acid residues separated by variable numbers of unrelated residues which are designated by X with a subscript indicating the number of residues in each case. Numbers in the bracket indicate the residue numbers of each sequence shown in the alignment. Abbreviations: GP5, fd bacteriophage gene 5 protein. GP32, T4 bacteriophage gene 32 protein, SSB, Escherichia coli ssb gene product, AdDBP, adenovirus single-stranded DNA-binding protein, HSV-1 ICP8, herpes simplex virus 1 infected cell protein 8 (Adapted from Wang and Hall, 1990).
native tertiary structure of the protein, but is not directly involved in the ssDNA binding activity.

Two research groups independently demonstrated that a carboxyl-terminal peptide containing the putative ssDNA-binding motif retains the same ssDNA-binding activity as the intact ICP8 protein (Leinbach and Heath, 1988; Leinbach and Heath, 1989; Wang and Hall, 1990). The result was further supported by deletion mutagenesis of the ICP8 gene (Gao and Knipe, 1989). To further define the role played by basic and aromatic amino acid residues in ssDNA binding, purified HSV-1 ICP8 was modified with reagents under conditions specific for these residues (Ruyechan and Olson, 1992). The subsequent ssDNA-binding assay showed that modification of lysine and tyrosine residues results in a loss of ssDNA-binding activity, suggesting that these residues are required for interaction of ICP8 with ssDNA. Due to the limitations of this approach, arginine modification was not found to affect ssDNA binding. Ruyechan and Olson (1992) also reported that tryptophan fluorescence of ICP8 was quenched upon binding to ssDNA, which implies that tryptophan is either involved in the interaction of ICP8 with ssDNA or reflects a conformational change in the protein upon ssDNA binding. The final understanding of the interactions of these motifs with ssDNA awaits data from site-directed mutagenesis experiments and solution of the three-dimensional structure of ICP8.

1.7.2.2 Cooperative binding region

ICP8 has an ability to melt duplexes consisting of short oligonucleotides annealed to ssDNA (Boehmer and Lehman, 1993a). On the other hand, it aids the renaturation of complementary DNA strands under proper conditions (Dutch and Lehman, 1993). These physical properties are associated with the cooperative binding activity of ICP8. Cooperativity is the phenomenon whereby an ssDBP has a higher affinity for ssDNA to
which an ssDBP is already bound than to one lacking ssDBP. Cooperativity of ICP8 can be demonstrated by Scatchard plot analysis, electron microscopy and agarose gel electrophoresis (Lee and Knipe, 1985; Ruyechan, 1983; Ruyechan et al., 1986). Dudas and Ruyechan (1998) recently identified a region required for cooperative DNA binding of ICP8 at its amino terminus centered around cystine residue 254, and separated from the previously known ssDNA-binding domain. They studied ssDNA-binding property of ICP8 by covalently modifying a cystein-specific fluorophore fluorescein-5-maleimide. An ICP8 mutant virus with an amino-terminal deletion encompassing this cooperative DNA binding region is unable to replicate. Nevertheless, the mutant protein is capable of localizing to the nucleus and its ssDNA-binding activity remains intact (Gao and Knipe, 1989). The result suggests that the amino-terminus of ICP8 is not directly involved in ssDNA-binding activity, but it confers upon ICP8 the cooperative binding feature that is essential for viral DNA replication.

1.7.2.3 Nuclear localization signal

The nucleus is surrounded by the nuclear envelope which is penetrated by the NPCs. The nuclear pore is an aqueous channel with a physical diameter of about 9 nm and allows small proteins in the cytoplasm to diffuse passively to the nucleus (Doye and Hurt, 1997). However, the channel is gated and selectively allows the import of larger proteins into the nucleus, usually more than 60 K that has a nuclear localization signal (NLS). The NLS-containing protein is recognized by a family of shuttling transport factors termed importins which target the proteins to the NPCs for energy-dependent translocation into the nucleus (Ohno et al., 1998; Silver, 1991).

Although no consensus sequence has been established for NLSs, the signal sequences often fall into one of the following two categories. The first, represented by the
NLS of the SV40 large T antigen (Kalderon et al., 1984), is a motif of seven or eight amino acids consisting of a single short basic sequence usually flanked by a proline or acidic residue (LaCasse and Lefebvre, 1995). The second is a bipartite basic structure where two adjacent clusters of basic residues are separated by about ten amino acids. The first cluster contains two basic residues, while the second cluster is composed of five residues, of which at least three are basic (Dingwall and Laskey, 1991). The motif, KRPAATKKAGQAKKKK, was initially identified in Xenopus nucleoplasmin (Robbins et al., 1991) and was later found to be a predominant NLS motif in many nuclear proteins. Besides these two major types of NLSs, a few other classes of NLSs have been identified. For example, a stretch of 38 amino acid residues present in the carboxyl-terminus of the heterogeneous nuclear ribonucleoprotein A1 (called M9 domain) functions in both nuclear import and export (Michael et al., 1995; Siomi and Dreyfuss, 1995). Recently, a novel NLS in the yeast Nab2 Poly(A)* RNA binding protein has been reported (Truant et al., 1998). The signal participates in a sequence-specific protein nuclear import pathway which is different from those of basic and M9 NLSs.

Following its synthesis in the cytoplasm, HSV-1 ICP8 is imported into the nucleus where it initially interacts with the nuclear matrix at prereplicative sites (Quinlan et al., 1984). The nuclear localization of ICP8 is partially mediated by a NLS within the protein. The NLS of HSV-1 ICP8 was mapped to the last 28 amino acid residues at the carboxyl terminus by genetic analysis of ICP8 mutants and ICP8-pyruvate kinase fusion proteins (Gao and Knipe, 1989; Gao and Knipe, 1992; Gao and Knipe, 1993). Examination of the amino acid sequence of the NLS of ICP8 shows a stretch of basic residues, but the sequence shares limited similarity to the classic NLS of SV40 large T antigen. Interestingly, sequences distant from the NLS can alter the nuclear localization function of ICP8, probably by affecting overall protein folding or assembly (Gao and Knipe, 1992).
The NLS of ICP8 was required for viral DNA replication during HSV-1 infection (Gao and Kinpe, 1993). Moreover, genetic analysis showed that a mutant ICP8 protein lacking the NLS had the same ssDNA-binding activity as ICP8. In contrast, a mutant ICP8 protein lacking the ssDNA-binding domain localized predominantly in the nucleus, but bound poorly to an ssDNA-cellulose column (Gao and Knipe, 1989). These results strongly suggest that nuclear localization and ssDNA binding are two separable functions of ICP8.

1.8 Assembly of HSV-1 DNA replication compartments

Similar to eukaryotic DNA synthesis (Newport and Yan, 1996), HSV-1 DNA replication takes place at discrete nuclear foci which are formed by the assembly of viral replication proteins and/or cellular structural or functional components. These foci were initially identified by Quinlan et al. (1984) and de Bruyn Kops and Knipe (1988). Two major types of nuclear foci were seen based on the immunofluorescence staining of HSV-1-infected cells with an antibody against ICP8. The first type of structure, termed prereplicative sites, has a punctate staining pattern and appears at early times of HSV-1 infection (2 to 3 hours postinfection). Similar structures were also found when DNA replication was blocked by Pol inhibitors. The second type of foci was termed replication compartments and they exhibit large globular staining patterns, which are localized to the nucleus 3 to 4 hours postinfection. The replication compartment contains all seven essential HSV-1 replication proteins and is a site where viral DNA replication takes place (de Bruyn Kops and Knipe, 1988; Goodrich et al., 1990; Liptak et al., 1996; Lukonis and Weller, 1996; Malik et al., 1996; Wilcock and Lane, 1991). Results from a confocal microscopy study suggested that the replication compartments are generated from structures equivalent to the prereplicative sites (de Bruyn Kops and Knipe, 1994).
Recently, two distinct types of HSV-1 prereplicative sites have been identified in infected cells at different stages of the cell cycle (Uprichard and Knipe, 1997). The first is present in large numbers in S-phase cells and is associated with cellular DNA replication proteins and host DNA synthesis activity. This structure contains all except one of the essential HSV-1 DNA replication proteins (the Pol accessory factor). However, HSV-1 DNA genomes are not preferentially deposited to these sites. Currently, it is not well understood why these prereplicative sites are generated in infected S-phase cells. One possibility is that the interaction between viral proteins and the cellular DNA synthesis machinery may relocalize cellular proteins required for viral DNA synthesis (Uprichard and Knipe, 1997). The second type of prereplicative site is found in limited numbers in cell cycle stages other than S-phase. This structure consists of HSV-1 DNA replication proteins which associate with cellular nuclear domain 10 (ND10), comprising a nuclear matrix structure that is present in 10 copies per nucleus in cultured cells. So far, no particular function has been assigned to ND10. It may be needed for cell proliferation (Ascoli and Maul, 1991). It appears that the HSV-1 DNA replication compartments develop from these ND10-associated prereplicative sites (Ishov and Maul, 1996; Liptak et al., 1996; Lukonis and Weller, 1996; Maul et al., 1996). In addition, DNA replication of adenovirus type 5 and simian virus 40 (SV40) is also associated with ND10 (Ishov and Maul, 1996). It has been speculated that the localization of viral DNA replication proteins to ND10 may provide an optimal environment for coupling of transcription and replication initiation (Lukonis and Weller, 1997; Maul et al., 1996).

A model for the assembly of HSV-1 DNA replication proteins into functional replication compartments was formulated by Liptak et al. (1996). The five viral DNA replication proteins, including the helicase, the primase, the primase-helicase associated factor, OBP, and ICP8 initially assemble into minimal prereplicative site structure. Then,
Pol and its accessory factor join the above complex to yield a complete structure. This model is well supported by biochemical studies, in which multiple protein-protein interactions among HSV-1 DNA replication proteins have been observed. For example, OBP can interact with ICP8, the primase-helicase associated factor, and the Pol accessory factor, respectively (Boehmer and Lehman, 1993b; Boehmer et al., 1994; Mclean et al., 1994; Monahan et al., 1998). A physical interaction between Pol and ICP8 has also been documented (O'Donnell et al., 1987). The helicase, the primase and their associated factor function as a three-subunit complex and a heterodimeric holoenzyme is formed between Pol and its accessory factor (Crute et al., 1989; Gottlieb and Challberg, 1994; Hernandez and Lehman, 1990).

The minimal requirements for the assembly of prereplicative sites can vary under different conditions. For example, the structure is generated without the helicase-primase complex or OBP when an HSV-1 Pol inhibitor is added to infected cells (Liptak et al., 1996). ICP8, the helicase-primase complex, and OBP are all essential for the assembly of prereplicative sites in infected cells (Liptak et al., 1996; Lukonis and Weller, 1996). However, the formation of prereplicative sites in transfected cells requires only ICP8 and the helicase-primase complex (Lukonis and Weller, 1997; Uritchard and Knipe, 1997). This discrepancy suggests that for transfected cells, a cellular OBP or other protein(s) may be capable of interacting with HSV-1 DNA replication proteins to assemble them into a replication complex. On the other hand, the HSV-1 OBP is required for binding to the viral DNA genome and it also interacts with other proteins in infected cells (Liptak et al., 1996).
1.9 Cytomegalovirus DNA replication

1.9.1 Viral gene loci required for transient complementation of DNA replication

A transient complementation assay has been used to successfully identify genes essential for HSV-1 and Epstein-Barr virus (EBV) DNA replication (Challberg, 1986; Fixman et al., 1992; Wu et al., 1988). Using this assay, Pari and Anders (1993) defined eleven viral gene loci that are required for the transient complementation of HCMV oriLyt-dependent DNA replication in susceptible human diploid fibroblast cells. The requirement of these loci for DNA replication is further confirmed by antisense oligonucleotide studies, in which the intracellular expression of an antisense mRNA that targets one of the loci inhibits viral DNA replication (Ripalti et al., 1995; Smith and Pari, 1995b).

On the basis of sequence comparisons with other herpesviruses, six of the eleven loci are likely to encode core replication proteins that assemble at the replication fork, including the DNA polymerase (Pol), the Pol accessory protein, the single-stranded DNA-binding protein (ssDBP), primase, helicase, and the primase-helicase associated factor (Chee et al., 1994; Table 1.1). By substituting the core proteins with those from another herpesvirus, Hayward's group demonstrated that the six EBV core replication genes are able to support HCMV oriLyt-dependent DNA replication in Vero cells in the presence of an auxiliary component derived from one of the loci essential for HCMV DNA replication (Sarisky and Hayward, 1996). Similar functional complementation among core replication components is also documented between HSV-1 and EBV (Fixman et al., 1995). Based upon these results, it appears that various core replication proteins made by herpesviruses exhibit similar functions in replication. Therefore, the common feature of lytic DNA
replication among herpesviruses is likely due to the conserved properties of this set of genes. Most of the HCMV loci required for viral DNA replication were characterized with respect to the analysis of transcripts and transcriptional regulation (Heilbronn et al., 1987; Kemble et al., 1987; Leach and Mocarski, 1989; Smith et al., 1996; Smith and Pari, 1995a). However, very few detailed biochemical studies of the protein products have been reported (Ertl and Powell, 1992; Ye and Huang, 1993).

Five additional loci were also found to be essential for HCMV DNA replication in the transient complementation assay. None of these five gene products share biochemical similarities to the HSV-1 origin-binding protein (OBP) and no homologs have been identified in HSV-1 or EBV (Boehmer and Lehman 1997; Fixman et al., 1992, 1995; Wu et al., 1988). These five auxiliary components consist of three sets of IE regulatory genes [UL36-38, the internal repeat short 1 (IRS1)/the terminal repeat short 1 (TRS1), UL122/UL123] that are required for the expression of the other DNA replication genes (Iskenderian et al., 1996); One early gene, UL84, that may promote the initiation of HCMV oriLyt-dependent DNA replication (Sarisky and Hayward, 1996); And the early genes UL112-113 that may organize the formation of HCMV DNA replication compartments (Penfold and Mocarski, 1997).

Limitations of the transient complementation assay should be noted in the identification of the loci essential for HCMV DNA replication. In this assay, the eleven HCMV loci contained the native promoters and the 3' untranslated control regions found in the replication genes. Some loci may encode transactivators which efficiently express other replication genes rather than directly participate in HCMV DNA replication. Indeed, when different combinations of the eleven essential loci were transiently expressed in diploid human fibroblast cells from a heterologous constitutive promoter, both UL112-113 and IRS1 auxiliary components proved to be nonessential for oriLyt-dependent DNA
synthesis (Sarisky and Hayward, 1996). The HCMV IE1-deficient virus replicates as efficiently as a wild-type virus at high multiplicity of infection (m.o.i.) in human fibroblast cells (Greaves and Mocarski, 1998). Thus, IE1, which is found to be essential for the viral DNA replication in the transient complementation assay, is dispensable during viral infection. Hence, more genetic and biochemical studies need to be carried out to further define a minimal set of genes required for HCMV DNA replication.

Murine cytomegalovirus homologs of the eleven loci required for HCMV DNA replication have been identified by sequence homology (Rawlinson et al., 1996). Six encode similar core DNA replication proteins, namely, Pol, the Pol accessory protein, the MDBP and the three subunits of the helicase-primase complex. Biochemical characterization of some of these proteins demonstrates that they play a key role in MCMV DNA replication (Loh et al., 1991, 1994; Ochiai et al., 1992; Pande et al., 1991). The homologs of five additional loci required for HCMV oriLyt-dependent DNA replication are found in MCMV, but little genetic and biochemical data is available for them.

1.9.2 Other viral proteins involved in DNA replication

The CMV genome contains several genes encoding proteins that are likely to be involved in nucleotide metabolism in the natural host in addition to the eleven loci required for the oriLyt DNA replication in the transient complementation assay. Based on comparisons with their HSV-1 counterparts, the predicted gene products include the large subunit of ribonucleotide reductase, deoxyuridine triphosphate nucleotidohydrolase, and deoxyribonuclease (Chee et al., 1990; Rawlinson et al., 1996). Cytomegaloviruses also encode a protein similar to the HSV-1 uracil-DNA glycosylase (UDG). This enzyme acts in the DNA repair pathway by removing uracil residues in DNA that result from the misincorporation of dUTP by Pol or spontaneous deamination of cytosine residues. Studies
from Prichard et al. (1996) showed that the initiation of viral DNA replication in a HCMV UDG-deficient virus is delayed by 48 hours compared to the wild-type virus. However, once DNA replication starts in the mutant virus, it proceeds normally, suggesting that the repair of damaged or modified DNA by UDG may be a critical step during initiation of HCMV DNA replication.

1.9.3 Cellular proteins and viral DNA replication

Cellular proteins also make significant contributions to CMV DNA replication. As described by Anders and McCue (1996), host transcriptional factors participate in the initiation of CMV DNA replication by binding to motifs within the oriLyt region. In addition, HCMV infection has an impact on progression through the cell cycle. Cellular DNA replication is blocked after G0 or G1 phase-cells are infected with HCMV (Salvant et al., 1998; Wade, et al., 1996). However, the activities of many cellular DNA synthesis enzymes are stimulated by the HCMV infection. They include dihydrofolate reductase, DNA polymerase, topoisomerase II, as well as the proliferating cell nuclear antigen (PCNA), a cellular DNA polymerase processivity factor (Benson and Huang, 1990; Dittmer and Mocarski, 1997; Ebert et al., 1994; Lu and Shenk, 1996; Wade et al., 1992; Wilcock and Lane 1991). Human cytomegalovirus may adopt a strategy whereby it hijacks the cellular DNA synthesis machinery for its own replication. The need for the expression of cellular genes for HCMV DNA synthesis may partially explain its relatively extended replication cycle.

1.9.4 The origin of DNA replication

The origin of HCMV DNA replication, oriLyt, was initially localized to a region adjacent to the gene encoding the ssDBP in the middle section of the U1 component of the
genome (Hamzeh et al., 1990). Later, two research groups (Anders et al., 1992; Masse et al., 1992) defined a 1.5 to 2.4-kb minimal oriLyt region by a transient replication assay in which plasmids containing sequence deletions around a putative oriLyt were introduced into HCMV-infected cells, and the replication of the plasmids was monitored by the accumulation of the DpnI-resistant DNA. Besides the minimal oriLyt, other sequences flanking this region substantially contribute to the overall DNA replication efficiency in the transient replication assay. The HCMV minimal oriLyt is distinct from other herpesvirus-replication origins because of its larger size and higher complexity. The region contains AT- and GC-rich sequences, many clustered repeat sequence elements, and binding sites for various transcription factors, such as ATF/CREB, MLTF/USF, and Sp1. Recently, insertion and deletion mutagenesis studies further defined a core segment within the minimal oriLyt (Zhu et al., 1998; Figure 1.4). The segment consists of two essential regions. Region I contains a 31-nucleotides (nt) homopyrimidine tract, termed the Y-block, which overlaps the small replicator transcript (srt), and several reiterated sequences, such as a 29-bp repeated element. This element is composed of an inverted pair of ATF-CREB binding motifs, which are separated by a direct repeat sequence. To date, only the Y-block in Region I has been demonstrated to be absolutely essential for oriLyt function. Region II has been found to: (i) encompass five binding sites for the transcription factor Sp1, (ii) overlap a portion of the highly conserved large dyad A sequence, (iii) contain the promoter for the srt transcript, and (iv) overlap a segment recently found to contain an RNA, termed vRNAs, which is covalently incorporated into packaged HCMV genomes. Collectively, these observations suggest that Region II may regulate the expression of transcripts that participate in oriLyt function, most likely the initiation of HCMV DNA replication (Huang et al., 1996).
Figure 1.4 Functional components of the 1.5-kb core lytic DNA replication origin of human cytomegalovirus. Black rectangles represent Regions I and II into which deletions result in completely abrogated replicator activity in transfection assays. Locations of the 29-bp repeated element (shaded circles) and the Y-block (shaded bar) are also illustrated. The open rectangle indicates the intervening deletable segment. The arrow shows the small replicator transcript (SRT) that terminate within the Y-block (Adapted from Zhu et al., 1998).
The MCMV minimal oriLyt of approximately 1.7 kb has been identified using a similar approach as described above (Masse et al., 1997). Similar to HCMV, the MCMV minimal oriLyt is mapped upstream of the M57 ORF encoding the MDBP, a homolog to the HCMV ssDBP. It displays the same complexity as that for HCMV. The region can be subdivided into two domains. Domain I includes a majority of inverted repeats, palindromes, AT-rich regions, and extensive consensus binding sites for transcription factors such as AP2, ATF, and Sp1. Domain II contains most of direct repeats. As previously shown for HCMV oriLyt, auxiliary sequences flanking the minimal region of MCMV oriLyt dramatically enhance DNA replication in a transient replication assay. Despite similarities in the overall structure and sequence characteristics, no sequence homology has been found between the oriLyts of MCMV and HCMV. Furthermore, the plasmid containing MCMV oriLyt is unable to replicate in HCMV infected human fibroblasts, or vice versa (Masse et al., 1997), indicating that the CMV oriLyt is virus specific and is not functionally exchangeable.

1.9.5 The initiation of DNA replication within oriLyt

The initiation of CMV DNA replication is a critical step in the switch from latency to productive infection. The mechanism of the initiation of CMV DNA replication is not well understood. It appears that the initiation process is more complex than that in HSV-1 due to the high complexity of CMV oriLyt and the absence of CMV homolog of the HSV-1 OBP (Chee, et al., 1990; Rawlinson, et al., 1996) that binds specifically to the origin and probably unwinds DNA by its intrinsic helicase activity (Boehmer et al., 1993).

The requirement of transcriptional control elements and/or transcripts for the initiation has been documented in many prokaryotic and eukaryotic DNA replication systems (Kornberg and Baker, 1991a). Recently, studies from Anders' laboratory (Huang
et al., 1996; Zhu et al., 1998) suggest that HCMV may utilize the similar mechanism to initiate its DNA replication within the oriLyt. A set of short transcripts, originating from the srt transcription unit within the essential region I of the core HCMV oriLyt, have been identified and characterized. These 0.2- to 0.25-kb transcripts, termed small replicator transcripts (SRT), are produced in the absence of the viral DNA replication. They are not polyadenylated and no ORF has been found within SRTs, indicating that they are unlikely to serve as messenger RNAs. It was found that the heterogeneous 3' ends of SRTs overlap the Y-block, a structure also identified in the oriLyt of EBV, but not in the oriLyt of HHV-6, a member of the betaherpesvirus family. Interestingly, EBV, like HCMV, lacks a homolog of the HSV-1 OBP (Fixman et al., 1995; Schepers et al., 1993), whereas HHV-6 requires a functional OBP for the initiation of DNA replication (Dewhurst et al., 1994; Gompels et al., 1995; Inoue and Pellet, 1995). Additionally, a very similar arrangement of transcripts and the Y-block-like element has been identified in the mitochondrial heavy-strand replication origin (Xu and Clayton, 1995). In mitochondria, an oligopyrimidine element, called CSBII, inhibits the release of nascent transcripts from the template strand and promotes the formation of a displacement loop in a region coincident with the origin of DNA replication.

