CHARACTERIZATION OF SIX MONOCLONAL ANTIBODIES AGAINST THE
MINUTE VIRUS OF MICE NS-1 PROTEIN, AND THE USE OF ONE IN THE
IMMUNOAFFINITY PURIFICATION OF NS-1 EXPRESSED IN INSECT CELLS

By
DOUGLAS EDWARD YEUNG

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We accept this thesis as conforming
to the required standard

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Department of Biochemistry

The University of British Columbia
Vancouver, Canada

Date April 19, 1990
I. Abstract

Six mouse monoclonal antibodies have been isolated which react against a bacterial fusion protein containing amino acids 364 to 623 of the NS-1 protein of the prototype strain of the Minute Virus of Mice (MVMp). All six were found to be of the IgG class of antibodies; five being IgG$_1$ and the sixth being IgG$_{2a}$. By immunoblot analyses, these antibodies all recognize an 83 kDa protein found only in MVM-infected mouse fibroblast cells, leading to the assumption that they are all NS-1 specific. Further evidence for this assumption is obtained from indirect immunofluorescence studies showing all but one of the mAbs react against a nuclear protein found in MVM-infected cells.

The epitopes of the antibodies were mapped using carboxy-terminal deleted bacterial fusion proteins derived from the plasmid encoding the original antigen. For the six monoclonal antibodies, four distinct epitopes were found (A - D). Three were clustered in a 16 amino acid region near the carboxy-terminal of the bacterial fusion protein, while the fourth was slightly more toward the amino-terminal side. Competition ELISAs against a 25 amino acid NS-1 specific peptide confirmed the mapping of the A epitope recognized by the CE10 and AC6 monoclonal antibodies.

Also in this thesis, the characterization of a NS-1 fusion protein and a non-fused NS-1 protein expressed in insect cells by recombinant baculoviruses is also described. The latter, a full-length NS-1 protein designated NS-1$_{Ac}$, was found to be an 84 kDa cytoplasmic protein. This protein was immunoprecipitated by all six monoclonal antibodies. A CE10 monoclonal antibody immunoaffinity column was employed in the single-step purification of NS-1$_{Ac}$ from insect cells. Four elution methods (alkaline, peptide, 6M guanidinium, and acid) were examined and the best purification was obtained using the acid elution.
# II. Table of Contents

I. Abstract.................................................................................................................. ii
II. Table of Contents................................................................................................... iii
III. List of Tables ........................................................................................................... v
IV. List of Figures .......................................................................................................... vi
V. List of Abbreviations ............................................................................................... vii
VI. Acknowledgements ................................................................................................. xii

VII. Introduction ........................................................................................................... 1
   A. Parvoviridae.......................................................................................................... 1
   B. The Autonomous Parvoviruses and the Minute Virus of Mice............................... 2
   C. The NS-1 Polypeptide............................................................................................. 5
   D. The Baculovirus Expression System...................................................................... 9
   E. Project Purpose...................................................................................................... 12

VIII. Materials and Methods ....................................................................................... 15
   A. Materials............................................................................................................... 15
   B. Bacterial Strains, Cells, Viruses, and Media......................................................... 16
      1. Plasmids............................................................................................................ 16
      2. Bacteria............................................................................................................. 18
      3. Hybridomas...................................................................................................... 18
      4. MVM and A9 cells........................................................................................... 19
      5. Baculovirus and Sf9 cells................................................................................ 20
   C. Purification of lacZ/NS-1.................................................................................... 22
      1. Mini-lysates....................................................................................................... 22
      2. Large scale purification..................................................................................... 22
   D. Production and Purification of Monoclonal Antibodies.......................................... 23
      1. Production of monoclonal antibodies............................................................... 23
      2. Sub-typing of monoclonal antibodies............................................................... 23
      3. Purification of monoclonal antibodies............................................................. 23
   E. ELISAs.................................................................................................................. 24
   F. SDS Polyacrylamide Gel Electrophoresis............................................................. 25
   G. Immunoblots........................................................................................................ 25
   H. Immunofluorescence............................................................................................ 26
   I. Epitope Mapping.................................................................................................... 27
      1. Basic Molecular Cloning Techniques............................................................... 27
      2. Attachment of the Termination Oligomer....................................................... 28
      3. Nested Deletions............................................................................................. 30
      4. Sequencing....................................................................................................... 31
   J. Immunoprecipitations............................................................................................ 32
   K. Immunoaffinity column construction.................................................................... 33
   L. Immunoaffinity purification of CP/NS-1 expressed in Sf9 cells............................. 33
   M. Immunoaffinity purification of NS-1Ac expressed in Sf9 cells.............................. 34
      1. Elution by peptide and alkali......................................................................... 34
      2. Elution by 6M guanidinium chloride............................................................... 35
      3. Elution by acid............................................................................................... 35

IX. Results ..................................................................................................................... 36
   A. Characterization of the mAbs............................................................................. 36
   B. Mapping of the epitopes...................................................................................... 40
Table 1: Distinctions between the three genera of the family Parvoviridae