Based on these results, Anders and McCue (1996) proposed a model for the initiation of HCMV DNA replication, where HCMV SRT initially cooperates with the Y-block element to mediate a local unwinding of the DNA at oriLyt, which would then serve as an entry point for the assembly of the replication machinery. As described above, Domain I of the minimal MCMV oriLyt contains consensus binding sites for transcription factors (Masse et al., 1997). However, it is not clear whether the HCMV-like SRT is generated during MCMV DNA replication and whether the transcript contributes to the initiation of MCMV DNA replication within oriLyt.
1.9.6 Human cytomegalovirus DNA replication compartments

During HCMV infection, nuclear structures analogous to the HSV-1 prereplicative sites and replication compartments were identified by the immunofluorescence staining of infected cells with an antibody against HCMV ssDBP (Anders et al., 1987; Kemble et al., 1987; Penfold and Mocarski, 1997). Similarly, the formation of replication compartments can be disrupted in the presence of the viral DNA synthesis inhibitor, phosphonoformic acid (PFA). However, several features unique to HCMV have recently been reported (Penfold and Mocarski, 1997). First, in addition to punctate and large globular staining patterns, cells infected with HCMV at early times (24 hours postinfection) exhibit bipolar foci, defined by staining infected cells with antibodies against the ssDBP, the Pol accessory factor and UL112-113. These structures may represent an intermediate phase in the development of replication compartments. Second, at late times (72 hours postinfection), punctate structures are regenerated at the nuclear periphery. The formation of these structures may reflect a virion maturation process adjacent to the nuclear membrane (Ward, et al., 1996). Third, at early times, the synthesis of viral DNA appears at the punctate and bipolar foci, and at sites that lack the uniform organization of the viral DNA replication proteins. Thus, HCMV may utilize both viral and cellular DNA replication machinery to synthesize its DNA during early phase of the infection.

Currently, very little is known about the pathway involved in the assembly of prereplicative sites and replication compartments in CMVs. The replication compartments of HCMV have been generated by cotransfection of Vero cells with plasmids containing the oriLyt and the eleven gene loci required for HCMV DNA replication (Sarisky and Hayward, 1996). In the absence of either the auxiliary component UL84 or the origin of replication, the remaining elements assemble into prereplicative sites.
1.10 Research objectives and approaches

The MCMV MDBP is a non-structural protein produced during early times of infection. The protein is a product of the M57 ORF, which is transcribed as a 4.2-kb mRNA and has a capacity of encoding a protein of 1191 amino acids with a calculated molecular weight of 131.4 K (Messerle, et al., 1992; Rawlinson et al., 1996; Figure 1.5). The amino acid sequence of the MCMV MDBP shares significant homology with that of HCMV ssDBP (Messerle et al., 1992), which has been demonstrated to bind ssDNA in vitro (Anders et al., 1986; Anders et al., 1987; Anders, 1990; Kemble et al., 1987; ). Extensive amino acid sequence homology is also found between the MCMV MDBP and the HSV-1 ICP8. Thus, it is likely that the MCMV MDBP shares similar biochemical properties with its HCMV and HSV-1 counterparts.

Although the ORF of the MCMV MDBP has been identified and characterized with respect to the analysis of its transcripts, no biochemical studies have been reported. This represents a gap in understanding the molecular mechanism of HCMV DNA replication. I began a characterization of the biochemical properties of the MCMV MDBP and have attempted to identify the ssDNA-binding domain and the NLS. The entire M57 ORF of the MCMV MDBP was first cloned into an expression vector and the MDBP fusion protein was overproduced in Escherichia coli (E. coli). A panel of monoclonal antibodies (MAbs) were then produced after immunization of mice with partially purified MDBP fusion protein. Next, ssDNA-cellulose column chromatography was carried out to characterize the ssDNA-binding property of the MDBP and to identify the ssDNA-binding domain of the MDBP by deletion mutagenesis. The NLS of the MDBP was characterized by transiently expressing the mutant MDBP protein in COS-1 cells and observing the subcellular localization of the expressed protein by indirect
immunofluorescence. Finally, gel filtration chromatography was performed to define the native molecular weight of the MDBP extracted from MCMV-infected cells.
Figure 1.5 Nucleotide sequence and deduced amino acid sequence of the M57 open reading frame encoding the murine cytomegalovirus major DNA-binding protein. The nucleotide sequence is shown from nucleotide number 88322 to 91894 of the M57 open reading frame and the deduced amino acid sequence is shown below as a single-letter. The putative zinc-finger like motif and the putative single-stranded DNA-binding motif are underlined and marked by descriptions. Stretches of basic residues (at least three basic residues in four) that could serve as nuclear localization signals are boxed. The location of restriction enzyme sites used in the cloning and deletion mutagenesis experiments are indicated. The GeneBank accession number for the sequence is X67021 (Adapted from Messerle et al., 1992).
ATGGCGGACGACGATCTCTCCAGCTGGCCCCGCTCGCTCCCGGCTGTGATGTTCTCTCTCAAAAGAA
H A D D O L S S L A P V A P A V W H F L K K
Sat 1
CGCGGGGAGCTGCGGGACATCGTGCGGACATGCTCTGGCAACAAGGCCAACCCTCCGTCATGCGCC
140
T R E L A I A A H S L C O K A T P V V I A P
Sal 1
GCTGTTGTAGATAGACGTAGCCTGGACCCGACCTTTTGCGCGGCGCGGTCCAGAAGCCGATGACACATAGAG
210
A F S P L A N R D O R T O V V D V L C O E L G I A
Sal 1
CCGCCTGATACACGGGGCAGTCTGTTGCGGCAAACGGCCCTCAAGGAGCTCTCTCTAGAGGGGGGCACGTCA
490
Y T L F S G G A D A A H A D G P S A A V A C D
Kho 1
GATCCGTTGGAATCTGCTCGAGACCAGGGGTTCTAGGGACCCCCGGCGTCAGCGAGGCGCCTTTCTACTTCTAGGTTCAC700
D P W V L E H G F Y O PAL SE A L F Y F MF
Pst 1
CCTCGGGGGCCAGTCGCTGGCCCTGCTCGCCAGACGCTCGGCTCATCGAGGGCGCGTCTGCAAGCTGGTCTGC
770
E D T G G T V K L T P F K K Y H G Y T S Q K L
Sal 1
ACGCCTGAGGACAGGACCTCATGACGCGCTCGCGGCTGCTCGAAGCTGGTCAAGCTGGTCTGCTG
910
T A V E R O Q L M T V D A V C S E L A F S Y A
EcoR 1
CCATCTATCTTGAATTCCGTCTACGAATTCCACGCGGCTCTCAGACTTTCTCGAATGGCGGCTGCTCAAGAA980
S I Y L O S V Y E F S T A S N F L E W P L V K N
2.0 MATERIALS AND METHODS

2.1 Reagents

Chemicals were purchased from Sigma (St. Louis, MO), BDH Inc. (Toronto, Ont.), and Bio-Rad (Richmond, CA). Cell culture media were purchased from Life Technologies Inc., Grand Island, NY. Bacterial culture medium was purchased from DIFCO Laboratories, Detroit, MI. Antibiotics were obtained from GIBCO/BRL. Restriction endonucleases and DNA modification enzymes were purchased from Life Technologies, Inc. (Gaithersburg, MD), New England Biolabs Inc. (Beverly, MA) and Boehringer-Mannheim (Germany). Enzymes were used according to manufacturers' instructions.

2.2 Viruses

2.2.1 Smith strain (Vancouver) of MCMV

The Smith strain of MCMV was originally obtained from Dr. J.B. Hudson of the University of British Columbia and plaque-purified in this laboratory. Except for an 8-kilobases (kb) deletion at one end of the genome, this virus has restriction profiles identical to the Smith strain of MCMV supplied by the American Type Culture Collection (ATCC) (Rockville, MD; ATCC VR-194) (Loh et al., 1991). In addition, it replicated to a limited extent in the spleen but failed to grow in the salivary glands of inoculated mice. Therefore, to avoid confusion, the virus used in this study has been referred to as the
Smith strain (Vancouver) of MCMV by Loh et al. (1991). The virus stock was previously prepared in this laboratory by propagation in 3T3-L1 cells and purified by differential centrifugation (Loh et al., 1988). The concentrated virus was stored at -70°C in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS). The frozen stock was quickly thawed at 37°C and diluted in DMEM supplemented with 2% FBS when used to infect susceptible cells.

2.2.2 Vaccinia viruses

The IHD strain of vaccinia virus (wild type) was originally obtained from Dr. William Britt of the University of Alabama. The virus was routinely propagated in CV-1 cells (ATCC CCL70) to produce high-titer virus stocks for infection experiments.

2.3 Cell lines

2.3.1 Balb/3T3 clone A31 (ATCC CCL 163)

Balb/3T3 cells, originally derived from Balb/c mouse embryo cells (Aaronson and Todaro, 1968), were used for virus-infection experiments and the preparation of infected-cell lysates.

2.3.2 CV-1 (ATCC CCL 70)

CV-1 cells, derived from the kidney of an African green monkey (Hay et al., 1992), were used for the propagation of vaccinia virus as well as DNA transfection and transient expression experiments.
2.3.3 COS-1 (ATCC CRL 1650)

COS-1 cells, established by transformation of simian CV-1 cells with an origin-defective simian virus 40 (SV40) genome (Gluzman, 1981), were used for DNA transfection and transient expression experiments. These cells constitutively express wild-type SV40 T-antigen and support the replication of plasmids containing the SV40 origin of replication.

2.3.4 FO (ATCC CRL 1646)

FO cells, derived from the Sp2/0-Ag14 myeloma cell lines (de St Groth and Scheigger, 1980), were used as a fusion partner for the production of hybridomas. These cells produce no endogenous immunoglobulin heavy or light chain molecules and fuse effectively with B-lymphoblasts in the presence of polyethylene glycol (PEG).

2.4 Cell culture and passage

All cell lines were maintained in DMEM supplemented with 10% FBS in a humidified NAPCO Incubator (Precision Scientific, Inc., Chicago, IL) at 37°C with 5% CO₂ and 95% air. Confluent BALB/3T3, CV-1, and COS-1 cells were passaged as follows. The monolayers were rinsed twice with GKNP and treated with trypsin-ethylenediaminetetraacetic acid (EDTA) solution consisting of 0.025% trypsin and 0.27 mM EDTA in GKNP. After the trypsin-EDTA solution was removed, appropriate number of cells were taken up with a pipette and deposited into fresh medium. BALB/3T3, COS-1, and CV-1 cells were split 1:10, 1:10, and 1:5 respectively at three-day intervals. The FO suspension cell culture was passaged by transferring 1/10th of the cell suspension into fresh medium every three days.
2.5 Infection of BALB/3T3 cells with MCMV

Two different protocols were used to infect Balb/3T3 cells with MCMV. To study the time course and the subcellular localization of MCMV MDBP, BALB/3T3 cells were grown in Nunclon 60-mm culture dishes (Nunc, Denmark) or seeded on 18 x 18 mm coverslips (Fisher Scientific, Canada) in Nunclon 35-mm culture dishes. The subconfluent cell monolayers were rinsed once with GKNP balanced salt solution and inoculated with 1.5 ml of the Smith strain (Vancouver) of MCMV at a multiplicity of infection (m.o.i.) of 5 plaque-forming units per cell (PFU/cell). The viruses were allowed to adsorb onto the cells at 37° C for 1 hour. The cell monolayers were washed three times with GKNP balanced salt solution to remove the unbound virus. The cells were incubated in DMEM supplemented with 2% FBS at 37° C. In studies with viral DNA synthesis inhibitors, the cells were infected with MCMV and subsequently incubated with 200 or 300 µg per ml of phosphonoformic acid (PFA) in DMEM supplemented with 2% FBS. The infected-cells were harvested at the indicated times post-infection.

In the second method, centrifugation was used to enhance the efficiency of MCMV infection by 10 to 20-fold (Osborn and Walker, 1968). This method was used to prepare MCMV-infected cell extracts for gel filtration chromatography. BALB/3T3 cells were grown in Nunclon 100-mm culture dishes until they were 90% confluent. The cell monolayers were rinsed once with GKNP balanced salt solution, inoculated with 15 ml of the Smith strain (Vancouver) of MCMV at an m.o.i of 100 PFU/cell, and centrifuged at 900 x g at room temperature for 20 minutes in a GLC-2B Centrifuge (Dupont Instruments). The unbound virus was removed by washing the cell monolayers three times with GKNP balanced salt solution. The infected-cells were incubated in DMEM supplemented with 2% FBS at 37° C and harvested at 24 hours post-infection.
2.6 Radiolabeling of MCMV-infected cells

For the preparation of cell extracts containing $[^35]S$methionine-labeled MCMV-specific proteins, cells grown in Nunclon 100-mm dishes were infected by the centrifugal enhancement method as described above. After 8 hours of infection, cell monolayers were washed once with methionine-free media and proteins were metabolically labeled with $[^35]S$methionine (50 μCi/ml; DuPont/New England Nuclear Corp., Boston, MA) in DMEM containing 1/10th the normal amount of methionine for 16 hours. Cells were collected and extracts were prepared as described in section 2.7.

2.7 Preparation of whole cell extracts

Cells grown in a Nunclon 100-mm dish were infected with MCMV or transfected with plasmid DNA. At the indicated times post-infection or transfection, cells were scraped into medium, transferred to a 15-ml Corning centrifuge tube (Corning Glass Works, Corning, NY), and pelleted. Cells were washed three times with cold phosphate-buffered saline (PBS) and transferred to a microfuge tube. Cells were solubilized in 250 μl of radioimmunoprecipitation assay (RIPA) buffer that included 1 mM of freshly prepared phenylmethylsulphonylfluoride (PMSF) for 1 hour on ice. Cell lysates were clarified by centrifugation at 12,000 x g at 4°C for 15 minutes. The supernatant was collected and proteins were analyzed by Western blotting as described in section 2.9.

2.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method described by Laemmli (1970). Protein samples were solubilized in SDS-PAGE sample buffer, heated at 100°C for 5 minutes, and analyzed on a 7.5% acrylamide separating gel and a 2.8% acrylamide stacking gel in a mini-
PROTEAN II Electrophoresis Cell (Bio-Rad). Sizes of proteins were deduced by comparison with molecular weight markers (Bio-Rad). The gel was run at a constant voltage of 100 volts until the dye front migrated to 1 cm from the bottom of the gel. After electrophoresis, if protein bands needed to be visualized, the gel was transferred to a clean glass container containing 20 ml of Coomassie blue staining solution and incubated at room temperature for 20 minutes on a slow-shaking platform. Then the gel was destained with several changes of destaining solution.

2.9 Western blotting

Western blotting was performed as described by Towbin et al. (1979) and Batteiger et al. (1982) with modifications (Loh et al., 1988). Proteins separated by SDS-PAGE were transferred electrophoretically to a nitrocellulose membrane in cold transfer buffer at 100 volts for two hours. The membrane was blocked in TBS containing 1% BSA at 4°C overnight and incubated with selected MAbs diluted appropriately in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) supplemented with 0.1% BSA at room temperature for 3 hours with gentle shaking. Then the membrane was washed three times with TBST and incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (AP; 1:3,000 dilution in TBST containing 0.1% BSA) at room temperature for 1 hour with gentle shaking. The membrane was washed again as before and developed in 10 ml of AP buffer containing 33 μl of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and 44 μl of p-nitro blue tetrazolium chloride (NBT). After a suitable period of development, the reaction was stopped by washing the membrane with several changes of ddH2O.
2.10 Immunoprecipitation

Immunoprecipitation was carried out as previously described (Loh, et al., 1988). Fifty microliters of [³⁵S]methionine-labeled MCMV proteins solubilized in immunoprecipitation buffer were incubated with 3 µl of MAb 4F11.21A at 4°C overnight. The immune complexes were then incubated with 40 µl of protein A-Sepharose CL-4B (1:1 dilution in immunoprecipitation buffer) at 4°C for one hour with gentle rocking. Subsequently, the sepharose beads were washed three times with immunoprecipitation buffer supplemented with 0.5 M NaCl and once with immunoprecipitation buffer. Immunoprecipitated proteins were eluted from the beads by boiling for 5 minutes in SDS-PAGE sample buffer and analyzed by SDS-PAGE. The proteins were stained with Coomassie blue as described in section 2.8 and enhanced for autoradiography with Entensify (NEN Research Products, Boston, MA) according to manufacturer's instructions. Finally, the gel was dried in a Bio-Rad Model 503 Gel Dryer for 2 hours at 80°C and exposed to Kodak XAR5 X-ray film (Eastman Kodak Company, Rochester, NY) at -70°C for an appropriate time.

2.11 Indirect immunofluorescence

2.11.1 Slide preparations

Two different methods for processing slides for indirect immunofluorescence were used (Loh et al., 1994). In the first method, which was used when only a few slides were required, cells infected with MCMV or transfected with plasmid DNA were seeded onto 18x18 mm glass coverslips. At the indicated time points, the coverslips were collected and cells were fixed in a 3.75% formaldehyde solution in cold PBS at room temperature for 5 minutes. The fixed cells were permeabilized by incubation in 1% Triton
X-100 in cold PBS for 5 minutes at room temperature. The coverslips were rinsed twice in PBS and blocked in 0.2% bovine serum albumin (BSA) in PBS at room temperature for ten minutes or stored in 0.2% BSA in PBS supplemented with 0.02% sodium azide at 4°C.

In the second method, toxoplasmosis slides were used to allow the rapid preparation of large number of samples for screening tissue culture supernatants from hybridoma cell lines. Briefly, CV-1 cells expressing MCMV MDBP (section 2.20) were collected by scraping cells into 1 ml of PBS and the cell suspension was transferred into a microfuge tube. The cells were pelleted, washed once with PBS, and resuspended in PBS at about 10^6 cells per ml. Fifteen microliters of the cell suspension were deposited onto each well of the 8-well toxoplasmosis slides (Bellco Biotechnology, Vineland, NJ) and air dried. The cells were fixed in methanol at -20°C for 3 minutes followed by permeabilization in acetone at -20°C for one and a half minutes. The slides were stored at -70°C and soaked in PBS for 5 minutes to rinse off salt deposits when used in indirect immunofluorescence experiments.

2.11.2 Indirect immunofluorescence assay

The coverslips and slides prepared above were incubated either with MAb 4F11.21A at 1:200 dilution in Tris-buffered saline (TBS) supplemented with 3% FBS or tissue culture supernatants from hybridoma cell lines at room temperature for 1 hour, washed three times with PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG antibody (1:75 dilution in TBS containing 3% FBS) for 1 hour. To identify the location of the nucleus, cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 2 minutes. Finally, the samples were rinsed twice in PBS and mounted onto glass slides with 50% (v/v) glycerol in PBS. The cells were examined
under a Nikon HFX-II A fluorescence microscope (Nikon Canada Inc., Mississauga, Ont.). Fluorescein and DAPI-stained cells were visualized under an interference filter (B-2A, DM510, Nikon) and an ultra violet filter (UV-2, DM400, Nikon) respectively. Photographs were taken with a Nikon FX-35WA Camera System (Nikon Corporation, Japan).

2.12 Bacterial strains and cultures

Two *E. coli* strains were used in this study. The first, DH5α [*supE44 lacU169(80 lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*], was purchased from Life Technologies, Inc. (Gaithersburg, MD) and used as the host for plasmids (Hanahan, 1983). The second, BL21(DE3) [*hsdS gal (cI857 ind1 Sam7 nin5 lacUV5-T7 gene1)*] was purchased from Novagen, Inc. and used for the expression of MCMV MDBG and its mutant proteins after transformation with appropriate expression plasmids. BL21(DE3) carries a lysogen of bacteriophage DE3 which contains the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase (Studier and Moffatt, 1991). Addition of isopropylthio-β-D-galactoside (IPTG) to a growing BL21(DE3) culture induces the expression of T7 RNA polymerase, which in turn transcribes the target gene in the expression plasmid. In addition, the BL21(DE3) strain lacks both the Ion protease and the ompT outer membrane protease that can degrade expressed recombinant proteins (Grodberg and Dunn, 1988).

Typically, bacteria were grown in Luria-Bertani (LB) medium at 37°C with vigorous shaking at 250 rotations per minutes (RPM) in an environmental shaker (New Brunswick Scientific Co., Inc., Edison, NJ). Ampicillin (50 µg/ml) was added as required.
2.13 Cloning vectors and plasmids used in this study

2.13.1 pTZ18R/19R

The plasmid vectors pTZ18R and pTZ19R were purchased from Pharmacia (St. Petersburg, FL). The plasmids contain the multiple cloning sequence (MCS) and the \textit{lacZ}' gene to facilitate blue/white screening of recombinants on X-gal plates.

2.13.2 pRSETA/B/C

The plasmid vectors pRSETA, pRSETB and pRSETC were purchased from Invitrogen Corporation. They were designed for high-level protein expression in bacteria. Based on the T7 promoter/T7 RNA polymerase system, the pRSET vectors allow the expression of recombinant proteins as fusion proteins with a leader peptide containing a hexahistidyl sequence for purification on nickel-chelated resins (Kroll \textit{et al}., 1993). The cloning of the protein-encoding sequence is facilitated by the presence of eleven unique restriction sites for all three vectors (Figure 2.1).

2.13.3 pM2

pM2 was a gift from Dr. Ivan Sadowski at the University of British Columbia (Sadowski \textit{et al}., 1992). The plasmid contains the SV40 early promoter/\textit{ori} region, the coding sequence for GAL4 [amino acid (aa) residues 1 to 147] and the SV40 early polyadenylation signal (Figure 2.2). The vector was used to construct plasmids for transient expression of MCMV MDBP under the control of the SV40 early promoter in COS-1 cells.
Figure 2.1. Schematic representation of the pRSET vectors. The multiple cloning sequence is illustrated at the top line with unique restriction endonuclease sites indicated. The significant regions are shown by open boxes or open arrows. The pRSETA/B/C differ only by the addition of a single base in the multiple cloning sequence, such that foreign genes can be inserted in-frame into one of these sequences. The phage T7 promoter directs the expression of the hexahistidyl-tagged fusion protein and transcripts are terminated within the phage T7 terminator. The hexahistidyl sequence allows purification of recombinant fusion protein on nickel-chelated resins by affinity chromatography. The vector also contains a gene encoding an ampicillin selectable marker β-lactamase and an f1 origin of replication for the production of single-stranded DNA. Abbreviations: Ap\(^R\), ampicillin resistance; f1 ORI, f1 origin of replication; MCS, multiple cloning sequence (Adapted from Kroll et al., 1993).
Nucleotide sequence encoding a leader peptide containing hexahistidyl tag

T7 terminator

T7 promoter

MCS

pRSETA/B/C

f1 ORI

Ap^R
Figure 2.2. Schematic representation of the pM2 vector. The multiple cloning sequence is illustrated at the top line with unique restriction endonuclease sites indicated. A unique \textit{BglII} site located immediately after the SV40 early promoter region and the origin of replication is also depicted. The significant regions are shown by open boxes or open arrows. The SV40 early promoter directs the expression of \textit{GAL4} (aa 1-147) and transcripts are terminated within the SV40 early polyadenylation region. Abbreviations: \textit{Ap}^R, ampicillin resistance; MCS, multiple cloning sequence; ori, origin of replication; poly A, polyadenylation (Adapted from Sadowski et al., 1992).
2.13.4 pSLAVE

The vector pSLAVE was a gift from Dr. Tim Zamb, Veterinary Infectious Disease Organization (V.I.D.O.), Saskatoon, Saskatchewan, Canada. The vector, containing a synthetic vaccinia late promoter and unique BglII and SmaI sites, was used to construct the plasmid pSL-M57 for expression of MCMV MDBP in CV-1 cells.

2.13.5 pAM121

Plasmid pAM121, kindly supplied by Dr. Ulrich Koszinowski at the University of Munich, Germany (Ebeling et al., 1983), contains the HindIII-D fragment of the Smith strain of MCMV. This fragment containing coding sequences of the genes for the MDBP, a putative assembly protein, the glycoprotein B (gB), and a portion of the viral DNA polymerase (pol) (Messerle et al., 1992).

2.14 General methods of molecular cloning

2.14.1 Plasmid DNA digestion and dephosphorylation

Plasmid DNA was digested by restriction endonucleases in an appropriate buffer supplied by the manufacturers at 37°C for 2 hours. To minimize self-ligation, 5'-phosphate groups from linearized plasmid vector DNA were removed by treatment with calf intestinal alkaline phosphatase (CIP) in CIP dephosphorylation buffer. Incubation was carried out with 1 unit of CIP at 37°C for 30 minutes. When restriction endonucleases that generate 5'-recessed termini were used, another unit of CIP was added to the above reaction and incubation was continued for a further 45 minutes at 55°C. After incubation, the dephosphorylation reaction mixture was extracted once with phenol and once with chloroform.
2.14.2 Agarose gel electrophoresis

Following digestion, DNA samples were mixed with agarose gel-loading buffer and loaded on 0.7% or 1% agarose gels (Bio-Rad) cast in a Horizontal Gel Electrophoresis System Model H6 (Bethesda Research Laboratories, Gaithersburg, MD). The *EcoRI/HindIII* fragments of bacteriophage lambda DNA (Life Technologies, Inc., Gaithersburg, MD) served as molecular weight markers. The DNA fragments were separated at a constant voltage of 75 volts in 1X Tris-acetate-EDTA (TAE) buffer. The gels were stained with 0.1 μg/ml of ethidium bromide in distilled, deionized water (ddH₂O) for 10 minutes. The DNA fragments were visualized on a Spectroline Model TC-302 Ultraviolet Transilluminator (Spectronics Corporation, Westbury, NY) and photographed with Polaroid type 667 film (Polaroid Corporation, Cambridge, MA).

2.14.3 Purification of DNA fragments from agarose gels and DNA ligation

To recover DNA fragments from agarose gels, the GENECLEAN II Kit (BIO 101 Inc., La Jolla, CA) was used according to manufacturer's instructions. After restriction endonuclease digestion and agarose gel electrophoresis, the desired DNA fragments were excised from the gel. Slices of the gels were transferred to a microfuge tube and weighed. Three volumes of sodium iodine stock solution were added to the tube and the agarose was dissolved by incubation at 48°C for 5 minutes. Each sample was then mixed with 7.5 μl of GLASSMILK® and incubated at room temperature for 10 minutes. The GLASSMILK®/DNA complex was pelleted by centrifugation at 12,000 x g at room temperature for 5 seconds and washed four times with 400 μl of NEW WASH® buffer. The pellet was resuspended in 5 μl of sterile ddH₂O and DNA was eluted by incubation of the sample at 48°C for 5 minutes followed by centrifugation at 12,000 x g at room temperature for 2 minutes. Five microliters of the purified DNA sample were
mixed with 1 μl of bacteriophage T4 DNA ligase in 1.5 μl of 5 x ligation buffer. The ligation reaction was carried out at 15°C for 4 hours or 4°C overnight.

2.14.4 Preparation of competent cells

Competent *E. coli* stocks were prepared as follows. A single freshly grown bacterial colony was inoculated into 100 ml of LB medium containing 50 μg/ml of ampicillin in a 2-liter flask. The culture was incubated at 37°C with vigorous shaking until the optical density at a wavelength of 600 nm (OD600) reached 0.4 as measured by SPECTRONIC 20 (BAUSCH & LOMB, USA). Then the culture was transferred to a cold centrifuge tube and cooled on ice for 10 minutes. Cells were pelleted by centrifugation at 4,000 RPM for 10 minutes at 4°C in a Sorvall SA600 rotor (RC-5B Refrigerated Superspeed Centrifuge, Dupont Instruments) and washed once with 10 ml of ice-cold 0.1 M calcium chloride solution. Cells were collected and resuspended in 2 ml of 0.1 M calcium chloride solution supplemented with 7% dimethyl sulfoxide (DMSO). Aliquots of 200 μl each were then dispensed into chilled microfuge tubes and frozen at -70°C.

2.14.5 Transformation of *E. coli*

For the transformation of competent bacteria with plasmid DNA, one tube of competent cells was quickly thawed by holding the tube in the palm of one's hand and then stored on ice for 10 minutes. An aliquot of 7.5 μl of DNA ligation reaction prepared from section 2.14.3 was gently mixed with the cells. The mixture was incubated on ice for 30 minutes followed by heat shock at 42°C for exactly 90 seconds. Cells were then chilled on ice for 2 minutes and resuspended in 800 μl of SOC medium followed by incubation at 37°C for 45 minutes with gentle shaking to allow the bacteria to recover and
express the ampicillin resistance gene. Finally, 150 µl of the transformed cells were spread over the surface of an agar plate containing ampicillin (50 µg/ml). The plate was inverted and incubated at 37ºC for 10-12 hours.

2.14.6 Analysis of plasmid constructs

The desirable plasmid constructs were identified by restriction mapping. To do this, plasmid DNA was purified from bacteria by the boiling method as described by Sambrook et al. (1989). Individual bacterial colonies were inoculated into 2 ml of LB medium containing 50 µg/ml of ampicillin in loosely capped tubes and grown at 37ºC overnight with vigorous shaking. Bacteria from each culture were then transferred into a microfuge tube, pelleted, and resuspended in 350 µl of STET buffer supplemented with 10 µl of freshly prepared lysozyme (20 mg/ml; Sigma, St. Louis, MO). The suspension was heated in a boiling water bath for exactly 50 seconds. The lysate was clarified by centrifugation at 12,000 x g at 4ºC for 15 minutes in a benchtop microcentrifuge (Microcentrifuge Model 235C, Fisher Scientific, Canada). After the pellet of bacterial debris was removed, 16 µl of a 5 M sodium chloride solution and 420 µl of isopropanol were added to the remaining supernatant. Plasmid DNA was allowed to precipitate at room temperature for 5 minutes and recovered by centrifugation at 12,000 x g at 4ºC for 15 minutes. The pellet was rinsed once with 70% ethanol and redissolved in 50 µl of Tris-EDTA (TE) buffer supplemented with 50 µg/ml of RNAase (Sigma, St. Louis, MO). To screen for the correct plasmid constructs, DNA was digested by appropriate restriction enzymes and analyzed by agarose gel electrophoresis as described in section 2.14.2. Plasmid DNA with the expected restriction map was used for subsequent cloning experiments or for transformation of E. coli BL21(DE3) for the expression of MCMV MDBP and its mutant proteins.
2.14.7 Purification of plasmid DNA

2.14.7.1 Preparation of plasmid DNA by the alkaline lysis method

Plasmid DNA was prepared by the alkaline lysis method as described by Sambrook et al. (1989). A single bacterial colony was cultured in 15 ml of LB medium supplemented with 50 μg/ml of ampicillin at 37°C overnight. The entire culture was inoculated into 500 ml of LB medium containing ampicillin (50 μg/ml) in a 2-liter flask, and bacteria were grown until the OD600 reached 0.8. To achieve high yields of plasmid DNA, 2.5 ml of chloramphenicol (34 mg/ml in ethanol) were added to the culture to achieve a final concentration of 170 μg/ml, and the incubation was continued for a further 16 hours at 37°C with vigorous shaking. The cells were collected by centrifugation, washed once with 100 ml of cold STE buffer, and lysed in 10 ml of Solution I (1 mg/ml of lysozyme) and 20 ml of Solution II. After incubation at room temperature for 10 minutes, 15 ml of cold Solution III was added, and the mixture was stored on ice for 10 minutes to allow chromosomal DNA, high-molecular-weight RNA, and potassium/SDS/protein/membrane complexes to precipitate. The lysate was clarified by centrifugation at 5,000 RPM for 20 minutes at 4°C in a Sorvall GSA rotor (RC-5B Refrigerated Superspeed Centrifuge, Dupont instruments). The supernatant was transferred to a centrifuge tube and DNA was precipitated with 0.6 volume of isopropanol at room temperature for 10 minutes. After centrifugation at 5,000 RPM for 15 minutes at room temperature in a Sorvall GSA rotor, the plasmid DNA pellet was rinsed with 70% ethanol and dissolved in 3 ml of TE buffer.
2.14.7.2 Purification of plasmid DNA by precipitation with polyethylene glycol

To prepare DNA for transfection and transient expression experiments, plasmid DNA prepared by the alkaline lysis method was further purified by precipitation with polyethylene glycol (PEG) as described by Sambrook et al. (1989). Plasmid DNA from section 2.14.6.1 was transferred to a 15-ml Corex centrifuge tube (Corning Glass Work, Corning, NY) and 3 ml of a 5 M lithium chloride solution (in ddH₂O) was added to precipitate the high-molecular-weight RNA. After centrifugation at 10,000 RPM for ten minutes at 4°C in a Sorvall SA600 rotor, the supernatant was transferred to a clean 30-ml Corex centrifuge tube and mixed with an equal volume of isopropanol. DNA was recovered by centrifugation at 10,000 RPM for 10 minutes at room temperature in a Sorvall SA600 rotor. The pellet was rinsed with 70% ethanol and dissolved in 500 μl of TE buffer that included 50 μg/ml of RNAase. The solution was transferred to a microfuge tube and incubated at room temperature for 30 minutes. Plasmid DNA was precipitated with 500 μl of a 1.6 M sodium chloride solution containing 13% (w/v) PEG 8000 followed by centrifugation at 12,000 x g at 4°C for 5 minutes. The DNA was dissolved in 500 μl of TE buffer, extracted once with phenol and chloroform, and precipitated with 100 μl of 10 M ammonium acetate and 3 volumes of ethanol at room temperature for 10 minutes followed by centrifugation at 12,000 x g at 4°C for 15 minutes. The DNA pellet was rinsed once with 70% ethanol and dissolved in 500 μl of TE buffer.

2.14.7.3 Purification of plasmid DNA by equilibrium centrifugation in cesium chloride-ethidium bromide gradients

Plasmid DNA was also purified by equilibrium centrifugation in cesium chloride (CsCl)-ethidium bromide gradients as described by Sambrook et al. (1989). Seven grams of UltraPure™ CsCl (GIBCO/BRL, Gaithersburg, MD) were dissolved in 7 ml of DNA
solution prepared in section 2.14.6.1 and subsequently mixed with 0.7 ml of ethidium bromide (10 mg/ml in ddH₂O). The mixture was transferred into a Beckman ultracentrifuge tube (Beckman Instruments) and centrifuged at 20°C at 55,000 RPM for 18 hours in an 80Ti rotor (Beckman L8-M Ultracentrifuge, Beckman Instruments) followed by 40,000 RPM for 1 hour. The DNA band was collected using an 18-gauge needle attached to a 3-ml syringe. To remove ethidium bromide and CsCl from the sample, the DNA solution was extracted several times with isoamyl alcohol followed by dialysis against TE buffer. DNA was collected and its concentration was determined as described in Section 2.14.7.4.

2.14.7.4 Determination of DNA concentration

The DNA concentration of purified samples was determined by measuring the absorbance of DNA in a standard cuvette at wavelengths of 260 nm and 280 nm respectively using a UV/VIS spectrometer (UNICAN 8700 series). The DNA sample was considered to be essentially free of contaminating proteins if the ratio of absorbance at 260 nm to that at 280 nm was about two. Since a DNA solution with an absorbance of one at 260 nm contains about 50 µg of double-stranded DNA per ml, the DNA concentration was calculated by the following formula: \[ [\text{DNA}] (\mu g/ml) = \text{dilution factors} \times 50 \times \text{absorbance (260 nm)}. \]

2.15 Cloning of the M57 open reading frame encoding MCMV MDBP

2.15.1 Construction of bacterial expression plasmid pRSET-M57

Plasmid pRSET-M57, used for the expression of MCMV MDBP in *E. coli*, was constructed in two major cloning steps (Figure 2.3A). First, a *SphI-EcoRI* fragment of pAM121, including about 0.2-kb of additional sequences 5’ of the M57 open reading
frame (ORF) and about 3.3-kb of additional sequences at the 3’ end, was subcloned into pTZ19R to generate pTSE737. To do this, a 4.5-kb BamHI fragment and a 5.2-kb EcoRI fragment were isolated from pAM121 and inserted into compatible cloning sites of pTZ19R to yield pTB448 and pTE1 respectively. Next, plasmids pTSB362 and pTB375 were generated by removing a 0.8-kb SphI fragment and an 1.4-kb BamHI fragment from pTB448 and pTE1 respectively followed by the religation of the resulting plasmids. Finally, a 3.6-kb SphI-BamHI fragment and a 3.8-kb BamHI-EcoRI fragment isolated from digestion of pTSB362 and pTB375 respectively, were ligated and cloned into SphI-EcoRI sites of pTZ19R to yield pTSE737.

Next, the entire M57 ORF encoding MCMV MDBP was cloned into pRSETB by following strategy. Plasmid pRSET-M57dC625 containing 5’ half of the M57 ORF was generated by inserting an 1.9-kb Ncol fragment from pTSB362 into compatible sites of pRSETB. Then pTSE737 was cut with MseI and end-filled with the Klenow fragment of E. coli DNA polymerase I and a 2.8-kb fragment containing the 3’ end of the M57 ORF was then generated by further cleavage of pTSE737 with MluI. The fragment was cloned into MluI and HindIII (end-filled) sites of pM2 to yield pM2-MH275. The blunt end ligation between the repaired MseI and HindIII sites recreated an intact HindIII site. Finally, pRSET-M57 was obtained by replacing a 2.8-kb MluI-HindIII fragment of pRSET-M57dC625 with an MluI-HindIII fragment from pM2-MH275. Thus the entire M57 ORF, from an Ncol site at 5’ end of the gene to an MseI site at the 3’ end of the
Figure 2.3. A. Construction of the expression plasmid pRSET-M57. The top line represents the HindIII cleavage map of the Smith strain of MCMV with the HindIII-D fragment expanded below. The positions of restriction endonuclease sites are indicated and the M57 open reading frame of the MCMV major DNA-binding protein is depicted by the hollow bar. The bottom lines delineate a series of subclones used for cloning the entire M57 open reading frame into the prokaryotic expression vector pRSETB. The size of the inserts is indicated above the lines. Abbreviations: kb, kilobases; ORF, open reading frame.

B. Schematic representation of prokaryotic expression plasmid pRSET-M57. The phage T7 RNA polymerase promoter directs the synthesis of a fusion protein comprising a 4.5 K leader peptide and a 131.4 K MCMV MDBP encoded by the M57 open reading frame. The restriction enzyme sites NcoI and HindIII used for cloning the M57 open reading frame, the ampicillin resistance gene, and the f1 origin of replication are indicated.
gene, was inserted into the MCS of pRSETB behind a polyhistidine coding sequence (Figure 2.3B). This plasmid was used to direct the synthesis of the histidine-tagged MCMV MDBP fusion protein after transformation of E. coli strain BL21(DE3).

2.15.2 Construction of eukaryotic expression plasmids pSV-M57 and pSL-M57

To express MCMV MDBP in COS-1 cells, the entire M57 ORF from pRSET-M57 was inserted into pM2 using BglII-HindIII cloning sites to generate the plasmid pSV-M57. Thereby, the M57 ORF was placed downstream of the SV40 early promoter, replacing the ORF containing the first 147 amino acids of the yeast transactivator GAL4.

For transient expression of MCMV MDBP in CV-1 cells, pSL-M57 was constructed by cloning the entire M57 ORF into the BglII site of the vector pSLAVE behind a synthetic vaccinia late promoter. Recombinant MCMV MDBP was expressed after transfection CV-1 cells with pSL-M57 DNA and subsequent infection with wild type vaccinia virus.

2.16 In-frame deletion mutagenesis of the M57 ORF

Two major groups of deletion mutants were made in this study by using convenient restriction enzyme sites within the M57 ORF (Table 2.1; Figure 2.4; Figure 2.5). The first, derived from the bacterial expression plasmid pRSET-M57, was used for mapping epitopes of MAbs and the ssDNA-binding domain within MCMV MDBP. The N-terminal or C-terminal deletion mutants were named pRSET-M57dNX or pRSET-M57dCX, where X refers to the first or last MDBP-specific aa residue present after the deletions were made. Internal deletion mutants were named pRSET-M57dX-Y, where X-Y refers to the aa sequence that was deleted. The second group, derived from the eukaryotic expression plasmid pSV-M57, was used for the identification of the NLS of
### Table 2.1. In-frame deletion mutants made in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Insert</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRSET-M57dN585</td>
<td>1.9-kb <em>EcoRI-HindIII</em> fragment of pRSET-M57</td>
<td>pRSETC cut with <em>EcoRI-HindIII</em></td>
</tr>
<tr>
<td>pRSET-M57dN625</td>
<td>1.8-kb <em>NcoI-HindIII</em> fragment of pRSET-M57</td>
<td>pRSETB cut with <em>NcoI-HindIII</em></td>
</tr>
<tr>
<td>pRSET-M57dN749</td>
<td>1.4-kb <em>BglII-HindIII</em> fragment of pRSET-M57</td>
<td>pRSETA cut with <em>BglII-HindIII</em></td>
</tr>
<tr>
<td>pRSET-M57dN1001</td>
<td>Deleting the 3.0-kb <em>NcoI</em> fragment of pRSET-M57</td>
<td>Deletion derivative of pRSET-M57</td>
</tr>
<tr>
<td>pRSET-M57dN1055</td>
<td>Deleting the 0.2-kb <em>BamHI</em> fragment of pRSET-M57dN1001</td>
<td>Deletion derivative of pRSET-M57dN1001</td>
</tr>
<tr>
<td>pRSET-M57dN1063</td>
<td>Deleting the 0.9-kb <em>SsrI</em> fragment of pRSET-M57dN749</td>
<td>Deletion derivative of pRSET-M57dN749</td>
</tr>
<tr>
<td>pRSET-M57dC625</td>
<td>1.9-kb <em>NcoI</em> fragment of pRSET-M57</td>
<td>pRSETB cut with <em>NcoI</em></td>
</tr>
<tr>
<td>pRSET-M57dC313</td>
<td>0.9-kb <em>BglII-EcoRI</em> fragment of pRSET-M57</td>
<td>pRSETB cut with <em>BglII-EcoRI</em></td>
</tr>
<tr>
<td>pRSET-M57dC254</td>
<td>0.8-kb <em>PstI</em> fragment of pRSET-M57dC313</td>
<td>pRSETB cut with <em>PstI</em></td>
</tr>
<tr>
<td>pRSET-M57dC216</td>
<td>0.6-kb <em>XhoI</em> fragment of pRSET-M57dC313</td>
<td>pRSETB cut with <em>XhoI</em></td>
</tr>
<tr>
<td>pRSET-M57dN54dC292</td>
<td>0.7-kb <em>SalI</em> fragment of pRSET-M57dC625</td>
<td>pRSETA cut with <em>BamHI-HindIII</em></td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Resultant Vector</td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------------------------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>pRSET-M57dN749d788-824</td>
<td>Deleting the 0.1-kb PstI fragment from pRSET-M57dN749</td>
<td>Deletion derivative of pRSET-M57dN749</td>
</tr>
<tr>
<td>pRSET-M57d55-686</td>
<td>Deleting the 1.9-kb SalI fragment of pRSET-M57</td>
<td>Deletion derivative of pRSET-M57</td>
</tr>
<tr>
<td>pRSET-M57d422-535</td>
<td>Deleting the 0.3-kb Scal-SmaI fragment of pRSET-M57</td>
<td>Deletion derivative of pRSET-M57</td>
</tr>
<tr>
<td>pRSET-M57d788-824</td>
<td>Deleting the 0.1-kb PstI fragment of pRSET-M57</td>
<td>Deletion derivative of pRSET-M57</td>
</tr>
<tr>
<td>SVdN312</td>
<td>2.7-kb EcoRI-HindIII fragment of pSV-M57</td>
<td>pM2 cut with BglII-HindIII</td>
</tr>
<tr>
<td>SVdC1056</td>
<td>3.2-kb BamHI fragment of pRSET-M57</td>
<td>pM2 cut with BglII-BamHI</td>
</tr>
<tr>
<td>SVdC625</td>
<td>1.9-kb BglII-HindIII fragment of pRSET-M57dC625</td>
<td>pM2 cut with BglII-HindIII</td>
</tr>
<tr>
<td>SVdC313</td>
<td>Deleting the 0.9-kb EcoRI fragment of SVdC625</td>
<td>Deletion derivative of SVdC625</td>
</tr>
<tr>
<td>SVdN625dC1064</td>
<td>1.3-kb NcoI-SstI fragment of pSV-M57</td>
<td>pM2 cut with BglII-HindIII</td>
</tr>
<tr>
<td>SVd422-535</td>
<td>1.9-kb MluI-BamHI fragment of pRSET-M57d422-535</td>
<td>pSVM57 cut with MluI-BamHI</td>
</tr>
<tr>
<td>SVdC1056d422-535</td>
<td>1.3-kb MluI-BamHI fragment of pRSET-M57d422-535</td>
<td>SVdC1056 cut with MluI-BamHI</td>
</tr>
</tbody>
</table>
Figure 2.4. Summary of deletion mutants derived from pRSET-M57. The top line indicates a sequence ruler with tick markers every 500 base pairs and restriction endonuclease sites illustrated below the line. The names of the deletion mutants are shown in the column on the left. The thick lines represent coding sequence for the MCMV major DNA-binding protein and its mutant proteins. Numbers above the lines depict amino acid residue numbers for the major DNA-binding protein.
Figure 2.5. Summary of mutants derived from SV-M57. The top line indicates a sequence ruler with tick markers every 500 base pairs and restriction endonuclease sites illustrated below the line. The names of deletion mutants are shown in the column on the left. The thick lines represent coding sequence for the MCMV major DNA-binding protein and its mutant proteins. Numbers above the lines depict amino acid residue numbers for the major DNA-binding protein. Striped bars indicate β-galactosidase and numbers below the bar represent residues numbers for β-galactosidase.
MCMV MDBP. The nomenclature of the constructs was similar to that described above except for the letters SV at the beginning of each construct (e.g. SVdN312).