Table 2: Immunoblot reaction of carboxy-terminal deleted *lacZ*/NS-1 proteins versus the monoclonal antibodies
IV. List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Map of the MVMp genome.</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>The construction of pUC19/D.</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Coomassie blue-stained gel of lacZ/NS-1 and immunoblots using the six monoclonal antibodies.</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>Coomassie blue-stained gel of mock- and MVM-infected cell lysates, and immunoblots using the six monoclonal antibodies.</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>Immunofluorescence time-course study of MVM-infected, synchronous LA9 cells using the CE10 monoclonal antibody.</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>Immunofluorescence in MVM-infected LA9 cells using the CE10, AC6, BE2, EA2, and CH10 monoclonal antibodies.</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
<td>The construction of pUC19/D.t and its unidirectional deleted clones.</td>
<td>44</td>
</tr>
<tr>
<td>8</td>
<td>Immunoblots of the six monoclonal antibodies against thirteen deletion clones of pUC19/D.t.</td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td>Predicted positions of the epitopes of the six monoclonal antibodies on lacZ/NS-1 and on MVM NS-1.</td>
<td>47</td>
</tr>
<tr>
<td>10</td>
<td>Immunoblots of the CH10 and BC4 monoclonal antibodies against a NS-1/NS-2 fusion protein.</td>
<td>49</td>
</tr>
<tr>
<td>11</td>
<td>Titration curves of the six monoclonal antibody solutions used in the competition ELISAs.</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>Graphs of the peptide 1 competition ELISAs showing absorbance at 405 nm versus the competitor concentration in each well.</td>
<td>52</td>
</tr>
<tr>
<td>13</td>
<td>Coomassie blue-stained gel of mock-, AcNPV- and AcSec-infected total cell lysates.</td>
<td>54</td>
</tr>
<tr>
<td>14</td>
<td>Immunofluorescence time course study of AcSec-infected Sf9 cells using the CE10 monoclonal antibody.</td>
<td>56</td>
</tr>
</tbody>
</table>
Figure 15: Coomassie blue-stained gel and immunoblot using the CE10 monoclonal antibody, of fractions taken during the immunoaffinity purification of CP/NS-1 expressed in insect cells.

Figure 16: Immunoblot of mock-, AcNPV-, and AcSec-infected Sf9 cell fractionation study

Figure 17: Coomassie blue-stained gel of mock-, AcNPV-, and AcNS-1-infected Sf9 cell lysates, and immunoblots using the CE10 monoclonal antibody.

Figure 18: AcNPV- and AcNS-1 infected Sf9 cell fractionation study.

Figure 19: Immunofluorescence time course study of AcNS-1-infected Sf9 cells using the CE10 monoclonal antibody.

Figure 20: Immunoprecipitation of AcNS-1-infected cell lysates using the six monoclonal antibodies.

Figure 21: Immunoprecipitation of AcNS-1- and AcNPV-infected cell lysates using the CE10 mAb and an anti-TP sera, and double immunoprecipitation studies on CE10 mAb immunoprecipitated AcNS-1 infected Sf9 cell lysates using the CE10 mAb and an anti-TP sera.

Figure 22: Coomassie blue-stained gel and immunoblot using the CE10 monoclonal antibody, of fractions taken during the immunoaffinity purification of NS-1Ac expressed in insect cells and eluted by peptide and alkali.

Figure 23: Coomassie blue-stained gel and immunoblot using the CE10 monoclonal antibody, of fractions taken during the immunoaffinity purification of NS-1Ac expressed in insect cells and eluted by 6M Guanidinium chloride.

Figure 24: Coomassie blue-stained gel and immunoblot using the CE10 monoclonal antibody, of fractions taken during the immunoaffinity purification of NS-1Ac expressed in insect cells and eluted by acid (pH 2.5).
V. List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A$_{280}$</td>
<td>absorbance at 280 nm</td>
</tr>
<tr>
<td>A$_{405}$</td>
<td>absorbance at 405 nm</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid (s)</td>
</tr>
<tr>
<td>AcNPV</td>
<td><em>Autographa californica</em> nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>bp</td>
<td>base pair (s)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>autoclaved, distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid (s)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EV</td>
<td>excluded virus</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>G</td>
<td>gauge</td>
</tr>
<tr>
<td>g</td>
<td>gram (s)</td>
</tr>
<tr>
<td>HGPRT$^-$</td>
<td>hypoxanthine-guanine phosphoribosyl transferase deficient</td>
</tr>
</tbody>
</table>
h.p.i.  hours post-infection
h.p.r.  hours post-release
Ig  immunoglobulin
Ile⁻  isoleucine deficient
IMDM  Iscove's Modified Dulbecco's Media
kb  kilobase
KCl  potassium chloride
kDa  kiloDalton
KH₂PO₄  potassium phosphate, monobasic
KOAc  potassium acetate
l  litre (s)
liq N₂  liquid nitrogen
M  molar
mA  milliampere (s)
mAb  monoclonal antibody
MCS  multiple cloning site
MgCl₂  magnesium chloride
MgSO₄  magnesium sulfate
min  minute
ml  millilitre (s)
mM  millimolar
mm  millimetre (s)
MOI  multiplicity of infection
MVM  Minute Virus of Mice
MVMp  Minute Virus of Mice, prototype strain
NaCl  sodium chloride
NaDOC  sodium deoxycholate
Na₂HPO₄  sodium phosphate, dibasic
NaN₃  sodium azide
NaOH  sodium hydroxide
(NH₄)₂SO₄  ammonium sulfate
nm  nanometer (s)
NP-40  Nonidet P-40
ORF  open reading frame
OV  occluded virus
P₄  MVMp promoter at 4 map units
P₃₈  MVMp promoter at 38 map units
pfu  plaque forming unit (s)
pmol  picomole
p-NPP  para-nitrophenyl phosphate
rαmIg  rabbit anti-mouse immunoglobulin
RNase  ribonuclease
rpm  revolution (s) per minute
RT  room temperature
SDS  sodium dodecyl sulfate
sec  second (s)
t  time
Tris  tris (hydroxymethyl) aminomethane
U  unit (s)
V  volt (s)
V/V  volume to volume ratio
W  watt (s)
W/V  weight to volume ratio
x g  times gravity
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ZnSO₄</td>
<td>zinc sulfate</td>
</tr>
<tr>
<td>μg</td>
<td>microgram (s)</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre (s)</td>
</tr>
<tr>
<td>°C</td>
<td>degree (s) Celsius</td>
</tr>
</tbody>
</table>
VI. Acknowledgements

First and most of all, I would like to thank my supervisor, Dr. Caroline Astell, for giving me the opportunity and the means to work in her lab. With her correct mix of guidance, advice, encouragement, and independence I was able to take this project (and my golf game) as far as I did.

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Thirdly, I owe a large debt of gratitude to the various students, faculty, and staff of the Biochemistry department at U.B.C. whom I have prodded and bothered for help, equipment, supplies, or participation in intramural teams. Thanks for all the friendship, memories, and support.

Finally, I’d like to thank the two other members of my graduate committee, Dr. R. Molday and Dr. P. Bragg for their suggestions and reading of this thesis; Dr. I. Clark-Lewis for synthesizing the peptides; Dr. P. Tattersall and S. F. Cotmore for their gifts of various sera against MVM proteins; Dr. J. W. Bodnar for the anti-TP polyclonal sera; Dr. R. Molday for the 2B2 monoclonal antibodies; Dr. F. Tufaro and his lab, and Dr. S. Gillam and her lab for their advice and harassment; and Michael "Doc" Weis for the painfully long hours he spent protecting the E.M. lab teaching me how not to blow up the Zeiss.

"Without the heart, there can be no understanding between the mind and the hand."

Anonymous
VII. Introduction

A. Parvoviridae

The family Parvoviridae consists of a group of physically similar viruses that are among the smallest known DNA viruses. These viruses infect a wide range of animals from insects to the higher vertebrates, including humans. They have been identified in many important veterinary diseases generally causing fetal and neonatal abnormalities by destroying rapidly dividing cell populations. In contrast, the human parvovirus, B19, causes a rather mild rash known as erythema infectiosum (Anderson et al. 1984) and a type of aplastic crisis in people with hemolytic anemias (Tijssen et al. 1990). Apart from the clinical and pathological importance of the parvoviruses, it was thought that the small size of its DNA genome and its limited coding capacity would allow these viruses to serve as a simple model for DNA replication and transcription. Rather than succumbing to expectation, these viruses have proven to be much more complex than first thought.

Parvoviruses have characteristic non-enveloped, isometric particles, 20 - 25 nm in diameter enclosing a linear, single-stranded DNA genome that is 4.7 to 6.0 kb long (Siegl 1984, Tijssen et al. 1990). The particles exhibit icosahedral symmetry and do not appear to contain carbohydrates or lipids. In terms of total mass, the DNA comprises 19 - 32% and the capsid proteins the rest. Recent reports indicate that there is, in fact, a very small proportion of viral encoded enzymes packaged as well (Cotmore and Tattersall 1989, Faust et al. 1989). The small size of the viruses makes them relatively resistant to environmental extremes, showing stability from pH 3 to pH 9, at 56 °C for at least 60 minutes (Siegl et al. 1984).

The family Parvoviridae is divided into three genera, Parvovirus, Dependovirus, and Densovirus. Classification into these three genera is dependent on three factors: 1) autonomous or helper virus-dependent replication, 2) encapsidation of mainly the negative sense strand or both positive and negative sense strand in equal proportion, and 3) host being vertebrate or invertebrate (see Table 1). Now that more is known about the
paroviruses, several of the distinctions outlined in Table 1 no longer absolutely define the three genera (reviewed by Tijssen 1990). Throughout the rest of this thesis, the genera will be referred to by their common names, autonomous for *Parvovirus*, helper-dependent for *Dependovirus*, and densonucleosis for *Densovirus*.

Table 1: Distinctions between the three genera of the family Paroviridae

<table>
<thead>
<tr>
<th>Parovirus</th>
<th>Dependovirus</th>
<th>Densovirus</th>
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<tbody>
<tr>
<td>autonomously replicating</td>
<td>helper virus-dependent</td>
<td>autonomously replicating</td>
</tr>
<tr>
<td>mainly negative sense strand packaged</td>
<td>both positive and negative sense strands packaged</td>
<td>both positive and negative sense strands packaged</td>
</tr>
<tr>
<td>vertebrate hosts</td>
<td>vertebrate hosts</td>
<td>invertebrate hosts</td>
</tr>
</tbody>
</table>

B. The Autonomous Paroviruses and the Minute Virus of Mice

The Minute Virus of Mice (MVM) is a representative member of the autonomously replicating paroviruses. Typical of all paroviruses, it has a linear, single-stranded DNA genome with palindromic sequences at the 3' and 5' termini capable of forming hairpin duplex structures, but unlike the helper-dependent subgroup of viruses, the 3' palindrome and the 5' palindrome are non-identical (Bourguignon et al. 1976). Like most of the members of the autonomous paroviruses, MVM does not require co-infection with helper virus for productive infection, packages predominantly negative sense DNA (i.e. DNA which is the complement of that encoded into RNA) greater than 99% of the time (Cotmore and Tattersall 1987), and infects a vertebrate host. The relatedness of the members of the autonomous sub-group becomes even more apparent in the genomic organization and polypeptides encoded.