2.17 Construction of plasmids expressing β-galactosidase-MDBP fusion proteins

The β-galactosidase (β-gal) gene was modified so that sequence containing unique restriction sites (NcoI, BamHI and SmaI) and encoding the residues MARDP replaced authentic β-gal sequence for the first 9 amino acid of the gene. A unique EcoRI site located at the junction of β-gal and pp50 (the polymerase accessory protein of MCMV) sequences from the plasmid pSVβgal-pp50dN202 (from this laboratory) was used for the construction of a β-gal-MDBP fusion protein. A list of plasmids expressing β-gal-MDBP fusion proteins is shown in Figure 2.5.

2.18 Expression of MCMV MDBP and its mutant proteins in *E. coli* strain BL21(DE3)

*Escherichia coli* strain BL21(DE3) was transformed with pRSET-M57 and various M57 mutants as described in section 2.14.5 and the expression of MCMV MDBP and its mutant proteins was induced by IPTG. Briefly, a single colony was inoculated into 2 ml of LB medium containing 50 µg/ml of ampicillin in a loosely capped tube. The culture was allowed to grow at 37°C to an OD₆₀₀ of 0.6 and stored at 4°C overnight. Cells were pelleted by centrifugation at 12,000 x g at 4°C for 30 seconds. The pellet was then resuspended in 1 ml of fresh LB medium and inoculated into 15 ml of LB media containing 50 µg/ml of ampicillin in a 50-ml Falcon centrifuge tube (Becton Dickinson and Co., Oxnard, CA). The culture was grown at 37°C until an OD₆₀₀ of 0.6. Protein expression was induced by adding IPTG to a final concentration of 0.8 mM and the culture was incubated a further 4 hours at 37°C to permit maximal protein expression. The cells were then collected and analyzed by Western blotting as described in section 2.9.
2.19 Preparation of the histidine-tagged MCMV MDBP fusion protein for immunization

The insoluble fraction of the histidine-tagged MCMV MDBP fusion protein expressed in *E. coli* BL21(DE3) was prepared by differential solubilization in 5 M urea. First, one milliliter of fresh bacterial culture containing pRSET-M57-transformed *E. coli* BL21(DE3) was prepared as described in section 2.18. The culture was inoculated into 50 ml of LB media containing 50 μg/ml of ampicillin in a 500-ml flask and grown at 37°C until an OD₆₀₀ of 0.6. The expression of the histidine-tagged MCMV MDBP fusion protein was induced by adding IPTG to a final concentration of 0.8 mM and the culture was incubated a further 4 hours at 37°C. The bacteria were pelleted and resuspended in 5 ml of lysis buffer supplemented with freshly prepared lysozyme (200 μg/ml) and 5 mM PMSF in a 15-ml Corning centrifuge tube (Corning Glass Work, Corning, NY). Cells were lysed at room temperature for 15 minutes on a rocking platform. To shear the *E. coli* chromosomal DNA, a Braunsonic model 1510 ultrasonic homogenizer with a 5-mm probe tip was used (B. Braun Instruments, San Francisco, CA). The cell suspension was subjected to six ultrasonic cycles of 300 Hertz, each cycle being 15 seconds in duration with 2 minutes cooling periods on ice between cycles. Then the lysates were transferred into microfuge tubes and the insoluble fraction was pelleted by centrifugation at 12,000 x g at 4°C for 15 minutes. The pellets were washed three times with 5 ml of 5 M urea in PBS at 4°C and rinsed twice in PBS. Finally, the insoluble proteins were suspended in 2 ml of PBS. Aliquots of 100 μl each were then dispensed into microfuge tubes and frozen at -70°C. The presence of MCMV MDBP in the preparations was verified by Western blotting using the MAb 4F11.21A and the purity of MCMV MDBP was assessed by Coomassie blue staining of the proteins separated by SDS-PAGE (section 2.8 and 2.9). The protein concentration of the antigen preparation

79
was estimated using a Bio-Rad DC protein assay kit according to the manufacturer's instructions.

2.20 Preparation of the histidine-tagged MCMV MDBP fusion protein for enzyme-linked immunosorbent assay

The histidine-tagged MCMV MDBP fusion protein was used as antigen for enzyme-linked immunosorbent assay (ELISA). For this purpose, the insoluble fraction of the bacterial pellet prepared as described in section 2.19 was washed once with 0.5 M urea and once with 1.5 M urea, each in 500 μl of washing buffer. Then the pellet was extracted with 3 M urea in 400 μl of washing buffer on ice for 10 minutes. The solubilized fraction was centrifuged at 12,000 × g at 4°C for 15 minutes and the supernatant containing the histidine-tagged MCMV MDBP fusion protein was collected. Finally, aliquots of 50 μl each were dispensed into microfuge tubes and stored at -70°C. The histidine-tagged MCMV MDBP fusion protein in the antigen preparation was identified by Western blotting using the MAb 4F11.21A and the purity of the histidine-tagged MCMV MDBP fusion protein extracted was assessed by Coomassie blue staining of the proteins separated by SDS-PAGE. Control antigens were also prepared by extracting the crude insoluble fraction from E. coli BL21(DE3) transformed with the cloning vector pRSETB in a similar manner. Protein concentrations of the antigen preparations were determined using a Bio-Rad DC protein assay kit according to the manufacturer's instructions.

2.21 Transient expression of MCMV MDBP and its mutant proteins in COS-1 cells

To express MCMV MDBP and its mutant proteins in COS-1 cells, plasmid DNA prepared by PEG precipitation or CsCl-ethidium bromide gradients (section 2.14.7) was
transfected into COS-1 cells by electroporation with the Gene Pulser apparatus (Bio-Rad) as previously described (Loh et al., 1994). COS-1 cells grown to about 90% confluence in Nunclon 100-mm culture dishes (GIBCO/BRL) were split 1:2 and incubated at 37°C for 24 hours. Cells were then trypsinized, suspended in DMEM containing 10% FBS and 1.6 μg/ml gentamycin, and counted. After centrifugation at 900 x g at room temperature for 5 minutes in a GLC-2B General Laboratory Centrifuge (Dupont Instruments), cells were washed once in 10 ml of N-2-Hydroxyethylpiperazine-N'2-ethanesulfonic acid buffered saline (HeBS) and finally resuspended in HeBS at a concentration of 3.2x10^6 cells/ml. For electroporation, 0.65 ml of the cell suspension was transferred to a sterile 0.4-cm cuvette (Bio-Rad) and 15 μg of DNA dissolved in 10 to 40 μl of HeBS were added. After incubation at room temperature for a further 10 minutes, the sample was electroporated at voltage and capacitance settings of 250 V and 500 μF respectively. The electroporated cells were incubated at room temperature for 10 minutes and resuspended at a concentration of 7x10^4 cells/ml in DMEM supplemented with 10% FBS and 1.6 μg/ml gentamycin. The cells were seeded onto 18x18 mm coverslips (Canlab Baxter) or in Nunclon 100-mm culture dishes, and incubated at 37°C. Twenty-four hours later, the medium was replaced with fresh DMEM supplemented with 10% FBS and 1.6 μg/ml gentamycin and cultured for another 24 hours. The cells were then harvested. The expression of the wild-type and mutant MCMV MDBP were monitored by Western blotting (section 2.9) and the subcellular location of the proteins was examined by indirect immunofluorescence (sections 2.11).

2.22 Transient expression of MCMV MDBP in CV-1 cells

CV-1 cells expressing recombinant MCMV MDBP were used for screening the tissue culture supernatants from hybridoma cell lines by indirect immunofluorescence.
For transient expression of MCMV MDBP, 15 µg of pSL-M57 DNA was transfected into CV-1 cells by electroporation as described in section 2.21. The transfected cells were seeded in Nunclon 100-mm culture dishes to allow the cells to attach. After incubation in DMEM supplemented with 10% FBS at 37°C for 4 hours, the cells were infected with 4 ml of wild-type vaccinia virus at an m.o.i. of 2 PFU/cell. Virus was adsorbed onto the cells at 37°C for 1 hour. The cell monolayers were then washed three times with GKNP balanced salt solution to remove unbound virus. The infected cells were incubated in DMEM supplemented with 2% FBS at 37°C for 20 hours and processed for immunofluorescence as described in section 2.11.

2.23 Production of MAbs against MCMV MDBP

2.23.1 Immunization

Four female Balb/c mice (6-8 weeks of age), purchased from Jackson Laboratory, Bar Harbour, Maine, were used for immunization. The mice were bred at the Animal Facility of the Department of Microbiology at the University of Saskatchewan and housed in accordance with the University of Saskatchewan Animal Care Committee Protocol #94-0046.

The insoluble fraction of the histidine-tagged MCMV MDBP fusion protein prepared in section 2.19 was emulsified with an equal volume of Freund's complete adjuvant (GIBCO/BRL) between two glass syringes via a two-way valve. The mice were each injected intraperitoneally with 500 µl of the emulsified antigen (total of 50 µg of antigen). Two weeks later, each mouse was boosted with 25 µg of antigen emulsified with 250 µl of Freund's incomplete adjuvant (GIBCO/BRL). Ten days after the boost, mice were test-bleed and the reactivity of the sera with MCMV MDBP was tested by
Western blotting. The mice were boosted four more times at three-week intervals and then rested for about one month. Four days prior to the fusion, the mouse was injected intraperitoneally with 25 μg of antigen in 200 μl of sterile PBS three times at one-day intervals.

2.23.2 Preparation of FO myeloma cells

About one week before the date of fusion, one vial of FO cells was removed from storage in liquid nitrogen, rapidly thawed in a 37°C water bath, and transferred to a tissue culture tube containing 10 ml of pre-warmed DMEM supplemented with 10% FBS. The cells were pelleted by centrifugation at 900 x g for 5 minutes (GLC-2B General Laboratory Centrifuge, Dupont Instruments), gently resuspended in 10 ml of fresh medium, and cultured in a Nunclon 100-mm culture dish. The next day, the medium was removed and replaced with fresh medium containing 20 μg/ml of 8-azaguanine. In the next 4 days, the cells were grown and split (1:2 or 1:3) in the presence of the drug to eliminate the hypoxanthine-guanine phosphoribosyl transferase gene revertants (i.e. HPRT+ cells). Two days before the fusion, the medium was replaced with fresh DMEM supplemented with 10% FBS. One day before the fusion, cells were split 1:4 and cultured in fresh medium. On the day of the fusion, cells were harvested and counted.

2.23.3 Preparation of feeder cells

Spleen cells from Balb/c mice were used as feeder cells during the initial cloning and subsequent subcloning of hybridoma cell lines. Feeder cells were generally prepared one day before they were required. Briefly, female Balb/c mice were sacrificed by cervical dislocation. Spleens were removed aseptically from the mice and each spleen was placed in a Nunclon 100-mm culture dish containing 5 ml of DMEM. The spleen was torn open
at one end and the cells were extruded into the medium. To make a single-cell suspension, the cell mixture was transferred to a sterile 5-ml syringe and pushed through a 26-gauge needle into a tissue culture tube. Five milliliters of DMEM were added to the above tube and the cells were pelleted at 900 x g for 5 minutes at room temperature. The red blood cells were lysed in 10 ml of a 0.167 M NH₄Cl solution (in ddH₂O) at room temperature for exactly 3 minutes. The remaining spleen cells were pelleted at 900 x g for exactly 4 minutes, washed twice with DMEM, and resuspended in an appropriate volume of hybridoma medium. Two drops of cell suspension were added to each well of the Nunclon 96-well culture plates. The plates were incubated in a humidified NAPCO Incubator at 37°C with 5% CO₂ and 95% air overnight. Typically, cells from each spleen were resuspended in 25 ml of media, providing enough feeder cells for two 96-well plates.

2.23.4 Fusion and feeding of hybridomas

On the day of fusion, the mouse which had the highest antibody titer in test bleedings was sacrificed by cervical dislocation. The spleen, usually 2 to 3 times normal size, was removed aseptically and placed in a Nunclon 100-mm culture dish containing 5 ml of DMEM. Spleen cells were prepared as described in the previous section and resuspended in 5 ml of DMEM. The total number of viable spleen cells was counted in a hemocytometer.

Next, FO myeloma cells were harvested, washed twice with DMEM, and counted. A total of 1.54 X 10⁷ FO cells were pelleted and resuspended in 5 ml of DMEM. Then, the FO cell suspension was mixed with an equal volume of spleen cells in a 50-ml Falcon centrifuge tube at a ratio of one FO cell per five spleen cells. The mixture was pelleted by centrifugation at 900 x g for 5 minutes at room temperature. The medium
was removed completely and the pellet was dislodged by tapping the tube. The uncapped tube was held in a beaker containing water at 37°C and 1 ml of prewarmed (37°C) DMEM containing 50% (w/v) PEG 1500 (Boehringer Mannheim, Germany) was added dropwise over a period of 2 minutes using a 5-ml pipette. A further 20 ml of prewarmed (37°C) DMEM was added over a period of 4 minutes with gentle shaking. The cells were pelleted by centrifugation at 900 x g for 5 minutes, resuspended gently in 15 ml of hybridoma medium, and cultured in a Nunclon 100-mm culture dish at 37°C. After 5 hours of incubation, the cells were mixed with 75 ml of hybridoma medium containing an additional 5% FBS and 3 x HAT (hypoxanthine, aminopterin, and thymidine; Gibco/BRL). One drop of the resulting cell suspension was added per well (using a 10-ml pipette) to 96-well culture plates containing feeder cells. The plates were incubated at 37°C.

Five days after the fusion, half of the plates were fed by adding one drop per well of hybridoma medium containing 2 x HAT using a 10-ml pipette and the remaining plates were fed the next day in a same manner. Eight days after the fusion, about one third of the medium was removed from each well of half of the plates by aspiration using a pasteur pipette. Then each well was replenished with one drop of hybridoma medium containing 1 x HAT using a 10-ml pipette. The procedure was repeated with the remaining plates on the following day.

Beginning with the ninth day after the fusion, the plates were checked daily for the presence of colonies of cells. When the colonies were about 2-3 mm in diameter, 125 µl of medium from each well was transferred to a sterile microfuge tube and stored at 4°C for screening by ELISA, indirect immunofluorescence and Western blotting (section 2.23.5). The well was replenished with fresh medium containing 1x HAT.
2.23.5 Screening for hybridoma cell lines secreting monoclonal antibodies specific for MCMV MDBP

Tissue culture supernatants from hybridoma lines were screened sequentially by ELISA, indirect immunofluorescence and Western blotting for the presence of MAbs specific for MCMV MDBP. A three-layer antibody capture enzyme-linked immunosorbent assay (ELISA) was developed for initial screening. The histidine-tagged MCMV MDBP fusion protein or control antigen prepared in section 2.20 was diluted to a concentration of 10 µg/ml with wash buffer supplemented with 3 M urea. An aliquot of 50 µl of antigen was added to each well of a 96-well Polysorp plate (Nunc, Denmark) and incubated at 4°C overnight. Unabsorbed antigen was removed and each well was rinsed twice with 200 µl of PBS. Then the wells were blocked with 100 µl of a 1% BSA solution in TBS at room temperature for 1 hour. The contents of the wells were removed by inverting the plate and tapping out the remaining fluid onto paper towels. Fifty microliters of tissue culture supernatant collected from hybridoma cells were added to each well and the plate was incubated at room temperature for 2 hours. Each well was washed three times with 200 µl of TBST supplemented with 0.1% BSA. Then 100 µl of AP-conjugated goat anti-mouse IgG (1:3,000 dilution in TBST containing 0.1% BSA) were added. After incubation at room temperature for 1 hour, the wells were washed and 100 µl of AP substrate solution (Bio-Rad) were added to each well. The color reaction was allowed to proceed at room temperature for 3 hours in the dark. Quantitation of the color reaction was done by reading the plates in a Bio-Rad model 2550 ELISA reader (Bio-Rad) at a wavelength of 405 nm. For each antibody sample, the ratio of absorbance readings obtained from wells coated with the histidine-tagged MCMV MDBP fusion protein and those coated with control antigen was used as a measure of the presence of
MCMV MDBP-specific antibody in the tissue culture supernatant. A value of 1.5 or more was considered to be positive.

Subsequently, indirect immunofluorescence was carried out as described in section 2.10 to test the ELISA-positive clones. Finally, positive clones identified in both ELISA and indirect immunofluorescence were further evaluated by Western blotting using a Miniblotter System (Immunetics, Cambridge, MA; Section 2.9).

2.23.6 Handling of hybridomas in 24-well plates

The hybridomas secreting MCMV MDBP-specific MAbs were maintained in Nunclon 96-well culture plates until the cells expanded to occupy roughly two-thirds of the well. At this point, the cells were removed by gently pipetting the contents of the well up and down with a plugged pasteur pipette, and transferred to a well containing 1 ml of feeder cells prepared the day before in a Nunclon 24-well culture plate (Nunc, Denmark). Two days after the clone was transferred, 1 ml of hybridoma medium supplemented with 1 x HT (hypoxanthine/thymidine) was added to the well. When the well was confluent, the cells were split 1:2 by transferring half of the cells to a second well. About 1 ml of supernatant was collected from each confluent well for use in indirect immunofluorescence and Western blotting. Cells from each well were pelleted, resuspended in 1 ml of DMEM supplemented with 20% FBS and 10% (v/v) DMSO, and stored in liquid nitrogen in a 2-ml freezing vial.

2.23.7 Subcloning of hybridoma cells by limiting dilution cloning

Hybridomas were subcloned by limiting dilution. Cells from one well of a Nunclon 24-well culture plate were harvested and resuspended in 0.5 ml of hybridoma medium. The cells were counted, diluted to a concentration of 1x10^6 cells per ml, and
0.1 ml of the cell suspension (1x10^5 cells) was transferred to a 10-ml tube (dilution tube #1) containing 9.9 ml of DMEM. Then 0.1 ml of cells from tube #1 was transferred to another tube containing 9.9 ml of DMEM (dilution tube #2). Finally, 1.5 ml of cells from tube #2 were transferred to a tube containing 13.5 ml of hybridoma medium (dilution tube #3). Two drops of the cell suspension from tube #3 were added to each well of a Nunclon 96-well culture plate containing feeder cells prepared one day before subcloning. After 7 to 14 days, supernatants from subclones were tested for the presence of MAbs by indirect immunofluorescence and Western blotting as described in sections 2.9 and 2.10. Tissue culture supernatants containing MAbs specific for MCMV MDBP were stored at -20°C.

2.23.8 Collection of ascitic fluids

Female Balb/c mice were primed with 300 µl of Freund’s incomplete adjuvant intraperitoneally. Twenty-four hours later, the mouse was injected intraperitoneally with 4x10^5 hybridoma cells using a 23-gauge needle attached to an 1-ml syringe. Cells from each hybridoma line were injected into a group of three mice. Ten days after injection, the abdomens of the mice became visibly distended. Ascitic fluid was drained by inserting an 18-gauge needle directly into the abdomen and fluid was collected in a conical 15-ml centrifuge tube. Each mouse was drained twice during a two-day period and sacrificed by cervical dislocation. The collected fluid was clarified by centrifugation at 900 x g at room temperature for 10 minutes. The supernatant was transferred to a 50 ml Falcon centrifuge tube and stored at 4°C overnight. The ascitic fluid was again centrifuged at 900 x g at room temperature for 10 minutes, dispensed into microfuge tubes in 500 µl aliquots, and frozen at -20°C. In general, tissue culture supernatants were used for indirect immunofluorescence and ascitic fluids were used at 1:2,000 to 1:4,000 dilution for Western blotting.
2.23.9 Determination of isotypes of monoclonal antibodies

The isotype of each MAb was determined using the mouse typer isotyping kit (Bio-Rad) according to manufacturer's instructions. Fifty microliters of antigen preparations (10 μg/ml) containing the histidine-tagged MCMV MDBP fusion protein were coated onto the wells of Immuno 96-well Polysorp plates (Nunc) as described in section 2.22.5. Unbound antigen was removed and the wells were rinsed twice with 200 μl of PBS and incubated with 1% BSA in TBS at room temperature for 1 hour. The contents of the wells were removed and 50 μl of tissue culture supernatant from each hybridoma were added and the plate was incubated at room temperature for 2 hours. Then each well was washed three times with 200 μl of TBST supplemented with 0.1% BSA. Fifty microliters of the undiluted rabbit anti-mouse subclass specific anti-serum (Bio-Rad) were added to each well. The plate was incubated at room temperature for 1 hour and washed five times with TBST supplemented with 0.1% BSA. Fifty microliters of horseradish peroxidase-conjugated, affinity purified goat anti-rabbit IgG (1:3000 dilution in TBST containing 0.1% BSA) were added to each well and the plate was incubated at room temperature for 1 hour. After the plate was washed three times with TBST supplemented with 0.1% BSA, the color reaction was initiated by adding 100 μl of peroxidase substrate solution to each well and the plate was incubated at room temperature for 20 minutes. Quantitation of color was done by reading the plate in a Bio-Rad model 2550 ELISA reader at a wavelength of 405 nm. Wells were considered positive if the value of absorbance was at least three times that of the negative control.