To date the autonomous parovirus genomes sequenced, or partially sequenced include the prototype strain of MVM (MVMp), the immunosuppressive strain of MVM (MVMi), the rat virus H-1, bovine parovirus (BPV), the human parovirus B-19, canine parovirus
(CPV-2), and feline panleukopenia virus (FPV). Comparisons of the sequences indicate that these parvoviruses show a high degree of similarity in their genomic organization with respect to hairpin sequences, promoter locations, protein gene position, intron positions, and polyadenylation signals (reviewed by Rhode III and Iversen 1990). For MVM, the highest degree of relatedness is shown with CPV, FPV, and the rodent parvoviruses (Cotmore and Tattersall 1987). Comparison of all seven genomes reveals a similar positioning of two large blocks of open reading frame (ORF) spanning approximately 80% of the genome, and a number of smaller ORFs (Shade et al. 1986). The major, right-hand ORF encodes the capsid polypeptides and the major left-hand ORF encodes the nonstructural polypeptides.

SDS-polyacrylamide gel analysis of MVM virions revealed the existence of three capsid proteins designated VP-1, VP-2, and VP-3 (for viral proteins 1 through 3 respectively). The apparent molecular weights of these proteins were 83 kDa, 64 kDa, and 62 kDa respectively (Tattersall et al. 1976). VP-1 and VP-2 have been shown to be primary translation products, while VP-3 was the result of proteolytic cleavage of VP-2 (Tattersall, Shatkin, and Ward 1977). The largest capsid protein, VP-1, makes up approximately 18% of total capsid protein; the rest being accounted for by the two smaller capsid proteins, although in variable proportions (Tattersall 1978). The exact structure of the capsomeres and the exact number of capsomeres forming the MVM virion is still unknown.

Identification of two nonstructural polypeptides distinct from the capsid proteins was obtained by in vitro translation of viral mRNAs (Cotmore et al. 1983). Three classes of MVM transcripts have been found, R1, R2, and R3 (Pintel et al. 1983) (see Figure 1). The R3 transcripts code for the capsid proteins and are initiated from a promoter at 38 map units (P38). Note that in MVM, one map unit corresponds to ~51 nucleotides (Astell et al. 1983, Astell et al. 1986). The R1 and R2 transcripts are initiated by a second promoter found at 4 map units (P4), with the R2 transcripts containing a large splice of 1476 nucleotides not found in the R1 class (Pintel et al. 1983). All three classes of transcripts contain a small splice of approximately 150 nucleotides at 43 map units and have polyadenylation sites clustered near
Figure 1: Map of the MVMp genome

The cytoplasmic transcripts of MVM, denoted R1, R2, R3, and R3', are aligned beneath a line diagram of the viral DNA strand which illustrates the extent of the 3' and 5' terminal hairpin palindromes and the positions of the two promoters at map units 4 and 38. The sequences encoded in the viral proteins NS-1, NS-2, VP-1, and VP-2 are illustrated with the reading frames expressed in each part of the molecule shade-coded (□ =1, □ =2, □ =3). Figure modified from S.F. Cotmore and P. Tattersall, 1987.
93 map units (Clemens and Pintel 1987, Pintel et al. 1983). Translation of the viral mRNAs in rabbit reticulocyte lysates and immunoprecipitation of the products with sera from animals infected with different autonomous parvovirus serotypes indicated the presence of two nonstructural polypeptides, NS-1 and NS-2. The NS-1 protein co-migrated with VP-1 (MW = 83 kDa) and NS-2 at an apparent molecular weight of 25 kDa (Cotmore et al. 1983).

C. The NS-1 Polypeptide

The large nonstructural protein was shown to be the primary translation product of the R1 transcripts (Cotmore et al. 1983). The first AUG codon in the R1 transcripts occurs at nucleotide 261 and the first in-frame stop codon at nucleotide 2277, just prior to the small splice site (Astell et al. 1983, Ben-Asher and Aloni 1984). Bacterial fusion proteins have been made including regions within the R1 transcript, in all three reading frames, and subsequently used to generate polyclonal antibodies. Antibodies directed against regions in the predicted large open reading frame immunoprecipitate NS-1 from in vitro translations making it highly unlikely that NS-1 contains sequences downstream of the 2277 termination codon, initiates at an AUG codon downstream of the 261 codon, or contains an undetected splice resulting in a reading frame shift (Cotmore and Tattersall 1986b, Cotmore and Tattersall 1987). Although the NS-1 protein has an apparent molecular weight of 83 kDa, the predicted protein encoded by the R1 transcripts would be 672 amino acids long and have a molecular weight of only 77 kDa. Sub-cellular fractionation and immunoprecipitation of MVM-infected LA9 cells identified the predominant NS-1 species as a nuclear-localized phosphoprotein with an apparent molecular weight of 83 kDa and a highly phosphorylated form running at 85 - 88 kDa (Cotmore and Tattersall 1986a).

Figure 1 illustrates the positions of the transcripts and the proteins encoded by the MVMP genome. To maximize the use of a limited size genome, the transcription strategy of MVM utilizes overlapping ORFs. NS-1 and NS-2 both have the same initiation codon and share a common amino-terminus, but due to the large splice present in the R2 transcripts and
the resulting reading frame shift, NS-2 has a carboxy-terminus different than NS-1. Similarly, the capsid proteins VP-1 and VP-2 are both translated from P38-initiated mRNAs, but due to alternative splicing patterns possible at the small splice site, the proteins initiate at different AUG codons starting at nucleotides 2287 and 2795 respectively (Jongeneel et al. 1986, Morgan and Ward 1986).

The NS-1 polypeptides of paroviruses show a high degree of structural conservation and amino acid homology. Sera from animals infected with canine parovirus, porcine parovirus, or the rodent paroviruses, H-1, H-3, or KRV all recognize the MVM NS-1 molecule. On the contrary, capsid polypeptides from different viruses rarely cross-react, indicating a higher degree of structural conservation of the NS-1 polypeptides than the capsid proteins. (Cotmore et al. 1983). The leftward open reading frame has been shown by heteroduplex mapping (Banjeree et al. 1983) and by amino acid homology (Shade et al. 1986) to be highly conserved among the paroviruses. The region of highest homology corresponds to a 405 nucleotide sequence (nt 1428-1833 in MVM) that, at the amino acid level, shares greater than 99% homology with H-1, 95.5% with FPV, 41% with the human parovirus B19, and 51% with the helper-dependent parovirus AAV (Astell et al. 1987, Cotmore and Tattersall 1987). A sequence corresponding to the purine triphosphate binding site consensus sequence, G(X)₄GKT₅G(X)₅₆L/V, has been identified in this region (Anton and Lane 1986, Astell et al. 1987). Downstream of the 405 nucleotide conserved region, the homology between paroviral NS-1 molecules drops to ~16% among B19, MVM, and AAV, but remains relatively high at ~60% among the rodent paroviruses. The highly conserved nature of NS-1, both in primary amino acid sequence and in antigenic structure points to the importance of NS-1 structure and function.

The role of NS-1 is not well understood although several experiments indirectly suggest possible functions for NS-1. First, co-transfection experiments with plasmids containing the NS-1 gene and a reporter gene under the control of either of the two viral promoters have shown that NS-1 up regulates the P38 capsid promoter and down regulates its own P₄.
promoter and several cellular promoters (Doerig et al. 1990, Doerig et al. 1988, Rhode III 1985, Rhode III and Richard 1987). As would be expected with this type of regulation, MVM-infected cells show the P38-derived R3 transcripts appearing slightly after the P4-derived R1 and R2 transcripts (Clemens and Pintel 1988, Tullis et al. 1988). The R3 transcripts are also approximately four times more abundant than the R1 and R2 transcripts late in the infection cycle (Pintel et al. 1983). Contrary to the expected pattern, expression of NS-1 and the capsid proteins is not found to be temporally regulated. They are detected almost simultaneously early in infection (Cotmore and Tattersall 1987) although later in infection, levels of NS-1 expression fall and capsid expression rises. In light of all of these studies, it has been proposed that NS-1 is a transcriptional regulator.

The second postulated function of NS-1 is in viral DNA replication. First, frameshift mutations which affect the NS-1 gene, but not the NS-2 gene, were made in an infectious plasmid containing the entire genome of MVM and found to prevent viral duplex DNA replication (Merchlinsky et al. 1983). In the related rodent virus, H-1, similar mutations also prevented DNA replication, but this function could be restored by co-transfection with a second plasmid containing an unmutated NS-1 gene (Rhode III 1982). Second, when some transformed cell lines are infected with MVM, there is increased viral DNA synthesis correlating with an increase in NS-1 production even though the full productive infection appears to be blocked at a later stage (van Hille et al. 1989). Third, a fraction of the NS-1 molecules have recently been found covalently bound to the 5' terminal of the MVM genome and certain replication intermediates (Astell et al. 1983, Cotmore and Tattersall 1988, Gunther and Tattersall 1988). The current model for the replication of the MVM genome proposes the requirement for a site-specific nickase/helicase function to be provided, similar to that of the gene A protein of ØX174, (Astell et al. 1983, Astell et al. 1985, Cotmore and Tattersall 1988). The fact that NS-1 is bound to the viral genome makes it an ideal candidate for this function. In the helper-dependent parvovirus AAV, the rep proteins are analogous to the nonstructural proteins of the autonomous parvoviruses, and have been shown to
specifically recognize and bind to the terminal hairpins (Ashktorab and Srivastava 1989, Im and Muzyczka 1989). Finally, Bodnar et al. (1989) have shown that MVM DNA is associated with the nuclear matrix of infected cells through the 5' end and a 60 kDa host encoded nucleolar antigen designated TP (Bodnar et al. 1989, Walton et al. 1989). Presumably, NS-1 is involved in this interaction as well. Bodnar suggests that the nucleoli are the functional sites of viral DNA replication with the MVM DNA being targeted to the nucleolar attachment sites by NS-1 (Walton et al. 1989).