2.24 In vitro transcription and translation

Using a TNT T7-coupled reticulocyte lysate system (Thompson et al., 1992; Promega, Madison, WI), DNA templates of pRSET-M57 and its deletion mutants were
transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate according to manufacturer's instructions. Briefly, one microgram of plasmid DNA was incubated in a reaction mixture containing rabbit reticulocyte lysate, reaction buffer, T7 RNA polymerase, an amino acid mixture minus methionine, RNAase inhibitor, and 40 to 50 μCi of [35S]methionine for 90 minutes at 30°C. Then the reaction mixture was frozen at -20°C until use.

### 2.25 DNA-cellulose chromatography

DNA-cellulose chromatography was performed by the method described by Loh et al. (1994). To determine the ssDNA-binding property of MCMV MDBP in infected cells, proteins extracted from MCMV-infected Balb/3T3 cells radiolabeled with [35S]methionine (100 μCi/ml) were applied to a Poly-Prep Chromatography Column (Bio-Rad) containing 2 ml of ssDNA cellulose or unmodified cellulose (Sigma) pre-equilibrated in binding buffer supplemented with 50 mM NaCl. The unbound material was reapplied to the column once to maximize the specific binding of proteins to the column. The column was washed a total of four times with 2-bed volumes of 50 mM NaCl in binding buffer. Bound proteins were eluted stepwise with 3 x 2-bed volumes of binding buffer supplemented with increasing concentrations of NaCl (100 mM, 250 mM, 500 mM, 1 M, and 2 M) and 2 ml fractions were collected during the process. The column was then regenerated by washing with three bed-volumes of 2 M NaCl in binding buffer, ten bed-volumes of 50 mM NaCl in binding buffer, and kept at 4°C in binding buffer containing 50 mM NaCl and 0.02% sodium azide. Finally, MCMV MDBP was immunoprecipitated from each fraction by MAb 4F11.21A and analyzed by SDS-PAGE.

To analyze ssDNA-binding activities of deletion mutants, the *in vitro* translation mixture was treated with 0.4 mg/ml of RNAase at room temperature for 15 minutes and
an aliquot (7 μl) was then diluted into 100 μl of binding buffer supplemented with 50 mM NaCl. The sample was clarified by centrifugation at 12,000 × g at 4°C for 15 minutes and analyzed by ssDNA-cellulose column in similar fashion with the exception that 0.5 ml of ssDNA-cellulose or cellulose were used. The relative amounts of [35S]methionine-labeled proteins in each fraction were monitored by liquid scintillation counting as follows. An aliquot (5 μl) from each fraction was spotted onto Whatman GF/C glass microfibre filters (Whatman, Inc.), placed in a scintillation vial (Kimble Glass, Vineland, NJ), and dried. Two milliliters of Amersham PCS® scintillation fluid (Amersham Corporation, Arlington Heights, IL), were added to the vial and counts were measured in a Beckman Liquid Scintillation Counter Model LS5000 TD (Beckman Instruments, Fullerton, CA). Then 300 μl of [35S]methionine-labeled proteins in each fraction were precipitated with 2 volumes of acetone in the presence of 20 μg of BSA at -20°C overnight, solubilized in 35 μl of SDS-PAGE sample buffer, and separated on SDS-PAGE. The precipitated proteins were visualized by autoradiography.

2.26 Gel-filtration chromatography

2.26.1 Calibration of the Bio-Gel A-1.5M gel column

The Bio-Gel A-1.5M gel column was calibrated with gel filtration protein standards (Bio-Rad) consisting of bovine thyroglobulin (670 K), bovine gamma globulin (158 K), chicken ovalbumin (44 K), horse myoglobin (17 K), and vitamin B-12 (1.35 K). One vial of molecular weight standards, containing about 18 mg of protein, was rehydrated by adding 0.5 ml of ddH2O. A 0.25 ml aliquot of the resulting protein solution was diluted at 1:1 with TBS supplemented with 0.1% (v/v) NP-40 and 0.02% (w/v) sodium azide and clarified by centrifugation at 12,000 × g for 5 minutes at 4°C. The
supernatant was removed (total of 0.5 ml) and loaded onto a prepacked Bio-Gel A-1.5M gel column (1 x 120 cm; fractionation range <10 to 1,500 kd) equilibrated at 4°C with TBS supplemented with 0.1% NP-40 and 0.02% sodium azide. Column chromatography was carried out at 4°C. Fractions of 130 drops each (about 1.6 ml) were collected with a GILSON Model FC-80K Fractionator (Gilson medical electronics, Inc., Middleton, WI). A 100-μl aliquot from each fraction was precipitated with three volumes of acetone at -20°C for 1 hour and the protein pellet was dissolved in 20 μl of ddH2O. To determine the elution profile of the standards, the protein concentration of each fraction was measured using a Bio-Rad DC protein assay kit according to the manufacturer's instructions and the presence of the gel filtration protein standards was verified by Coomassie blue staining of the proteins separated by SDS-PAGE. A graph was drawn by plotting the fraction number on the X axis and logarithm of the molecular mass on the Y axis. From this graph, the molecular masses of the eluted proteins were extrapolated.

2.26.2 Separation of MCMV-infected cell lysate

Murine cytomegalovirus-infected cell extract from a Nunclon 100-mm culture dish was prepared as described in section 2.7 and diluted with TBS to bring the final NP-40 concentration to 0.1%. One milliliter of the diluted sample was applied to and fractionated on a prepacked Bio-Gel A-1.5M column and gel filtration column chromatography was carried out at 4°C as described in section 2.26.1. Proteins in an 800-μl aliquot from each fraction were precipitated in two volumes of acetone in the presence of 40 μg of BSA at -20°C for 1 hour and pelleted at 12,000 x g for 20 minutes in a microfuge at 4°C. Pellets were solubilized in SDS-PAGE sample buffer and the elution profile of MCMV MDBP was determined by Western blotting using MAb 4F11.21A. Similarly, [35S]methionine labeled MCMV-infected cell extracts prepared in sections 2.6
and 2.7 were fractionated on a Bio-Gel A-1.5M gel column. Proteins in each fraction were immunoprecipitated by MAb 4F11.21A and analyzed by SDS-PAGE and autoradiography as described in section 2.10.
3.0 RESULTS

3.1 Expression of the histidine-tagged MCMV MDBP fusion protein gene in *E. coli*

To obtain large quantities of MCMV MDBP for use as antigen to produce MAbs, I expressed the histidine-tagged MCMV MDBP fusion protein gene in *E. coli* strain BL21(DE3). The entire M57 ORF encoding MCMV MDBP was cloned into the prokaryotic expression vector pRSETB. The resultant plasmid pRSET-M57 encodes a fusion protein consisting of MDBP and hexahistidyl residues at the N-terminus of the protein (Figure 2.3B). *Escherichia coli* strain BL21(DE3) carrying this plasmid was tested for the expression of the fusion protein after induction with IPTG. Figure 3.1A shows Coomassie blue staining of proteins from whole-cell lysates after separation by 7.5% SDS-PAGE. As seen, when BL21(DE3) carrying plasmid pRSET-M57 was induced with 0.8 mM IPTG for four hours, a major protein band with an apparent molecular weight of 137 K was revealed (Figure 3.1A, lane 2). The size of the protein was in good correlation with the predicted molecular weight of the histidine-tagged MCMV MDBP fusion protein (136 K), but slightly larger than that of MDBP expressed in MCMV-infected Balb/3T3 cells (133 K; Loh et al., 1991). The protein was not found in BL21(DE3) (Figure 3.1A, lanes 3 and 4), BL21(DE3) [pRSETB] (Figure 3.1A, lanes 5 and 6) and uninduced BL21(DE3) [pRSET-M57] (Figure 3.1A, lane 1). These results suggested that the protein was a specific gene product expressed from BL21(DE3) [pRSET-M57] after IPTG induction.
Figure 3.1. A. Expression of the histidine-tagged MCMV MDBP fusion protein in *E. coli*. Two different concentrations of SDS-PAGE were used to analyze the histidine-tagged MCMV MDBP fusion protein expressed in *E. coli* strain BL21(DE3) (Lanes 1 to 6, 7.5% SDS-PAGE; Lanes 7 and 8, 5% SDS-PAGE). Except where noted, whole-cell lysates representing 0.1 ml of bacterial culture at an optical density at 600 nm equal to 1.0 were analyzed by 7.5% SDS-PAGE and visualized by staining with Coomassie blue. Lane 1, BL21(DE3) [pRSET-M57] without IPTG induction; Lane 2, BL21(DE3) [pRSET-M57] with IPTG induction; Lane 3, BL21(DE3) lysate; Lane 4, BL21(DE3) lysate prepared from the bacteria culture in the presence of IPTG; Lane 5, BL21(DE3) [pRSETB] without IPTG induction; Lane 6, BL21(DE3) [pRSETB] with IPTG induction; Lane 7, 2 ml of resuspended insoluble fraction of the histidine-tagged MCMV MDBP fusion protein; Lane 8, 2 ml of resuspended insoluble fraction of the histidine-tagged MCMV MDBP fusion protein after being washed with 5.0 M urea. The numbers on the left represent sizes of molecular weight markers.

B. Western blotting analysis of the histidine-tagged MCMV MDBP fusion protein expressed in BL21(DE3). Whole-cell lysates representing 5 µl of bacteria at an optical density at 600 nm equal to 1.0 were separated by 7.5% and probed by Western blotting with monoclonal antibody 4F11.21A. Lane 1, BL21(DE3) lysate; Lane 2, BL21(DE3) lysate prepared from the bacteria culture in the presence of IPTG; Lane 3, BL21(DE3) [pRSETB] without IPTG induction; Lane 4, BL21(DE3) [pRSETB] with IPTG induction; Lane 5, BL21(DE3) [pRSET-M57] without IPTG induction; Lane 6, BL21(DE3) [pRSET-M57] with IPTG induction. The numbers on the left represent sizes of molecular weight markers.
To determine whether the expressed protein contained MCMV MDBP, whole-cell lysates were analyzed by Western blotting with MAb 4F11.21A specific for MDBP (Loh et al., 1991). Figure 3.1B shows that a 137 K protein was detected by the MAb in the lysates from IPTG-induced BL21(DE3) [pRSET-M57] (Figure 3.1B, lane 6), but not in the lysates from the uninduced bacteria (Figure 3.1B, lane 5). Additionally, no protein band was found in BL21(DE3) or BL21(DE3) [pRSETB] in the absence or presence of 0.8 mM IPTG (Figure 3.1B, lanes 1, 2, 3, and 4). Therefore, these data confirmed that the histidine-tagged MCMV MDBP was produced in pRSET-M57-transformed BL21(DE3) after IPTG induction.

The expression of foreign proteins in E. coli often results in generation of inclusion bodies, suggesting insoluble protein aggregates which are accumulated in the cytoplasm of bacteria (Schein, 1989). It is not clear what causes the formation of these structures. Rudolph and Lilie (1996) proposed that overproduction of foreign proteins might exhaust the bacterial chaperone machinery, resulting in protein misfolding and precipitation. The formation of inclusion bodies may be advantageous to protein purification (Schoner et al., 1985). Having established that the histidine-tagged MCMV MDBP fusion protein was produced in E. coli, I next investigated whether the protein was also present in inclusion bodies. To do this, the insoluble fraction of whole-cell lysates from BL21(DE3) [pRSET-M57] after IPTG induction was prepared as described in section 2.19. Proteins in the insoluble fraction were separated by 5% SDS-PAGE and stained by Coomassie blue. As seen in Figure 3.1A, the histidine-tagged MCMV MDBP fusion protein existed as a predominant component in insoluble fraction (Figure 3.1A, lane 7), indicating that the protein was likely misfolded and aggregated to form inclusion bodies after overproduction in E. coli. The insoluble fraction which contained large amounts of the histidine-tagged MCMV MDBP fusion protein was washed with 5.0 M
urea. After this washing procedure, the majority of the aggregated histidine-tagged MCMV MDBP fusion protein still remained in the insoluble fraction (Figure 3.1A, lane 8).

3.2 Isolation of monoclonal antibodies specific for MCMV MDBP

The availability of only one MAb 4F11.21A specific for an epitope within the C-terminus of MCMV MDBP prompted us to make MAbs which could recognize additional epitopes on the protein. This would facilitate the analysis of functional domains of MDBP. To produce these MAbs, Balb/c mice were immunized with the insoluble histidine-tagged MCMV MDBP fusion protein that had been washed with 5.0 M urea. Hybridomas were produced as described in section 2.23. Supernatants collected from hybridoma cultures were first screened by ELISA in 96-well plates coated with the histidine-tagged MCMV MDBP fusion proteins. These soluble proteins were prepared by extracting the insoluble fraction of whole-cell lysates from IPTG-induced BL21(DE3) [pRSET-M57] with 3 M urea. The lower concentration of urea was used in an attempt to minimize possible effects of urea in ELISA. To exclude hybridomas secreting antibodies against E. coli proteins, proteins extracted from BL21(DE3) [pRSETB] were also included during initial ELISA screening. About 680 clones were tested and 60 were positive. The positive clones were screened by indirect immunofluorescence using CV-1 cells expressing recombinant MCMV MDBP. Forty-six clones remained positive after this procedure. Western blotting was then used to test whether these clones were capable of production of MDBP-specific antibodies. A total of 22 clones were positive. Subcloning these clones by limiting dilution cloning finally yielded 21 hybridomas secreting MAbs against MDBP.
To produce large quantities of MAbs, hybridomas were injected into the peritoneal cavities of Balb/c mice and ascitic fluids were collected. I injected 21 hybridoma cell lines. Of these, 16 induced the production of ascitic fluids, while five formed solid tumors that failed to generate ascitic fluids. Western blotting was performed to test the presence of MDBP-specific antibodies in ascitic fluids. Of the 16 samples tested, eight were positive at 1:3000 dilution, one was positive at 1:1000 dilution, and the rest were negative. The eight MAbs which showed a strong reaction (1:3,000 dilution) in Western blotting were designated as 1F1.12B, 3C2.11B, 5F7.11B, 7A4.11B, 10H10.11B, 2H12.11B, 6C11.11B, and 8B12.11B and were further characterized. The immunoglobulin (Ig) classes and subclasses of these eight MAbs were determined by ELISA using standard mouse monoclonal typing reagents. As shown in Table 3.1, all MAbs were of the IgG class with κ-type light chains. In addition, the MAbs covered a wide spectrum of IgG subclasses, showing a great diversity of immune responses against the histidine-tagged MCMV MDBP fusion protein.
Table 3.1 Subclasses of monoclonal antibodies

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<th>10H10.11B</th>
<th>2H12.11B</th>
<th>6C11.11B</th>
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3.3 Mapping of continuous epitopes on MCMV MDP

To determine the epitopes on MCMV MDP recognized by MAbs, I constructed a library of N-terminal, C-terminal, or in-frame internal deletion mutants by using convenient restriction enzyme sites within the M57 ORF encoding MDP (section 2.16). The mutant proteins were expressed in *E. coli* strain BL21(DE3) and analyzed by Western blotting with the eight MAbs described in section 3.2 and MAb 4F11.21A previously made in this laboratory. The molecular weights of the expressed mutant proteins were determined from Western blotting in the presence of molecular weight markers. As seen in Table 3.2, mutant proteins with apparent sizes that were consistent with the predicted molecular weights were expressed in bacteria harboring the plasmid listed on the left. The results for the epitope-mapping experiments are summarized in Figure 3.2. Representative Western blotting experiments are shown in Figures 3.3 and 3.4. Monoclonal antibody 1F1.12B reacted with the truncated MDP proteins possessing the N-terminal 625, 313, 254, and 216 residues respectively (Figure 3.3A, lanes 1, 2, 3, and 4), while it failed to detect a mutant protein of MDP with an internal deletion between amino acid residues 55 and 686 (Figure 3.3A, lane 5). In addition, MAb 1F1.12B was found to be reactive with a truncated protein containing amino acid residues 54 to 292 (Figure 3.2A; pRSET-M57dN54dC292). Taken together, these data locate the 1F1.12B epitope to an area between residues 54 to 216. The epitope for MAb 5F7.11B was mapped using similar analysis and it detected all the fragments containing the N-terminal 625, 313, and 254 residues, respectively (Figure 3.3B, lanes 1, 2, and 3), but not the one consisting of residues 1 to 216 (Figure 3.3B, lane 4). Monoclonal antibody 5F7.11B reacted with a protein containing residues 54 to 292 of MDP (Figure 3.2A; pRSET-M57dN54dC292), but failed to detect a mutant protein lacking amino acid residues 55 to 686 (Figure 3.3B, lane 5).
a Refer to Figure 2.4 for the construction of deletion mutants.

b Numbers represent residues of the amino acid sequence of MCMV MDBP. All the mutant proteins were expressed as the fusion protein containing a leader peptide at the N-terminus. The calculated size of the peptide ranges from 3.5 to 4.7 K based upon the cloning sites used for fusion. Additional amino acid residues resulting from the presence of the linker oligonucleotides are shown in single letter code.

c Determined from Western blotting in the presence of molecular weight markers.
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<th>Apparent molecular weight (K) &lt;sup&gt;c&lt;/sup&gt;</th>
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<sup>a</sup> Indicates the plasmid used for expression.  
<sup>b</sup> Represents the amino acid sequence truncated at the specified positions.  
<sup>c</sup> Obtained from gel analysis.
Figure 3.2. Mapping of epitopes by analysis of deletion mutants derived from the prokaryotic expression plasmid pRSET-M57. Mapping results for epitopes near the N- (A) and C-termini (B) are shown. Top line represents a sequence ruler with tick marks every 200 amino acid residues. The hollow bar below the ruler indicates the amino acid sequence of MCMV MDBP. The names of the deletion mutants are shown in the left column. Expressed portions of MCMV MDBP are depicted as black bars with an extra leader sequence attached in the N-terminus of the protein (small hatched box). Numbers above black bars indicate the first or last M57-specific residue which is present. Names of monoclonal antibodies and reactivities of these monoclonal antibodies against the truncated proteins in Western blotting are tabulated on the right. The "+" and "-" signs represent positive and negative reactivities respectively.
<table>
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Figure 3.3. Mapping of epitopes in the N-terminus of MCMV MDBP. Truncated proteins expressed from *E. coli* strain BL21(DE3) were separated by 12% SDS-PAGE and probed by Western blotting with monoclonal antibody 1F1.12B (panel A) or 5F7.11B (panel B). Lane 1, pRSET-M57dC625 (aa 1 to 625); Lane 2, pRSET-M57dC313 (aa 1 to 313); Lane 3, pRSET-M57dC254 (aa 1 to 254); lane 4, pRSET-M57dC216 (aa 1 to 216); and lane 5, pRSET-M57d55-686 (aa 1 to 54 and 687 to 1191). The numbers on the left represent sizes of molecular weight markers.
Therefore, these results suggested that MAb 5F7.11B was specific for an epitope located within the region containing residues 54 to 254.

Epitopes in the C-terminal half of MDBP were determined by the analysis of series of N-terminal truncated proteins. Western blotting results revealed that the C-terminal 128 residues were the minimal length of fragment with which MAb 4F11.21A reacted (Figure 3.4A, lane 6), suggesting that the epitope for 4F11.21A was located within residues 1063 to 1191. While MAb 10H10.11B detected a mutant protein possessing the C-terminal 136 amino acids (Figure 3.4B, lane 5), it failed to react with a truncated protein containing only the 128 residues in the C-terminus (Figure 3.4B, lane 6). Therefore, the region encompassing residues 1055 to 1191 contains the 10H10.11B epitope. Similarly, the reaction profile of MAb 2H12.11B in Western blotting (Figure 3.4C) suggested that the epitope is present within residues 1001 to 1191.

In summary, at least five epitopes recognized by these nine MAbs were distributed in two different regions located near the N- and C-termini of MDBP. These five potential epitopes were localized to residues 54 to 216 (1F1.12B), 54 to 254 (5F7.11B and 3C2.11B), 1001 to 1191 (2H12.11B), 1055 to 1191 (7A4.11B and 10H10.11B), and 1063 to 1191 (4F11.21A, 6C11.11B, and 8B12.11B) (Figure 3.5). None of my MAbs recognized epitopes in the large mid-portion of MDBP encompassing the residues 255 to 1000.
Figure 3.4. Mapping of epitopes in the C-terminus of MCMV MDBP. Truncated proteins expressed from *E. coli* strain BL21(DE3) were separated by 12% SDS-PAGE and probed by Western blotting with monoclonal antibody 4F11.21A (panel A), 10H10.11B (panel B) or 2H12.11B (panel C). Lane 1, pRSET-M57dN585 (aa 585 to 1191); Lane 2, pRSET-M57dN625 (aa 625 to 1191); Lane 3, pRSET-M57dN749 (aa 749 to 1191); Lane 4, pRSET-M57dN1001 (aa 1001 to 1191); Lane 5, pRSET-M57dN1055 (aa 1055 to 1191); and lane 6, pRSET-M57dN1063 (aa 1063 to 1191). The numbers on the left represent sizes of molecular weight markers.
Figure 3.5. Distribution of epitopes on MCMV MDBP. The top line indicates a sequence ruler with tick markers every 200 residues. The hollow bar below the ruler indicates the amino acid sequence of MCMV MDBP. The regions containing epitopes recognized by MAbs are indicated as black bars. The essential region required for reactivity with MAbs is shown as the hatched bar. Numbers shown above the bars represent amino acid residues. Names of monoclonal antibodies which recognize each region are shown under each bar.
3.4 Murine cytomegalovirus MDBP is an early protein

Messerle and coworkers (Messerle et al., 1992) have previously demonstrated that a 4.2-kb mRNA encoding MCMV MDBP is detectable as early as two hours post-infection, persists throughout the replication cycle, and accumulates in the presence of the viral DNA synthesis inhibitor phosphonoacetic acid. To determine the time course of expression of MDBP in MCMV-infected cells, the presence of MDBP in virus-infected cells was monitored by Western blotting. Balb/3T3 cells were infected with the Smith strain (Vancouver) of MCMV. At different times post-infection, the cells were collected and whole-cell lysates were prepared. Proteins extracted from infected cells were separated by SDS-PAGE and MDBP was detected by Western blotting with MAb 4F11.21A. The MDBP was detected with MAb 4F11.21A four hours post-infection, although the reactive band was too faint to be reproduced in Figure 3.6A. However, six hours post-infection the 133 K band is clearly visible and continues to increase in intensity throughout the duration of the experiment. Murine cytomegalovirus DNA replication usually does not commence until eight hours post-infection (Keil et al., 1984). Thus the time of appearance of the MDBP confirmed its classification as an early protein.