Two other functions have been postulated for NS-1. The first is a role in packaging of the viral DNA into the virions. NS-1 was found on the outside of the virions, still covalently bound to the 5' end of the genome (Cotmore and Tattersall 1989, Faust et al. 1989). Removal of the NS-1 molecule by nuclease digestion or protease digestion did not affect the infectivity of the virions, ruling out that NS-1 is involved in receptor recognition (Cotmore and Tattersall 1989). Therefore, Cotmore and Tattersall believe that NS-1 may be directly involved, or at the very least complicate, the packaging mechanism. The second function is as an ATPase. The purine nucleotide binding sequence mentioned previously shows the highest degree of homology to the large T antigen of polyoma viruses and SV40 (Anton and Lane 1986, Astell et al. 1987). This region in SV40 large T antigen has been implicated in an ATP-dependent helicase function, leading to the postulation that MVM NS-1 may have a similar function (Astell et al. 1987). The large size of NS-1 may permit it to be a multi-functional protein, with phosphorylation playing a role in the regulation of these activities.

In order to determine the functions of NS-1, several attempts have been made to overexpress NS-1. Attempts at producing mammalian cell lines that constitutively express H-1 NS-1 under the control of its own P_4 promoter failed. H-1 NS-1 under the control of the P_38 promoter was constitutively silent, but when induced by exogenous NS-1, the P_38/NS-1 cell line expressed normal levels of NS-1 transiently prior to cell death (Rhode III 1987). Attempts at producing stable HeLa cell lines capable of expressing the left hand open reading frame of B19 also failed unless the NS gene was mutated to prevent synthesis of the protein.
product (Ozawa et al. 1988). Similar results were obtained for MVM NS-1 (Moir et al. 1987, Pintel et al., unpublished results, St. Amand and Astell, unpublished results). These results have led to the belief that NS-1 may be the cytotoxic agent responsible for MVM-mediated cell lysis, and that high concentrations of NS-1 impair long-term viability of cell lines expressing NS-1.

D. The Baculovirus Expression System

The baculovirus expression system developed by Smith et al. (1983) has been used to produce high levels of foreign proteins in insect cells. This helper-independent viral vector system utilizes the strong polyhedrin gene promoter of Autographa californica (multiple nucleocapsid) nuclear polyhedrosis virus (denoted AcNPV or AcMNPV) to produce functional, post-translationally modified proteins (reviewed by Luckow and Summers 1988b, Miller 1988). The advantages of this system are that it carries out post-translational modifications, does not employ the use of transformed cells or transforming elements, and that the virus has been found to infect only invertebrates and is therefore of relatively low safety hazard.

The family Baculoviridae has over 400 known members characterized by an enveloped, rod-shaped nucleocapsid enclosing an 80 - 200 kb double-stranded DNA genome (reviewed by Doerfler and Bohm 1986, Granados and Federici 1986). Subclassification of these viruses is based on the mechanism by which the viral envelope is obtained. The subgroup A viruses (or nuclear polyhedrosis viruses) obtain their lipid envelope by an intranuclear envelopment process and are capable of being occluded into proteinaceous nuclear organelles known as polyhedra. These occlusion bodies contain several virions each (Miller 1988) and will be discussed later with regard to the baculovirus life cycle. Further classification into single nucleocapsid polyhedrosis viruses (SNPVs) or multiple nucleocapsid polyhedrosis viruses (MNPVs) is possible depending on the number of nucleocapsids packaged in each lipid envelope. A member of the latter group, AcNPV, is the best characterized of all the
baculoviruses.

The nuclear polyhedrosis viruses produce two types of infectious virions, excluded virus (EV) and occluded virus (OV). The EV form is produced first, budding off from infected cells at around eighteen hours post-infection (h.p.i.). This form of the virus is responsible for the systemic infection of the insect via cell to cell transmission through the hemolymph. The OV, on the other hand, is responsible for the horizontal transmission from insect to insect usually via the ingestion of contaminated leaves. Late in infection, OVs are packaged into a paracrystalline matrix which protect the viruses from environmental factors. A highly alkaline environment such as an insect's midgut breaks down the polyhedra and releases the viruses. There, they then infect the new host (Granados and Federici 1986).

The major component of the protective polyhedra is a 29 kDa polyhedrin protein (Rohrman 1986). The gene encoding this protein is classified as a 'very late' phase gene, with transcription occurring 18 - 24 h.p.i. and protein expression being detected from 20 h.p.i. to 70 h.p.i. when cell lysis occurs. In insect cell culture, AcNPV forms greater than 30 polyhedra per nuclei and the polyhedrin protein consists up to 60% of total 'Coomassie blue-stainable' protein (Matsuura et al. 1987, Potter et al. 1976). Most baculovirus expression systems work by the substitution or insertional inactivation of the polyhedrin gene, placing foreign genes under the control of the strong polyhedrin promoter. In this case, only OV production is disrupted, not EV production. Because the polyhedrin gene is non-essential for viral replication and is a late-expressed gene, there is minimal pressure to eliminate foreign genes put in its place (Miller 1988).