3.5 Murine cytomegalovirus MDBP binds ssDNA.

I set out to define whether MCMV MDBP was capable of binding ssDNA because this property is essential for the overall function of ssDNA-binding proteins (Kornberg and Baker, 1991b). To examine the ssDNA-binding property of the viral protein, Balb/3T3 cells infected with MCMV were radiolabeled with [35S]methionine for 16 hours beginning at 10 hours post-infection. Infected cell proteins were extracted and
Figure 3.6. A. Time courses of expression of MDBP in MCMV-infected cells. Total proteins extracted from mock-infected (M) or MCMV infected Balb/3T3 cells at different time points after infection were analyzed by 5% SDS-PAGE and the major DNA-binding protein was detected by Western blotting with monoclonal antibody 4F11.21A. The numbers on the left represent sizes of molecular weight markers.

B. MCMV MDBP is a ssDNA-binding protein. Proteins extracted from MCMV-infected Balb/3T3 cells radiolabeled with [35S] methionine (100 μCi/ml) were applied to ssDNA-cellulose or cellulose control columns (2 ml bed volume per column), washed with buffer containing 50 mM NaCl, and eluted in a stepwise fashion with buffers containing 100 mM, 250 mM, 500 mM, and 1 M NaCl as shown above the lanes. The eluate from the first and last fractions of each elution step was immunoprecipitated with MAb 4F11.21A and analyzed for protein content by SDS-PAGE and autoradiography.
applied to a ssDNA-cellulose column or an unmodified cellulose column. The column was washed with buffer containing 50 mM NaCl and proteins were eluted in a stepwise fashion with 3x 2-bed volumes of binding buffer containing different salt concentrations (100 mM, 250 mM, 500 mM, and 1 M). The eluate from the first and last fractions of each elution step was collected and immunoprecipitated with MAb 4F11.21A. The protein was analyzed by autoradiography after separation by SDS-PAGE. As shown in Figure 3.6B, a 133 K band was eluted from the ssDNA-cellulose column but not the control cellulose column at 500 mM NaCl, indicating that MCMV MDBP is a ssDNA-binding protein.

Next, I wished to determine whether the histidine-tagged MCMV MDBP fusion protein had similar ssDNA-binding property as the MDBP from MCMV-infected cell extracts. To do this, the histidine-tagged MCMV MDBP fusion protein was synthesized in an in vitro transcription-translation reaction in the presence of [35S]methionine (section 2.24). Aliquots of the [35S]methionine-labeled fusion proteins were diluted in washing buffer containing 50 mM NaCl and applied to a ssDNA-cellulose column before stepwise elution with 3 x 2-bed volumes of binding buffer containing increasing concentrations of NaCl. The first and last fractions of 2-bed column volume each were collected. The proteins in each fraction were precipitated by acetone and detected by autoradiography after separation by SDS-PAGE. As shown in Figure 3.7A, the majority of [35S]methionine-labeled proteins was eluted in the presence of 250 mM NaCl. The apparent size of the eluted protein (137 K) is consistent with the predicted molecular weight of the histidine-tagged MCMV MDBP fusion protein, indicating that the protein synthesized in this in vitro transcription-translation reaction contained the full-length fusion protein. To exclude the possibility that the cellulose may contribute to the binding of the fusion proteins to the column, control experiments using unmodified cellulose as a
Figure 3.7. ssDNA-binding properties of MCMV MDBP deletion mutants. [35S]methionine-labeled pRSET-M57 (A), pRSET-M57d422-535 (B), and pRSET-M57d788-824 (C) gene products synthesized in a T7 coupled in vitro transcription-translation system were applied to ssDNA-cellulose or unmodified cellulose columns, washed with buffer containing 50 mM NaCl, and eluted in a stepwise fashion with buffers containing 100 mM, 250 mM, 500 mM, and 1 M NaCl. Proteins in the first and last fractions in each elution step were precipitated with acetone and visualized by autoradiography after separation by SDS-PAGE. The lane labeled “input” to the right of each panel represents 1/20th of the total amount of protein loaded onto each column.
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A

B

C

ssDNA-Cellulose

Cellulose
column matrix were performed. The result revealed no significant binding of the fusion protein to the unmodified column (Figure 3.7A). Furthermore, as shown in Figures 3.7B, the histidine-tagged fusion proteins which lack residues 422 to 535 did not bind to the ssDNA-cellulose column. Therefore, it is very unlikely that the binding of the fusion protein to ssDNA-cellulose column is due to interactions between the leader peptide in the N-terminal protein and ssDNA molecules. Taken together, these data suggest that the histidine-tagged MCMV MDBP fusion protein that was synthesized in the in vitro transcription-translation reaction was able to bind to ssDNA.

3.6 Identification of the ssDNA-binding domain of MCMV MDBP

Having demonstrated that MCMV MDBP binds ssDNA, I attempted to identify the ssDNA-binding domain within the protein. Genetic and biochemical studies of the HSV-1 ssDNA-binding protein ICP8 have mapped the ssDNA-binding domain to a region encompassing amino acid residues 300 to 849 (Gao et al., 1988; Gao and Knipe, 1989; Leinbach and Heath, 1988; Leinbach and Heath, 1989; Wang and Hall, 1990). It is believed that the zinc-finger like motif and the consensus DNA-binding sequence in this region are important for ssDNA-binding. Amino acid sequence analysis of MCMV MDBP revealed that a zinc-finger like motif and a putative ssDNA-binding domain were present within residues 472 to 486 and 782 to 831 respectively (Figure 1.5).

Two internal deletion mutants were constructed to determine whether the regions containing the zinc-finger like motif and the putative ssDNA-binding domain contribute to the ssDNA-binding activity of MCMV MDBP. The first, pRSET-M57d422-535, encodes a mutant protein with a deletion of residues 422 to 535 of MDBP containing the zinc-finger like motif and its flanking regions. The second, pRSET-M57d788-824, possesses an internal deletion from residues 788 to 824 in the MDBP sequence, resulting
in removal of part of the putative ssDNA-binding domain. These plasmids were used as a template to produce the histidine-tagged mutant proteins in an in vitro transcription-translation system. The ability of resultant proteins to bind ssDNA was analyzed by ssDNA-cellulose column chromatography as described in section 2.25. Figure 3.7B shows that the bulk of protein expressed from deletion mutant pRSET-M57d422-535 was present in the wash fractions containing 50 mM NaCl, suggesting that the protein lacking residues 422 to 535 has very little affinity for ssDNA. While the majority of the protein expressed from deletion mutant pRSET-M57d788-824 was retained by ssDNA-cellulose column in the presence of 50 mM NaCl, they were mostly eluted from the column at 100 mM NaCl (Figure 3.7C). The result indicated that loss of a sequence encoding a large part of the putative ssDNA-binding domain affected the ssDNA-binding affinity of MCMV MDBP.

To further determine which portion of MCMV MDBP possesses ssDNA-binding activity, plasmids pRSET-M57dC625 and pRSET-M57dN625 were used to synthesize truncated proteins which contain the N- and the C-terminal half of MDBP respectively. The ssDNA-binding activity of the proteins were tested by ssDNA-cellulose column chromatography. As seen in Figure 3.8, the proteins possessing the N-terminal residues 1 to 625 (pRSET-M57dC625) were not retained by the column at 50 mM NaCl, indicating that they failed to bind ssDNA (Figure 3.8A). On the other hand, the C-terminal half of MDBP consisting of residues 625 to 1191 (pRSET-M57dN625) behaved similarly to full-length MCMV MDBP in which most of the protein was retained by the column in the presence of 50 mM NaCl and then eluted at salt concentration of 250 mM (Figure 3.8B). As shown in Figure 3.8C, more than half of the bound proteins (pRSET-M57dN749) was eluted at 100 mM NaCl; However, 250 mM NaCl was still required for the complete elution of mutant protein from the column. This result indicated that further deletion of N-
Figure 3.8. The C-terminal half of MCMV MDBP possesses ssDNA-binding activity. [\textsuperscript{35}S]methionine-labeled pRSET-M57dC625 (A), pRSET-M57dN625(B), pRSET-M57dN749 (C), and pRSET-M57dN749d788-824 (D) gene products synthesized in a T7 coupled \textit{in vitro} transcription-translation system were applied to ssDNA-cellulose or unmodified cellulose columns, washed with buffer containing 50 mM NaCl, and eluted in a stepwise fashion with buffers containing 100 mM, 250 mM, 500 mM, and 1 M NaCl. Proteins in the first and last fractions in each elution step were precipitated with acetone and visualized by autoradiography after separation by SDS-PAGE. The lane labeled "input" to the right of each panel represents 1/20th of the total amount of protein loaded onto each column.
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ssDNA-Cellulose       | Cellulose
terminal sequences slightly impaired ssDNA binding. Finally, when residues 788 to 824 that contain a large portion of the putative ssDNA-binding domain were removed (pRSET-M57dN749d788-824), the ability of the truncated protein to bind ssDNA was severely impaired (Figure 3.8D). Similar to the mutant pRSET-M57d788-824 (Figure 3.7C), the protein was mostly eluted in the presence of 100 mM salt. It should be noted that small amount of the N-terminal deleted protein bound non-specifically to the cellulose column (Figure 3.8).

The results of experiments designed to map the ssDNA-binding domain of MCMV MDBP are summarized in Figure 3.9. Several conclusions could be drawn from these data. First, the zinc-finger like motif and its adjacent regions (residues 422 to 535) in the N-terminal half significantly affected the ssDNA-binding properties of MCMV MDBP (pRSET-M57d422-535). However, this region appeared not to be directly involved in binding, because the truncated protein possessing the N-terminal half of MDBP (pRSET-M57dC625) had no affinity for ssDNA. Second, the C-terminal half of MCMV MDBP (pRSET-M57dN625) possessed ssDNA-binding activity. Finally, deletion of amino acid residues 788 to 824 which contain part of the putative ssDNA-binding domain affected binding of the C-terminal half to ssDNA (pRSET-M57dN749d788-824), suggesting that these residues may be directly involved in interaction between MCMV MDBP and ssDNA.
Figure 3.9. Summary of ssDNA-binding property of deletion mutants of MCMV MDBP. A sequence ruler is indicated below the main headings, with tick marks every 200 amino acid residues. The amino acid sequence of MCMV MDBP is denoted as the hollow bar. The vertical line and striped box within the bar show the zinc-finger like motif and the ssDNA-binding domain respectively. Amino acid sequences containing these motifs are indicated at the bottom. Black dots above the amino acids denote cysteine and histidine residues within the zinc-finger like motif that are conserved among herpes simplex virus type 1 and betaherpesviruses. The "+" sign denotes aromatic and basic amino acid residues that might contribute ssDNA-binding. In addition, aromatic and basic amino acid residues within the ssDNA-binding domain that are conserved or conservative substitutions among betaherpesviruses are boxed. The names of the deletion mutants are shown in the column on the left. The truncated proteins encoded by these mutants are represented as black bars. Numbers above the bars indicate the last or the first residue present. The ability of the truncated proteins to bind ssDNA is summarized in the column on the right. The "+++" sign represents the protein was eluted at 250 and 500 mM NaCl; The "++" represents the protein was eluted at 100 mM and 250 mM NaCl; "+" represents the protein was eluted at 100 mM NaCl, and the "-" denotes inability to bind ssDNA in the presence of 50 mM NaCl.
Zinc-finger like motif

\[
\text{LCGACGGRCCHTCYA}
\]

472 to 486

ssDNA-binding domain

\[
\text{FFKKGALQRKPIKGCLSFLFRCHEKLPRCGLSCLEFWQRVLQNSLFR}
\]

782 to 831

Mutant | Protein encoded | ssDNA-binding
---|---|---
pRSET-M57 | | ++ +
pRSET-M57 d422-535 | | -
pRSET-M57 d788-824 | | +
pRSET-M57 dC625 | | -
pRSET-M57 dN625 | | ++
pRSET-M57 dN749 | | ++
pRSET-M57 dN749d788-824 | | +
3.7 Nuclear distribution of MDBP after infection of cells with MCMV

A previous study has shown that MCMV MDBP is homologous to the HSV-1 ssDNA-binding protein ICP8 (Messerle et al., 1992). ICP8 is translocated to the nucleus of cells during HSV-1 infection and participates in viral DNA replication (Boehmer and Lehman, 1997). Therefore, one would predict that MCMV MDBP should also enter the nucleus where MCMV DNA synthesis takes place. To test this hypothesis, Balb/3T3 cells were infected with the Smith strain (Vancouver) of MCMV. The infected cells were collected and fixed at various periods of time after virus inoculation. Subcellular location of MCMV MDBP was determined by indirect immunofluorescence with the MAb 4F11.21A specific for an epitope near the C-terminus of MCMV MDBP. Figure 3.10A shows a typical example of the immunofluorescence staining pattern observed in infected cells at 24 hours post-infection. The fluorescence was observed in large globular structures within the nucleus, which were reminiscent of HSV-1 DNA replication compartments described by de Bruyn Kops and Knipe (1988).

Quinlan et al. (1984) have demonstrated that the intranuclear distribution of HSV-1 ICP8 is affected by the status of viral DNA synthesis. I wished to investigate whether inhibition of MCMV DNA replication can also alter the subcellular distribution of MDBP. To do this, Balb/3T3 cells were infected with the Smith strain (Vancouver) of MCMV and incubated in the presence of 300 μg/ml of PFA, an inhibitor of the CMV DNA polymerase (Anders et al., 1987; Huang, 1975; Kemble et al., 1987). At 24 hours after infection, the cells were fixed and processed for indirect immunofluorescence with the MAb 4F11.21A. As seen in Figure 3.10B, MDBP-specific fluorescence remained in the nucleus, but was present in distinct, punctate foci spaced throughout the nucleus. A similar nuclear distribution of ICP8 was observed in HSV-1 infected cells when viral DNA replication was blocked (Quinlan et al., 1984). It was believed that these nuclear
Figure 3.10. Subcellular location of wild-type (wt) MCMV MDBP and its mutant proteins. Balb/3T3 cells were grown on coverslips and infected with MCMV in the absence (A) and presence of 300 µg/ml of phosphonoformic acid (B). Alternatively, COS-1 cells were grown on coverslips and transfected with the plasmid encoding wt MDBP (C), SVdC1056 (D), SVdC625 (E), SVdN625dC1064 (F), SVdC313 (G), SVdN312 (H), SVd422-535 (I), or SVdC1056d422-535 (J). The subcellular location of wt and mutant proteins in infected or transfected cells were examined by indirect immunofluorescence with monoclonal antibody 4F11.21A and a mixture of monoclonal antibodies specific for epitopes located within the N-terminus of MCMV MDBP.
structures may serve as a reservoir for ICP8 not bound to replicating or progeny DNA. Therefore, like HSV-1 ICP8, MCMV MDBP can be observed in different locations in the cell nucleus based on the status of viral DNA replication.

3.8 Nuclear distribution of MCMV MDBP after transient expression in cells

To determine whether import of MCMV MDBP to the nucleus was independent of other viral proteins, an eukaryotic expression plasmid SV-M57 was constructed as described in section 2.15.2, in which the full-length of MDBP coding sequence was placed immediately downstream of the SV40 early promoter. The plasmid DNA was then transfected into COS-1 cells by electroporation (section 2.21). To confirm that the plasmid expressed the predicted MDBP, whole cell extracts prepared at 48 hours after transfection were examined by Western blotting with MAb 4F11.21A. As shown in Table 3.3, the apparent size of wild-type MDBP expressed from the plasmid was consistent with the predicted molecular weight. Next, indirect immunofluorescence was performed to determine the subcellular location of MDBP. COS-1 cells grown on coverslips were transfected with the plasmid SV-M57. At 48 hours after transfection, the cells were collected and processed for immunofluorescence and stained with the MAb 4F11.21A. As shown in Figure 3.10C, nuclear staining was observed in cells transfected with the plasmid encoding MDBP, indicating that nuclear translocation of MCMV MDBP was an inherent property of the protein. Moreover, in contrast to MDBP in infected cells (Figure 3.10A), the distribution of the protein in the transfected cells appears to be diffuse throughout the nucleus (Figure 3.10C), which supports the idea that compartmentalization of MDBP into large globular areas in cell nucleus may require the presence of additional viral proteins.
a Refer to Figure 2.5 for the construction of mutants.

b Numbers indicate residues of the amino acid sequence of MCMV MDBP. Additional amino acid residues resulting from the presence of the linker oligonucleotides are shown in single letter code. For the SVβgal-M57dNX and SVβgal-M57dCX series, where X stands for residue number, the mutant proteins were expressed as a fusion protein containing a truncated β-galactosidase (residues 10 to 1008) at the N-terminus. The predicted molecular mass of the truncated β-galactosidase is 114 K.

c Determined from Western blotting in the presence of molecular weight markers.
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3.9 Identification of nuclear localization signals of MCMV MDBP

3.9.1 The N-terminus of MCMV MDBP contains a nuclear localization signal that is absolutely required for nuclear distribution of the protein

Proteins less than 40 to 60 K in size can diffuse passively into the cell nucleus through nuclear pore complexes (Pante and Aebi, 1996). However, it is very unlikely that MCMV MDBP enters the nucleus by simple diffusion because of its larger molecular weight (133 K). Furthermore, if diffusion accounts for subcellular location of MDBP, one would expect the protein to be equally distributed throughout the cell rather than accumulating predominantly in the nucleus as shown above (Figure 3.10A). Numerous studies have demonstrated that active import of large proteins (more than 40 to 60 K) to the nucleus is conferred by distinct amino acid sequence(s) known as nuclear localization signals (NLSs). The ability of MCMV MDBP to localize to the nucleus in the absence of other viral proteins suggests that such NLSs may be present within MDBP.

The best characterized NLS contains one or more clusters of basic amino acid residues which are exemplified by the SV40 large T antigen NLS (PKKKRKV) and the NLS of Xenopus nucleoplasmin, KRPAATKKAGQAKKKK (Dingwall and Laskey, 1991). Analysis of the amino acid sequence of MCMV MDBP revealed several clusters of basic amino acid residues that resemble these classical NLSs. Of these, two (LKKTRE at positions 21-26 and ARRRF at positions 110 to 114) located in the N-terminus. Additional clusters of basic residues were also found within the C-terminal 262 amino acids of MCMV MDBP (Figure 1.5). To determine whether regions containing these basic residues were involved in nuclear transport of MCMV MDBP, I first constructed two plasmids SVdC1056 and SVdC625 that encode mutant proteins possessing the N-terminal 1056 and 625 residues respectively (Figure 2.5). The plasmids were transfected
into COS-1 cells and the cells were collected 48 hours after transfection. Examination of the total protein samples by Western blotting revealed that cells transfected with each plasmid expressed a product detected by MAb 5F7.11B, which recognizes an epitope located between the N-terminal residues 54 to 254. As shown in Table 3.3, the apparent molecular weight of these proteins closely matched their predicted size, indicating that the desired mutant proteins were present.

Next, subcellular location of these proteins were studied by indirect immunofluorescence. COS-1 cells transfected with the plasmids were collected 48 hours after transfection and the cells were processed for immunofluorescence. The transfected cells were stained with hybridoma tissue culture supernatants containing MAbs specific for epitopes within the N-terminus of MCMV MDBP. In addition, to increase the likelihood of a positive fluorescence signal, a mixture of MAbs 1F1.12B, 5F7.11B, and 3C2.11B was used. Figure 3.10D shows that removal of the C-terminal residues 1057 to 1191 (SV-dC1056) did not affect nuclear distribution. In addition, a mutant containing a further C-terminal deletion, SV-dC625, behaved similarly to SV-dC1056 (Figure 3.10E), indicating that the C-terminal half of MCMV MDBP was not essential in mediating the import of the protein into the nucleus.

The results of these experiments also suggested that a functional NLS was located in the N-terminal half of the protein between residues 1 and 625. To test this hypothesis, I next examined the effects of the set of deletion mutants on the nuclear localization of MDBP. Figure 3.10F shows that the truncated protein possessing residues 625 to 1064 only (SV-dN625dC1064) was primarily localized to the cytoplasm, supporting the idea that the N-terminus of the protein participates in nuclear translocation of MCMV MDBP. To further define this, plasmid SV-dN312 was made which encodes a protein lacking the N-terminal 311 residues. As seen in Figure 3.10H, the subcellular distribution of the
truncated protein was completely cytoplasmic, suggesting that the N-terminal region of MDBP was absolutely required for nuclear localization. The data also further confirmed that the C-terminus did not contain sequences that could function as a NLS for MCMV MDBP.