A number of AcNPV transfer vectors have been designed for the insertion of foreign genes into the baculovirus genome. They all rely on the in vivo recombination of homologous regions in the vector with the AcNPV genome for the replacement of the polyhedrin gene with the gene of interest. The virus can be grown in insect cell culture in Sf9 cells, a clonal isolate of the IPLB-SF-21 AE cell line of Spodoptera frugiperda (fall army worm) (Summers and Smith 1987). The recombination event occurs during the co-transfection of the vector DNA
with viral genomic DNA into uninfected Sf9 cells. Cells infected with recombinant viruses can be selected for based on the lack of occlusion bodies in the nuclei of infected cells (occ⁻ phenotype) and the recombinant viruses can then be purified from these cells in the EV form (Summers and Smith 1987). All of the transfer vectors contain the polyhedrin flanking sequences including the upstream promoter sequences and the downstream poly-adenylation site, as well as a unique restriction site placed just downstream of the polyhedrin promoter (reviewed by Kang 1988, Luckow and Summers 1988b, Miller 1988). Various vectors differ in the amount of upstream and downstream flanking sequences retained.

As with most other expression systems, the level of protein expression of individual foreign genes is highly variable. A number of factors have been found to affect protein expression levels in insect cells; the most well-studied being the upstream and downstream sequences required for high level expression (Luckow and Summers 1988a, Matsuura et al. 1987). The region from -60 to -1 of the polyhedrin promoter appears to be sufficient for expression. The most popular baculovirus vector, pAc373, deletes the -7 to +670 region of the polyhedrin gene (Smith et al. 1983), although higher levels of expression have been reported if the -7 to +1 nucleotides are retained such as D. H. Bishop's pAcYM1 vector or M. D. Summers' new pVL941 vector (Matsuura et al. 1987, Summers 1988). The polyhedrin coding region itself does not appear to contain sequences required for high-level expression. The pAcYM1 vector actually contains a deletion from the +2 nucleotide to a point 13 nucleotides past the polyhedrin termination codon (Matsuura et al. 1987). Other factors found to affect protein expression are the leader and downstream sequences of the inserted gene. The length of the leader and trailing sequences (Kang 1989, pers. comm.), the ribosome binding site, and the sequence immediately upstream of the initiation codon (Miller 1988) appear to play some role in determining the level of expression. Whether or not these are the only factors affecting expression is not known and is being actively investigated.

The efficiency of this system has been demonstrated by the very abundant expression of numerous non-invertebrate proteins in the invertebrate host. These diverse proteins range in
source from bacteria [E.coli β-galactosidase, (Summers and Smith 1987)] to higher vertebrates [humans β-interferon (Smith et al. 1983)] and in size from 15.5 kDa [human interleukin-2 , (Smith et al. 1985)] to 160 kDa [HIV env, (Hu et al. 1987)]. Correct post-translational modifications have been shown for many of the proteins including phosphorylation, N-glycosylation, O-glycosylation, myristylation, and palmitylation (see reviews by Summers 1989, Kang 1988, Luckow and Summers 1988b, Miller 1988). Although these post-translational modification systems are present in insect cells, whether these systems operate identically to those in mammalian systems has yet to be proven. The Sf9 glycosylation pathway has been shown to process high mannose sugars in a manner similar to the mammalian processing pathway, but not as efficiently nor identically (Jarvis and Summers 1989, Kuroda et al. 1990). Correct proteolytic cleavage of recombinant precursor proteins, and of several mammalian signal sequences has also been seen, as has correct targeting of the proteins to cellular locales. It is therefore not surprising and in fact very encouraging that many of the expressed proteins retain their original antigenicity, immunogenicity, and biological activities (see reviews by Kang 1988, Luckow and Summers 1988b, Miller 1988).

E. Project Purpose

Prior to the beginning of this project, G. Brown constructed the pUC19/D plasmid (Figure 2), which contains the PstI D fragment of MVM cloned into the PstI site of pUC19 (Brown 1987). This fragment, spanning nucleotides 1349 - 2125 of the MVMp genome, codes for a fragment of NS-1 as well as partially overlapping exon 2 of the NS-2 gene. Due to the reading frame shift required for NS-2, the PstI D fragment is specific for NS-1 if the correct NS-1 reading frame is maintained. Transformation of E.coli JM83 with the pUC19/D plasmid results in the expression of a 33 kDa β-galactosidase-NS-1 fusion protein.
Figure 2 The construction of pUC19/D.
The sequences encoding NS-1 and NS-2 are shown above the line diagram of the MVM viral DNA strand illustrating the PstI sites in the genome. The reading frame of each expressed part of the molecule is shade-coded. Below the line diagram shows the position of the PstI D fragment into the unique PstI site within the multiple cloning site of pUC19 just downstream of the lacZ promoter.
designated lacZ/NS-1 (Brown 1987). Brown purified this fusion protein from SDS-polyacrylamide gels and used it to immunize Balb/C mice for the production of monoclonal antibodies against lacZ/NS-1 (Yeung et al. 1990, manuscript in prep.) Six positive colonies secreting antibodies against lacZ/NS-1 were isolated, two of which were subcloned to monoclonality by C. R. Astell. The other four colonies were frozen away at the primary subcloning stage. Concurrently, initial work on the construction and isolation of a NS-1 encoding baculovirus was carried out by R. Russnak of this laboratory. During the course of my work, the baculovirus project was continued by W. Chen and G. Wilson in this laboratory (Wilson et al. 1990, manuscript in prep.)