I wish to further determine whether the N-terminal fragment containing residues 1 to 313 is localized to the nucleus. To this end, plasmid SV-dC313 was constructed and examination of the subcellular location of the protein revealed a predominantly nuclear localization, suggesting that a NLS was present in this region (Figure 3.10G). However, as the protein synthesized from this construct has an apparent molecular weight of 40 K (Table 3.3), passive diffusion could not totally be excluded. Therefore, a chimeric protein was made by fusing the N-terminal 254 residues of MDBP to the C-terminus of a modified version of E. coli β-gal as described Section 2.17. The latter protein, with a predicted molecular weight of 114 K has been frequently used to study the NLS of nuclear proteins (Efthymiadis et al., 1997; Rous et al., 1993; Schmolke et al., 1995). As a control, the plasmid SVβgal encoding E. coli β-gal was also made. Expression of proteins from these plasmids was confirmed by Western blotting with MAb 5F7.11B specific for an epitope located within the N-terminus of MDBP and a commercially available MAb against β-gal. Subsequently, subcellular location of fusion proteins expressed in transfected cells was determined by indirect immunofluorescence. As expected, β-gal expressed from the plasmid SVβgal was found in the cytoplasm (Figure 3.11A). In contrast, fusion of the N-terminal residues 1 to 254 to β-gal in plasmid SVβgal-M57dC254 resulted in predominantly nuclear staining (Figure 3.11C). Additionally, further N-terminal truncation (SVβgal-M57dC57) did not impair nuclear localization of the fusion protein (Figure 3.11D). Therefore, these data suggested that the N-terminal 55 residues contained a NLS that was sufficient for mediating the nuclear
Figure 3.11. The amino terminal and carboxyl terminal sequences of MCMV MDBP could direct the import of β-galactosidase into the nucleus. COS-1 cells were grown on coverslips and transfected with the plasmid encoding β-galactosidase (A), SVβgal-M57dN1063 (B), SVβgal-M57dC254 (C), or SVβgal-M57dC55 (D). The subcellular location of β-galactosidase and β-galactosidase fusion proteins in transfected cells were examined by indirect immunofluorescence with a commercially available monoclonal antibody specific for β-galactosidase.
import of a heterologous cytoplastic protein. Representative photographs of the subcellular location of the fusion proteins (Figure 3.11) were taken on cells stained with a MAb specific for β-gal. However, the cells transfected with plasmid SVβgal-M57dC254 were also stained with pooled MAbs (1F1.12B, 5F7.11B, and 3C2.11B) specific for epitopes located between residues 54 and 254. The staining pattern of these MAbs was similar to that of anti-β-gal MAb. Due to the lack of MAbs specific for epitopes located within the extreme N-terminus of MDBP (residues 1 to 55), immunofluorescence staining of cells transfected with plasmid SVβgal-M57dC57 were carried out only with the MAb specific for β-gal.

Two clusters of basic amino acid residues (YRKMR at positions 1093 to 1098 and SKRSRL at positions 1186 to 1191) were found in the extreme C-terminus of MCMV MDBP. To analyze whether these sequences could function as NLS, I tested the subcellular location of the chimeric protein that contained the C-terminal 128 residues fused to the C-terminus of β-gal at residue 1008 (SVβgal-M57dN1063). The fusion protein was examined by both Western blotting and indirect immunofluorescence with MAb 4F11.21A as well as the a MAb against β-gal. As shown in Figure 3.11B, complete nuclear staining of the fusion protein was observed. Therefore, like the N-terminal sequence, the C-terminus of MDBP could also target a heterologous cytoplastic protein to the nucleus.

3.9.2 Sequences distant from the N-terminus of MCMV MDBP also affect the nuclear localization of the protein

Having established that the N-terminal region of MCMV MDBP played a critical role in mediating the import of the protein into the nucleus, I turned to identify additional sequences that might also contribute to nuclear localization. Sequence analysis revealed
that MCMV MDBP contained a zinc-finger like motif \textit{LFGACGGRCCCHTLEY} between amino acid residues 472 to 486. I have demonstrated that the region encompassing this motif had a significant impact on ssDNA-binding (section 3.6). Therefore, it would be interesting to see whether the region also affected the nuclear localization of the protein. To test this, plasmid SVd422-535 was made which encoded a mutant protein with a deletion of the zinc-finger like motif and additional flanking sequences. Transfection of the plasmid into COS-1 cells and immunofluorescence staining of the expressed protein with MAb 4F11.21A resulted in both cytoplasmic and nuclear fluorescence (Figure 3.10I). A significant percentage of MDBP-expressing cells also showed exclusive nuclear staining. The nuclear staining of the protein was unlikely to be caused by passive diffusion due to its large apparent size as determined by Western blotting (120 K; Table 3.3). Unexpectedly, further deletion of the C-terminal residues 1057 to 1191 of the mutant protein (SV-dC1056d422-535) apparently restored nuclear localization (Figure 3.10J). These results suggested that although the extreme N-terminal portion of MDBP is necessary for the nuclear localization of the protein, sequences distant from this region could also influence this process.

The results of immunofluorescence study of subcellular location of MCMV MDBP are summarized in Figure 3.12. The data suggest that i) the N-terminus (aa 1 to 311) of MCMV MDBP is absolutely necessary for targeting the protein to the nucleus; ii) a nuclear localization signal is present in this region that can direct a heterologous cytoplasmic protein to the nucleus; iii) the region from aa 422 to 535 that contained the zinc-finger like motif influences the nuclear localization of MCMV MDBP; and iv) while the C-terminus (aa 1063 to 1191) is not required for nuclear localization of MCMV MDBP, it is sufficient for mediating nuclear translocation of a heterologous cytoplasmic protein.
Figure 3.12. Structure and subcellular location of deletion mutants of MCMV and the β-galactosidase-MDBP chimeric proteins. A sequence ruler is indicated below the main headings, with tick marks every 200 amino acid residues. The amino acid sequence of MCMV MDBP is denoted as a hollow bar. The vertical line within the bar show the zinc-finger like motif. Amino acid sequence containing the zinc-finger like motif is indicated below the hollow bar. The “+” signs above the amino acids denote cysteine and histidine residues within the zinc-finger like motif that are conserved among betaherpesviruses. The names of plasmids are shown in the column on the left while the truncated proteins encoded by these plasmids are represented as black bars. Numbers above the bars indicate the last or the first residue present on the major DNA-binding protein. Striped bars indicate β-galactosidase and numbers below the bar represent residues numbers for β-galactosidase. The subcellular location of mutant proteins is tabulated on the right. The abbreviations used are: N=nuclear; C=cytoplasmic; N/C=nuclear or cytoplasmic.
3.10 Determination of native molecular weight of MCMV MDBP

Previous works have revealed that HSV-1 ICP8 binds to other viral DNA replication proteins and modulates their activities (Boehmer et al., 1994; Crute et al., 1989; O'Donnell et al., 1987). Knowledge of interactions between ICP8 and other proteins have provided valuable information for understanding HSV-1 DNA replication. To elucidate whether MDBP forms stable complexes with other macromolecules during MCMV infection, the native molecular weight of the protein was determined by gel filtration chromatography. To do this, Balb/3T3 cells were infected with the Smith strain (Vancouver) of MCMV. Proteins were extracted from the cells at 24 hours post-infection and applied to a pre-equilibrated Bio-Gel A-1.5M column. Gel filtration chromatography was performed as described in section 2.26. Fractions of 1.6 ml each were collected. Proteins were precipitated with acetone and separated by SDS-PAGE. The amount of MDBP in each fraction was estimated by Western blotting with MAb 4F11.21A. As shown in Figure 3.13A, column fractions reactive with MAb 4F11.21A spanned fractions 61 and 87 with peak fractions centered around fractions 71 to 79. Compared to molecular standards, these peak fractions were located between bovine gamma globulin (158 K) and bovine thyroglobulin (670 K). The positions of peak fractions indicated that the protein had apparent molecular weights ranging from 200 K (fraction 71) to 400 K (fraction 79). Therefore, the size of native MDBP appeared larger than that of a monomer (133 K; Loh et al., 1991), implying that MDBP might exist predominantly as multimeric complexes during MCMV infection. It was noted that MDBP in fractions 61 to 65 had larger apparent molecular weight than that of bovine thyroglobulin marker (670 K), suggesting that part of the protein may form aggregates under these experimental conditions.

To determine the possible components of MDBP-containing complexes, [35S]methionine-labeled, MCMV-infected cell lysates were fractionated on a Bio-Gel
Figure 3.13. Size fractionation of MDBP in MCMV-infected Balb/3T3 cells by gel filtration chromatography. A. Proteins extracted from Balb/3T3 cells infected with MCMV were fractionated on a Bio-Gel A-1.5M column and 1.6 ml fractions were collected. The amount of MDBP present in each fraction was analyzed by Western blotting with MAb 4F11.21A after electrophoretic separation by 7.5% SDS-PAGE.

B. [³⁵S]methionine-labeled proteins extracted from Balb/3T3 cells infected with MCMV were fractionated on a Bio-Gel A-1.5M column and 1.6 ml fractions were collected. Proteins immunoprecipitated with MAb 4F11.21A from each fraction were separated by 7.5% SDS-PAGE and visualized by autoradiography.

C. [³⁵S]methionine-labeled proteins extracted from MCMV-infected Balb/3T3 cells in immunoprecipitation buffer containing 0.5 M NaCl were fractionated on a Bio-Gel A-1.5M column and 1.6 ml fractions were collected. Proteins immunoprecipitated with MAb 4F11.21A from each fraction were separated by 7.5% SDS-PAGE and visualized by autoradiography.

The numbers on the left represent sizes of molecular weight markers. Fraction numbers are indicated at the bottom and arrows on the top denote peak fractions where the gel filtration molecular weight standards were located. The peak fraction for the 670 K marker for panel C (fraction #64) is beyond the lowest fraction number shown.
A-1.5M column as described in section 2.26.2. Proteins in each fraction were immunoprecipitated with MAb 4F11.21A and the resultant immunoprecipitates were analyzed by 7.5% SDS-PAGE and visualized by autoradiography. As seen in Figure 3.13B, a predominant protein band with the apparent size consistent with MDBP (133 K) was revealed between fractions 60 and 84 with the highest reactivity centered around fractions 74. Compared to molecular weight standards, fraction 74 was located between bovine gamma globulin (158 K) and bovine thyroglobulin (670 K). This extrapolated to a molecular weight of 250 K, significantly larger than the size of a MDBP monomer. Examination of the polypeptides immunoprecipitated from the peak fraction by MAb 4F11.21A revealed that in addition to the predominant 133 K band, which corresponds to the MDBP, fainter bands with apparent molecular weights of 50 to 90 K were co-precipitated (Figure 3.13B). This result indicated that other viral or host-derived proteins may contribute to the formation of MDBP-containing complexes in infected cells.

Using an alternative approach, [35S]methionine-labeled infected cell proteins were extracted under high salt conditions (immunoprecipitation buffer containing 500 mM NaCl). Size fractionation of the extracted proteins on a Bio-Gel A-1.5M column was also carried out in the presence of 500 mM NaCl. This is designed to serve two purposes. First, non-specific aggregation of the solubilized proteins is minimized under high salt conditions. However, lower affinity but biological important protein-protein interactions may be disrupted. Second, high molecular weight MDBP-DNA complexes should be disrupted in the presence of 500 mM salt since MDBP was completely eluted from ssDNA columns under those conditions (Figure 3.6B). Figure 3.13C shows that MDBP was mainly precipitated from fractions 75 to 87, which correspond to an average molecular weight of 225 K. A 50 K protein co-precipitated with MDBP from fractions
77 to 85 whereas a 90 K protein co-precipitated with MDBP from fractions 75 and below, suggesting that MDBP may be associating with these two proteins in separate complexes. Taken together, these data suggested that MCMV MDBP was present as high molecular weight complexes in infected cells. However, the exact identity of the MDBP-associated proteins needs further investigation.
4.0 DISCUSSION

4.1 Production of monoclonal antibodies specific for MCMV MDBP

Monoclonal antibodies have played an important role in studies on CMV (Britt and Vugler, 1992; Loh, 1989, 1991). Previous work in this laboratory has led to the isolation of MAb 4F11.21A which recognizes an epitope located within the C-terminal portion of MDBP. This MAb is useful for the studies of expression kinetics of MDBP during MCMV infection (Loh et al., 1991) and MCMV replication in permissive and non-permissive systems (Yan, 1996). In order to define structure-function relationship of MCMV MDBP, it is desirable to have MAbs which are capable of recognizing a wide spectrum of epitopes on MDBP molecule. In the present investigation, eight additional MAbs have been developed against MDBP. Of these, six recognize epitopes that are different from MAb 4F11.21A. These MAbs were used in this study for characterizing the NLS of MDBP in immunofluorescence experiments. I would assume that these MAbs will also be useful in the analysis of specific interactions of MDBP with other viral or cellular proteins and in the study of MDBP bound in different spatial or conformational states.

Because MDBP is not produced in MCMV-infected cells in sufficient quantity to be conveniently purified, bacterial expression system was used to produce recombinant MCMV MDBP for immunization. The full-length M57 ORF encoding MDBP was cloned into plasmid vector pRSETB and high level expression of the histidine-tagged MCMV MDBP fusion protein was achieved by induction of bacterial culture with IPTG.
The presence of large amounts of fusion proteins in the insoluble fraction of bacterial whole-cell lysates allowed convenient isolation of the protein for use as antigens. High titers (at 1:2000 dilution) of MDBP-specific antibodies were obtained after immunization of a mouse with 50 μg of antigen followed by a single boost with 25 μg. Moreover, MAbs generated from immunization with fusion proteins contain most mouse IgG subclasses (Table 3.1) and recognize epitopes located within five different regions of MDBP (Figure 3.5). Taken together, these results demonstrate that the insoluble histidine-tagged MCMV MDBP fusion protein is highly immunogenic. This is consistent with previous studies which have shown that insoluble protein overexpressed in bacteria is an excellent antigen (Carroll and Laughon, 1987).

The screening procedures used in the generation of MAbs in this study warrant further discussion. First, given the fact that large numbers of hybridoma culture supernatants must be tested for the presence of MDBP-specific antibodies in a short period of time, I developed a three-layer antibody capture ELISA for primary screening. Due to the fact that the insoluble histidine-tagged MCMV MDBP fusion protein used for immunization was contaminated with E. coli proteins, the highly purified proteins should be required in initial screening in order to eliminate clones producing antibodies specific for E. coli proteins. To obtain such antigens for use in ELISA screening, I have tried to purify the histidine-tagged MCMV MDBP fusion proteins using nickel-chelated affinity column chromatography. However, such attempts have proven unsuccessful because the fusion protein was unable to bind to the column. The reason for this is not clear. Probably the hexahistidyl tag is masked due to misfolding of the fusion protein after overproduction in E. coli. To bypass this problem, I have prepared the antigen by extracting the insoluble fraction of bacterial cell lysates with 3 M urea. This procedure released small amount of the histidine-tagged MCMV MDBP fusion protein together with
*E. coli* proteins from the protein aggregates. The presence of contaminating *E. coli* proteins in the antigen preparation requires the inclusion of a control antigen containing whole cell lysates of *E. coli* strain BL21(DE3). This excludes hybridomas that secrete antibodies specific for *E. coli* proteins. The assay worked well in this study, in which 60 positive clones were identified.

Next, to eliminate false positive hybridomas obtained from the primary screening procedure, I have tested the ELISA-positive clones by indirect immunofluorescence with recombinant MCMV MDBP expressed in CV-1 cells. This assay allows us to identify positive clones by examination of the specific nuclear staining pattern of MCMV MDBP with MAbs. Finally, Western blotting was used as a last screening procedure to identify clones that secreted antibodies specific for linear epitopes on MDBP. Further studies, such as immunoprecipitation, are needed to determined whether clones showing positive reaction in indirect immunofluorescence secrete MAbs specific for conformational determinants on MDBP.

4.2 Distribution of continuous epitopes on MCMV MDBP

In this study, I have attempted to map epitopes recognized by MAbs on MDBP of MCMV by analysis of a library of deletion mutants. Because the mapping experiments were conducted by immunoblotting, the epitopes that were defined were limited to linear amino acid sequences without appreciation for discontinuous or conformation-dependent ones that could depend on secondary, tertiary, or quaternary structures of the protein. Therefore these results provide only a minimal estimate of the number of antigenic determinants on the molecule. As indicated in Figure 3.5, the majority of MAbs (five out of eight) produced in this work together with MAb 4F11.21A previously made in this laboratory all recognize epitopes present in or in close proximity to the extreme C-
terminus of MDBP, suggesting that dominant epitopes for the entire MDBP are present in these regions.

I was unable to obtain MAbs that react with the middle portion of MCMV MDBP in Western blotting. In addition, polyclonal antibodies produced in this laboratory by immunization of a rabbit with the insoluble fraction of the histidine-tagged MCMV MDBP fusion protein did not react with this region either. Such an uneven distribution of epitopes on molecules has been documented for other viral proteins. For example, during the generation of MAbs specific for the replicase protein nsP2 of Semliki Forest virus, Kujala et al. (1997) immunized mice with the histidine-tagged nsP2 fusion protein produced in E. coli. Epitope-mapping experiments showed that all 33 MAbs which they produced recognized the N-terminus of the protein. None of their MAbs reacted to the C-terminal half of the molecule.

As seen in Figure 4.1, several antigenic regions are predicted in the middle portion of MCMV MDBP (aa 255 to 1000) based on the Jamson-Wolf antigenic index plot. Then why did I fail to obtain MAbs that could recognize epitopes located within this portion of MDBP? Previous work has demonstrated that foreign proteins overproduced in E. coli are often present as misfolded protein aggregates (Rudolph and Lilie, 1996; Schein, 1989; Schoner et al., 1985). Consistent with these finding, I have demonstrated that the histidine-tagged MCMV MDBP fusion protein is a major component in the insoluble fraction of cell lysates (Figure 3.1A, Lane 7 and 8). Because of protein misfolding, epitopes in the middle portion of MDBP may not be exposed properly on the surface of the protein. In order to differentiate into plasma cells which secrete antibodies, B lymphocytes are required to bind antigen through specific Ig molecules on their cell surfaces (Alt et al., 1987; Blackwell and Alt, 1989; Burnet, 1957). According to this fact,
Figure 4.1. Jameson-Wolf antigenic index plot of MCMV MDBP. The plot is generated from the amino acid sequence of murine cytomegalovirus major DNA-binding protein using the PROTEAN program (DNASTAR Inc., 1990-1994). The Jameson-Wolf method calculates an index of antigenicity by combining values for hydrophilicity, surface probability, flexibility, and secondary structure predictions of Chou-Fasman and Garnier-Robson. These values generate a graph that indicates putative antigenic sites at peak valued residues. The top line represents a sequence ruler with tick numbers every 100 residues. Black bars show regions containing epitopes recognized by a panel of monoclonal antibodies made in this study. Numbers below black bars represent amino acid residues.
one would predict that when the misfolded MDBP are introduced into the peripheral lymphoid tissue such as the spleen, epitopes on the middle segment of the protein are likely to be inaccessible to the membrane Ig on B lymphocyte cell surfaces. Therefore, the immunized spleen cells used as fusion partners may not contain differentiated B lymphocytes that can produce antibodies recognizing epitopes within the central region of MDBP.

4.3 ssDNA-binding property of MCMV MDBP

In this study, I have demonstrated that MCMV MDBP from virus-infected cell extracts was completely eluted from ssDNA-cellulose column at 500 mM NaCl (Figure 3.6B). However, when MCMV MDBP synthesized in an in vitro transcription-translation reaction was examined, most of the bound proteins were eluted at 250 mM NaCl (Figure 3.7A). Several possibilities may contribute to the lower affinity of the in vitro translation product. First, in the absence of normal post-translational modifications such as phosphorylation, proteins synthesized in vitro may fold differently, resulting in lower affinity for ssDNA. Second, the presence of other viral proteins in the extracts of infected cells may enhance the ssDNA-binding property of MCMV MDBP. Third, the small amount of protein synthesized in vitro may not be sufficient for promoting cooperative binding, a characteristic for ssDNA-binding proteins such as HSV-1 ICP8 (Dudas and Ruyechan, 1998). These possibilities remain to be tested.

I have further identified that the C-terminal residues 749 to 1191 (pRSET-M57dN749) possess ssDNA-binding activity. Preliminary results of deletion mutagenesis experiments suggested that the region containing residues 788 to 824 may contribute to the ssDNA-binding property of MDBP (Figure 3.7C). Inspection of amino acid sequence of MDBP reveals that the region encompassing residues 784 to 831 is rich
in aromatic and basic amino acid residues. Sequence alignment data shows that these aromatic and basic residues are well conserved among betaherpesviruses (Figure 4.2A), indicating that mutations of these residues may be lethal to these viruses. Additionally, overall arrangement of these consensus basic and aromatic residues in the amino acid sequence, \textbf{K-X5-R-X9-F-X2-F-X6-F-X1-R-X8-W-X9-R}, fits the predicted ssDNA-binding motif that was proposed by Wang and Hall (1990) (Figure 1.3). The crystal structure of the ssDNA-binding domain of the protein gp32 from bacteriophage T4 has been solved (Shamoo \textit{et al.}, 1995). It reveals that a ssDNA-binding cleft is formed by several twisted $\beta$-sheets. The cleft consists of a positively charged surface and a series of hydrophobic pockets formed by clusters of aromatic side chains. Similar structures have also been documented for other ssDNA-binding proteins, such as adenovirus DBP and human RPA (Bochkarev \textit{et al.}, 1997; Kanellopoulos and van der Zandt, 1995; Tucker \textit{et al.}, 1994). A model has been proposed for the binding of a ssDNA-binding protein to a ssDNA molecule, which suggests that bases and the phosphate backbone of the DNA chain interact respectively with the aromatic and positively charged side chains within the ssDNA-binding cleft. As indicated in Figure 4.3, potential $\beta$-sheets are present within the putative ssDNA-binding domain of MCMV MDBP. Additionally, several aromatic and basic residues conserved among betaherpesviruses appear to be present on these structures. Therefore, one would be inclined to predict that the interaction of the ssDNA-binding domain of MCMV MDBP with ssDNA may be similar to that of bacteriophage T4 gp32. Further site-directed mutagenesis experiments should determine whether the basic and aromatic residues within the predicted ssDNA-binding domain of MDBP (Figure 4.2) play an important role in binding.
Figure 4.2. Amino acid sequence alignment among ssDNA-binding proteins from herpesviruses. Sequences from human cytomegalovirus (HCMV), simian cytomegalovirus (SCMV), human herpesvirus 6 (HHV6), and herpes simplex virus 1 (HSV1) were aligned pairwise with the murine cytomegalovirus sequences containing residues 780 and 833 (A) and 470 and 490 (B) respectively using the MegAlign program (DNASTAR Inc., 1993). The zinc-finger like motif and the putative ssDNA-binding domain are boxed and the black dots above amino acids denote cysteine, histidine, aromatic, and basic residues that are conserved or conservative substitutions among herpesviruses.
Figure 4.3. β-sheets within the putative ssDNA-binding domain of MCMV MDBP as predicted by the algorithm of Chou-Fasman (Chou and Fasman, 1978). The plot is generated from the amino acid sequence of murine cytomegalovirus major DNA-binding protein encompassing residues 780 to 833 using the PROTEAN program (DNASTAR Inc., 1990-1994). The top line represents a sequence ruler with tick numbers every 5 residues. The hollow bar represents the putative ssDNA-binding domain. The predicted β-sheets are shown as black bars with correspondent residues indicated above. Aromatic and basic residues that are present in the predicted β-sheets are shown with small dots above them. Numbers at the bottom of plot shows the position of amino acid residues.
I have also demonstrated that while the N-terminal half lacks ssDNA-binding activity, the region encompassing residues 422 to 535 significantly affects ssDNA-binding property of the protein (Figure 3.7B). There are at least three possible explanations for this result. First, deletion of residues 422 to 535 may disrupt the correct folding of the protein and the N-terminal half of such a misfolded protein may interfere with the function of the ssDNA-binding domain in the C-terminal half of the protein. I prefer this idea for the following reasons. My data from the analysis of the ssDNA-binding properties of deletion mutants showed that the C-terminal half of the protein had very little affinity for ssDNA when the region containing residues 422 to 535 was deleted (Figure 3.7B). However, further deletion of entire N-terminal half (pRSET-M57dN625) partially restored binding (Figure 3.8B). Analysis of amino acid sequences of ssDNA-binding proteins indicates that a zinc-finger like motif is present in most of these proteins. For example, Shamoo et al. (1995) have shown that such motif in bacteriophage T4 protein gp32 is required for maintenance of the overall tertiary structure of the protein, but not involved in direct interaction with ssDNA. It has also been reported that HSV-1 ICP8 is a zinc metalloprotein and mutations within the zinc-binding motif disrupt ssDNA-binding (Gao and Knipe 1989; Gupte et al., 1991). Interestingly, a C-X2-C-X5-H-X1-C type zinc-finger like motif (Berg, 1986) is present within residues 472 to 486 of the amino acid sequence of MCMV MDBP (Figure 1.5) and the motif is also highly conserved among beta herpesviruses (Figure 4.2B). Therefore, it is tempting to speculate that the putative zinc-finger like motif of MCMV MDBP may be involved in ssDNA-binding activity by maintaining the overall tertiary structure of the protein.

The second interpretation is that the N-terminus of MDBP participates in cooperative ssDNA-binding. Therefore, deletion of the region may disrupt a function that is required for the binding of the protein to ssDNA with high affinity. Recently, the
amino acid sequences required for cooperative ssDNA-binding of ICP8 has been located to the region in the N-terminus of the protein (Dudas and Ruyechan, 1998). Chemical modification of a cysteine residue in this region results in alteration of the cooperative binding property of the protein. Sequence analysis of amino acid residues 422 to 535 of MCMV MDBP reveals several cysteine residues conserved among betaherpesviruses. Further studies are needed to determine whether these residues play a role in cooperative binding.

Last, the N-terminus of MDBP may be a substrate for phosphorylation that is important for regulation of ssDNA-binding activity. This speculation is based on the following information. Inspection of amino acid sequence of MDBP reveals two putative serine phosphorylation sites SGD and SRE from residues 422 to 535 in the N-terminus. These sites are in good correlation with the consensus sequence Ser(Thr)-X-Glu(Asp) for casein kinase II (Pinna, 1990). Experimental data from this laboratory have demonstrated that MDBP is phosphorylated. Evidence from the study of p53, a transcriptional factor associated with the suppression of cell growth, has shown that its DNA-binding activity is regulated by phosphorylation with casein kinase II (Hupp et al., 1992). However, it is not clear whether the potential casein kinase II sites in the region encompassing residues 422 to 535 of MDBP are phosphorylated and whether phosphorylation affects the ssDNA-binding property of the protein.

4.4 Identification of amino acid sequences affecting nuclear distribution of MCMV MDBP.

Immunofluorescence studies of MCMV-infected cells revealed that MDBP is localized to the nucleus and displays either punctate or globular staining patterns depending upon the progression of viral DNA synthesis (Figures 3.10A and 3.10B).
These staining patterns are similar to prereplicative sites and DNA replication compartments described previously for HSV-1 ICP8 (de Bruyn Kops and Knipe, 1988; Quinlan et al., 1984). The availability of MAbs recognizing different epitopes on MDBP allowed us to identify amino acid sequences required for the translocation of MDBP into the nucleus. I have demonstrated that deletion of the N-terminal 311 residues (SV-dN312) completely prevented the nuclear import of the protein (Figure 3.10H), suggesting that this region is absolutely required for the nuclear localization. The location of the sequence necessary for nuclear localization of MCMV MDBP is consistent with adenovirus DBP (Morin et al. 1989), but different from ICP8 in which the C-terminal 28 residues serve as a NLS for the protein (Gao and Knipe, 1989, 1992).

Why is the N-terminal sequence so important for the nuclear localization of MCMV MDBP? A simple explanation is that the region contains a NLS that is necessary for targeting MDBP to the nucleus. As seen in Figure 4.4, two stretches of basic amino acid residues are found in the N-terminus. One, referred to as basic box A, contains the sequence LKKTRE (aa 21 to 26). The other, named box B, consists of residues ARRRF (aa 110 to 114). These boxes show limited homology to the classical NLSs described by Dingwall and Laskey (1991). However, the surface probability predictive plot (Emini et al., 1985) indicates that box A is likely to be exposed on the surface of the protein (Figure 4.4), suggesting that these basic residues may be accessible to the nuclear transport apparatus. I have also demonstrated that the N-terminal 55 residues containing box A directed a heterologous cytosolic protein to the nucleus (Figure 3.10D). Due to the limitations of the present study, I am not yet in a position to confirm that the N-terminal 55 residues are also necessary for the import of MDBP into the nucleus. Taken together, these data strongly suggested that box A in the N-terminus of MDBP may serve as a potential NLS for the protein. Therefore, the basic residues within this box are logical
Figure 4.4. Distribution of stretches of basic residues (at least three basic residues in 4) on murine cytomegalovirus major DNA-binding protein. The top line indicates a sequence ruler with tick marks every 200 residues. The hollow bar below the ruler indicates the open reading frame of the murine cytomegalovirus major DNA-binding protein. The regions containing stretches of basic residues that could serve as nuclear localization signals are depicted as black stripes and designated by single letters (A, B, and C). The "+" signs represent basic box with high surface probability (from Emini surface probability plot; Emini et al., 1985). Comparison of basic residues in each box with nuclear localization sequences from the SV40 large T antigen, Xenopus nucleoplasm, and the heterogeneous nuclear ribonucleoprotein A1 M9 domain are shown at the bottom. Numbers represent positions of amino acid residues. Abbreviations: MCMV MDBP, murine cytomegalovirus major DNA-binding protein; SV40, simian virus 40; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1.
starting points for future deletion and oligonucleotide-directed mutagenesis studies.

Another possible explanation is that the amino acid residues in the N-terminus of MDBP may contribute part of a discontinuous NLS. To assemble a fully functional NLS, the residues in the N-terminal portion juxtapose those in the other portion of the protein via proper protein folding. Therefore, disruption of the N-terminal sequence could prevent these separated clusters of basic residues from the formation of such NLS. It has been reported that the nuclear transport of the HSV-1 tripartite complex including DNA primase, helicase, and their associated proteins requires an NLS that formed from parts of each protein (Barnard et al., 1997). As shown in Figure 4.4, MCMV MDBP consists of several small basic regions clustered in its amino and carboxyl termini. Therefore, it is possible that these regions may join together to create a single functional NLS similar to the classical NLS (Dingwall and Laskey 1991). However, the nuclear staining of mutant protein with a deletion in the C-terminal MDBP (SVdC1056; Figure 3.10D) makes this assumption very unlikely. Finally, the requirement of the N-terminal section for nuclear targeting may be due to the possibility that this region can bind to factors other than importin that facilitate the transport of MDBP into the nucleus. Therefore, the deletion might abolish this function.

While the N-terminal 311 residues are necessary for nuclear localization of MDBP, it appears not to be the only sequence that can affect nuclear localization. I have noted that deletion of residues 422 to 535 results in partial retention of the mutant protein in the cytoplasm (Figure 3.10I), suggesting that sequences in this portion of the protein may contribute to nuclear import as well. The importance of this region for the overall functions of MDBP is also reflected by its critical role in ssDNA-binding (Figure 3.7B). It is unlikely that a functional NLS is present in this region because a mutant protein lacking the N-terminus but retaining residues 422 to 535 displayed exclusively
cytoplasmic staining (Figure 3.10H). As discussed in section 4.3, removal of the zinc-finger like motif from this region may affect the tertiary structure of the protein. Recently, Conti et al. (1998) have demonstrated that binding of NLS to karyopherin α, a nuclear transport protein, is highly dependent on the overall folding of the imported protein. Therefore, the impaired nuclear import property of the protein after deletion of residues 422 to 535 might result from the impaired ability of misfolded protein to bind nuclear transport proteins. Moreover, it is interesting to note that when the C-terminal 135 residues were removed (SVdC1056d422-535), deletion of residues 422 to 535 no longer affects the nuclear localization (Figure 3.10J). Probably, the NLS may be masked by the sequence in the C-terminus in the context of a misfolded protein, thereby removing the C-terminus results in restoration of a functional NLS.

In the present study, I have also found that the C-terminus targeted a heterologous cytoplasmic protein to the nucleus (Figure 3.11B). It is not clear why the sequence cannot serve as a NLS for MDBP (Figure 3.11H). Previous study has shown that protein context affects the presentation of NLS (Nelson and Silver, 1989). Therefore, it is likely that the NLS within the C-terminus of MDBP is masked in the context of the protein.

4.5 MDBP complexes are found in MCMV-infected cells

Extensive studies of HSV-1 have demonstrated that viral DNA replication requires assembly of a multimeric protein complex. Interactions between ICP8 and other viral DNA replication proteins have been documented in numerous studies (Boehmer and Lehman, 1997). In the present work, I have presented evidence that MDBP could form complexes in the virus-infected cells. While MDBP migrated as a monomer with an apparent molecular weight of 133 K in SDS-PAGE (Loh et al., 1991), my data from gel filtration chromatography (Figure 3.13A) revealed that it existed mainly as complexes
with apparent molecular weights ranging from 200 K to 400 K, indicating that the protein is present either as a dimer, or is complexed with other viral/cellular proteins. In contrast to this finding, Makhov et al. (1996) have revealed that HSV-1 ICP8 is present primarily as monomers when the molecular weight of the protein particles was evaluated by electron microscopy. However, the HSV-1 proteins used in their study were purified from nuclear extracts of sf9 cells infected with ICP8-expressing recombinant baculoviruses in the absence of other HSV-1 replication proteins. Therefore, protein complexes that may contain HSV-1 DNA replication proteins were not expected to be present. By further analysis of [35S]methionine-labeled MCMV-infected cell lysates, I have found that MDBP complexes consisted predominantly of MDBP molecules (Figure 3.13B). However, additional proteins were found to be associated with MDBP. Previous work from this laboratory has provided preliminary evidence that the viral DNA polymerase accessory protein pp50 may form complexes with MDBP in the virus-infected cells. The absence of such interactions in cells co-transfected with plasmids encoding MDBP and pp50 suggested that the formation of these protein complexes may be dependent on additional viral proteins.

4.6 Conclusions and future studies

In the present investigation, the M57 ORF encoding MCMV MDBP was cloned into a prokaryotic expression vector and the histidine-tagged MCMV MDBP fusion protein was produced in E. coli. A panel of MAbs recognizing epitopes located in the N- and C-termini of the protein was generated by immunization of mice with the fusion protein. Monoclonal antibodies were used in Western blotting, immunofluorescence, immunoprecipitation, and gel filtration chromatography to biochemically characterize MCMV MDBP. I have drawn the following conclusions based on experimental results
presented in this thesis: i) Overproduction of the histidine-tagged MCMV MDBP fusion protein in *E. coli* leads to formation of insoluble aggregates; ii) The majority of MAbs generated by using histidine-tagged MCMV MDBP fusion protein were specific for linear epitopes located in the C-terminus of MCMV MDBP; iii) Murine cytomegalovirus MDBP encoded by the M57 ORF binds ssDNA, indicating the protein is functionally related to the HSV-1 ssDNA-binding protein ICP8; iv) Although the C-terminus of MCMV MDBP containing the putative ssDNA-binding domain possesses ssDNA-binding activity, the N-terminal half of the protein also influences this property. It is very likely that the zinc-finger like motif in the N-terminus is involved in the proper folding of the putative ssDNA-binding domain in the C-terminal MDBP; v) Murine cytomegalovirus MDBP is a nuclear protein and the intranuclear distribution pattern in the presence or absence of viral DNA replication is similar to HSV-1 ICP8; vi) The N-terminus of MCMV MDBP is absolutely required for the import of MCMV MDBP into the nucleus, while sequences distant from this region also influence this process. A NLS is identified in the N-terminus which is capable of directing a heterologous cytoplasmic protein to the nucleus. Therefore, it appears that domains responsible for ssDNA-binding and nuclear import are located in distinct regions on MDBP. These findings are different from those in HSV-1 ICP8 in which the C-terminal half of the protein contains both the ssDNA-binding domain and NLS; vii) Protein complexes are found in MCMV-infected cells which contain MDBP and other viral or cellular proteins. The apparent sizes of these complexes suggest that the protein is present either as a dimer, or high molecular weight complexes associated with additional macromolecules from the virus and/or host cell. The results of the initial characterization of MCMV MDBP showed that it shares similarities as well as differences with other herpesvirus ssDNA-binding proteins, particularly HSV-1 ICP8. Because of the higher degree of sequence homology between
the MCMV and HCMV proteins, the conclusions may also be applicable to the HCMV major DNA-binding protein.

Based on the results of the present investigation, several questions are immediately apparent. First, minimal amino acid sequences required for ssDNA-binding activities and nuclear localization of MCMV MDBP are unclear. For example, we do not know which residues within the putative ssDNA-binding domain are involved in ssDNA-binding activities and whether the putative zinc-finger like motif in MCMV MDBP plays a role in ssDNA binding. Does the cluster of basic residues in the N-terminal region of MCMV MDBP serve as NLS for the protein? Further construction of MDBP mutants through deletion and site-directed mutagenesis should help to provide answers to these questions. Second, MCMV MDBP is present as high molecular weight complexes in infected fibroblasts. Then what are the exact identities of the MDBP-associated proteins? Does pp50, the DNA polymerase processivity factor, interact with MDBP during viral infection? Third, the molecular mechanism of assembly of MCMV DNA replication compartments in infected cells have yet to be elucidated. Is formation of such compartment associated with ND10 domains of host cells? Therefore, answers to these questions will not only extend our understanding of viral DNA replication in the betaherpesviruses, but also provide insights into DNA replication in eukaryotic cells.
REFERENCES


APPENDIX I - Buffers and Solutions

Agarose gel-loading buffer 10X: 50% glycerol, 100 mM EDTA, and 1% (w/v) bromophenol blue.

Alkaline phosphatase buffer (AP buffer, pH 9.5): 100 mM Tris base, 100 mM NaCl, and 5 mM MgCl₂.

AP substrate solution (Bio-Rad): 1 ml 5 X diethanolamine buffer, 4 ml ddH₂O and one p-nitrophenylphosphate tablet.

Binding buffer (pH 7.4): 50 mM Tris base, pH 7.4, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM PMSF.

CIP dephosphorylation buffer (pH 8.3): 100 mM Tris-HCl, pH 8.3, 10 mM ZnCl₂, and 10 mM MgCl₂.

Coomassie blue staining solution: 0.25% (w/v) Coomassie brilliant blue R250, 50% (v/v) methanol, and 10% (v/v) acetic acid.

BCIP: 5% (w/v) BCIP in absolute dimethylformamide.

DAPI: 0.1% (w/v) p-phenylenediamine, 0.0001% (w/v) DAPI, and 50% (v/v) glycerol.

Destain solution: 5% (v/v) methanol and 7.5% (v/v) acetic acid.

GKNP balanced salt solution (pH 7.0), 10X: 8% (w/v) NaCl, 0.4% (w/v) KCl, 0.06% (w/v) Na₂HPO₄·H₂O, 0.06% (w/v) KH₂PO₄, 1% (w/v) glucose, 0.02% (w/v) phenol red, and 0.004% (v/v) chloroform.

HeBS (pH 7.1), 2X: 40 mM HEPES, 1.6% (w/v) NaCl, 0.074% (w/v) KCl, 0.054%(w/v) Na₂HPO₄·12H₂O, and 1% (w/v) D-glucose.

HT 50 X: 68 mg hypoxanthine and 19 mg thymidine in 100 ml ddH₂O.

Hybridoma medium: DMEM supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 0.1 μM selenium, 1.6 μg/ml gentamycin, and 1% (v/v) 100 X penicillin/streptomycin from GIBCO.

Immunoprecipitation buffer (pH 7.4): 50 mM Tris base, pH 7.4, 150 mM sodium chloride, 1% NP-40, 1% sodium deoxycholate, and 1 mM PMSF.

Immunoprecipitation washing buffer (pH 7.4): TBS containing 0.5 M NaCl plus 1% NP-40, 1% deoxycholate, and 1 mM PMSF.

Lysis buffer (pH 8.0): 50 mM Tris base, pH 8.0, 2 mM EDTA, and 0.1% (v/v) Triton X-100.

NBT: 7.5% (w/v) NBT in 70% (v/v) dimethylformamide.
**PBS (pH 7.2-7.4):** 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.02% KH2PO4, and 0.22% (w/v) Na2HPO4·7H2O.

**RIPA buffer (pH 8.0):** 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) deoxycholate, and 0.1% (w/v) SDS.

**SDS-PAGE sample buffer, 2X:** 100 mM Tris-HCl, pH 6.8, 10% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue.

**Separating gel mixture (7.5%):** 2.5 ml mixture containing 30% acrylamide and 0.8% bis-acrylamide, 2.5 ml lower-Tris buffer, 30 μl 10% (w/v) ammonium persulfate, 10 μl N,N',N''-tetramethylethylenediamine (TEMED), 5 ml ddH2O (total volume 10 ml).

**Solution I:** 50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA.

**Solution II:** 0.2 N NaOH and 1% SDS.

**Solution III:** 3 M potassium and 5 M acetate.

**SOC medium (pH 7.0):** 2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.05% NaCl, and 20 mM glucose.

**Stacking gel mixture (2.8%):** 0.5 ml mixture containing 28% acrylamide and 1.6% bis-acrylamide, 1.3 ml upper-Tris buffer, 22.5 μl 10% (w/v) ammonium persulfate, 7.5 μl N,N',N''-tetramethylethylenediamine (TEMED), 3.2 ml ddH2O (total volume 5 ml).

**STE buffer (pH 8.0):** 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

**STET buffer (pH 8.0):** 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5% Triton X-100.

**Transfer buffer:** 10% (v/v) 10X Tris-glycine buffer, 20% methanol, 70% ddH2O.

**TAE buffer (pH 8.0):** 40 mM Tris-acetate, pH 8.0, and 1 mM EDTA.

**Tris-buffered saline (TBS, pH 8.0):** 50 mM Tris base and 150 mM NaCl.

**Tris-buffered saline tween 20 (TBST, pH 8.0):** 0.05% (v/v) Tween 20 in TBS.

**Tris-EDTA (TE) buffer (pH 8.0):** 10 mM Tris base and 1 mM EDTA.

**Tris-glycine electrophoresis buffer (pH 8.3), 1X:** 10% (v/v) 10X Tris-glycine buffer, pH 8.3, 0.1% (w/v) SDS.

**Trypsin-EDTA solution (pH 8.0), 20X:** 0.5% trypsin and 5.3 mM EDTA.
Washing buffer (pH 7.5): 50 mM Tris base, pH 7.5, 100 mM NaCl, 0.5 mM dithiothreitol, and 10% glycerol.
**APPENDIX II - List of the Major Suppliers of Reagents Used in This Study**

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>American Type Culture Collection</td>
<td>- Cultured cells of many types</td>
</tr>
<tr>
<td>Rockville, MD</td>
<td></td>
</tr>
<tr>
<td>BDH Inc.</td>
<td>- Chemicals</td>
</tr>
<tr>
<td>Toronto, Ont.</td>
<td></td>
</tr>
<tr>
<td>Bio-Rad</td>
<td>- Chemicals</td>
</tr>
<tr>
<td>Richmond, CA</td>
<td>- Goat anti-mouse IgG conjugated to alkaline phosphatase</td>
</tr>
<tr>
<td></td>
<td>- Goat anti-rabbit IgG conjugated to horseradish peroxidase</td>
</tr>
<tr>
<td></td>
<td>- Bio-Gel A-1.5M gel column</td>
</tr>
<tr>
<td>Boehringer-Mannheim</td>
<td>- Calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>Germany</td>
<td>- PEG 1500</td>
</tr>
<tr>
<td>DIFCO Laboratories</td>
<td>- Bacterial culture media</td>
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<td>Detroit, MI</td>
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<tr>
<td>Dupont/New England Nuclear Corp.</td>
<td>- $^{35}$S)methionine</td>
</tr>
<tr>
<td>Boston, MA</td>
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<tr>
<td>GIBCO/BRL</td>
<td>- Cell culture media</td>
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<tr>
<td>Life Technologies Inc.</td>
<td>- Antibiotics</td>
</tr>
<tr>
<td>Grand Island, NY</td>
<td>- Restriction endonucleases</td>
</tr>
<tr>
<td></td>
<td>- DNA modification enzymes</td>
</tr>
<tr>
<td></td>
<td>- <em>E. coli</em> strain DH5α</td>
</tr>
<tr>
<td>Invitrogen Corp.</td>
<td>- Plasmid vectors pRSETA/B/C</td>
</tr>
<tr>
<td>Jackson ImmunoResearch Laboratories, Inc.</td>
<td>- fluorescein isothiocyanate-conjugated donkey anti-mouse IgG antibody</td>
</tr>
</tbody>
</table>
New England Biolabs Inc.  
Beverly, MA  
- Restriction endonucleases  
- DNA modification enzymes

Novagen, Inc.  
- E. coli stain BL21(DE3)

Pharmacia, Inc.  
Piscataway, NJ  
- Protein A-Sepharose CL-4B  
- Plasmid vectors pTZ18R/19R

Sigma  
St. Louis, MO  
- Chemicals  
- ssDNA cellulose or unmodified cellulose