This thesis will describe the characterization of the six monoclonal antibodies and demonstrate their use in the detection of MVM NS-1. As well, use of the antibodies in the characterization of a NS-1 fusion protein and of a full-length NS-1 protein, expressed in insect cells, will be described. One of the monoclonal antibodies (CE10) was purified from mouse ascitic fluid, coupled to cyanogen bromide-activated Sepharose 4B, and used in the immunoaffinity purification of both proteins from recombinant baculovirus-infected Sf9 cells.
VIII. Materials and Methods

A. Materials

All chemicals were analytical or reagent grade and were either from BDH Inc., Fisher Scientific, or Sigma Chemical Co. unless otherwise specified. All polyacrylamide gel electrophoresis reagents and protein molecular standards were obtained from Bio-Rad Laboratories; except for Ultrapure SDS, Ultrapure Tris, pre-stained High Molecular Weight Standards, and unstained High Molecular Weight Standards which were from Bethesda Research Laboratories; and Dalton Mark VII-L unstained molecular weight markers and SDS-6H high molecular weight standard mixture from the Sigma Chemical Co. E. M. grade paraformaldehyde was obtained from JBEM.

Bactotryptone, yeast extract, and bactoagar were purchased from Difco Laboratories. Penbritin-1000 (ampicillin) was from Ayerst Laboratories. All cell culture reagents and chemicals were Sigma Chemical Co. tissue culture grade unless otherwise specified. Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle's Medium (DMEM), Met-DMEM, TC-100 medium, and TNM-FH medium were obtained from Gibco Canada Inc. as was the fetal bovine serum (FCS). DMSO was from the Eastman Kodak Co.

Guinea pig anti-TP polyclonal sera was the kind gift of Dr. J. W. Bodnar (Northeastern University). The anti-rhodopsin mouse monoclonal antibody 2B2 was the generous gift of Dr. R. S. Molday (University of British Columbia). The mouse monoclonal sub-isotyping kit was from Hyclone Laboratories and secondary antibodies were purchased from Bethesda Research Laboratories or from Jackson Immunoresearch through Promega Biotec. Nitrocellulose and Optibind NC were obtained from Schleicher & Schuell. The 5-bromo-4-chloro-3-indoyl phosphate / nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase development system was purchased from Bethesda Research Laboratories. Falcon MicroTest III flexible assay plates were from Becton-Dickson.

$^{[35}S\text{-Methionine (1100 Ci/mmol), cell labelling grade [}^{35}S\text{-Methionine (600 Ci/mmol),}$
and $[\alpha^{32}\text{P}]-\text{dATP}$ (3000 Ci/mmol) were obtained from NEN Research Products. Deoxynucleotide triphosphates and $\alpha$-phosphorothioate deoxynucleotide triphosphates were from Pharmacia P-L Biochemicals.

Restriction enzymes, React buffers, RNase T$_1$, and T4 DNA Ligase were obtained from Bethesda Research Laboratories. DNA Polymerase I Klenow fragment and Exonuclease III were purchased from Promega Biotec and S1 nuclease from Pharmacia P-L Biochemicals. Sequenase, sequencing mixes, and sequencing reagents were from the United States Biochemical Corp.

Ultrapure Agarose was also from Bethesda Research Laboratories. Sep-Pak C$_{18}$ cartridges were obtained from the Millipore Corp., and acetonitrile was from the Aldrich Chemical Co. Geneclean was purchased from Bio101 or Promega Biotec. Curix RP-1 film was from Agfa-Gevaert.

DEAE-Sephacel, CNBr-activated Sepharose 4B, Protein A Sepharose CL-4B, and Protein G Sepharose 4 FF were from Pharmacia P-L Biochemicals. X-OMAT AR film was obtained from the Eastman Kodak Co.

B. Bacterial Strains, Cells, Viruses, and Media

1. Plasmids

Plasmid pUC19/D, was obtained from Grant Brown (this laboratory) and its construction has been described elsewhere (Brown 1987).

Plasmids pAcRP6/$\Delta X$ and CPss/NS1/pAcYM1 were constructed by Dr. Roland Russnak (this laboratory). Schematic diagrams of the construction of these two baculovirus transfer vectors, and the pAcNS1 construct to be described later are given in Appendix I. For the construction of pAcRP6/$\Delta X$, the 2.3 kb Hgal fragment of MVM was first isolated from a Hgal digest of pMM984, an infectious clone containing the entire genome of MVM (Merchlinsky et al. 1983). The ends of this fragment were filled-in with T4 DNA Polymerase and blunt-end cloned into the filled-in BamH1 site of the baculovirus transfer vector, pAcRP6 (Matsuura et
producing pAcNS1. Finally, this construct was digested with XhoI, filled-in with T4 DNA Polymerase, and religated creating a frame-shift mutation at the unique XhoI site in the NS-1 gene (refer to Appendix Ia).

In order to construct CPss/NS1/pAcYM1, the 2.3 kb Hgal fragment of MVM was obtained by a Hgal digest of pMM984. The fragment's ends were filled-in with T4 DNA Polymerase and blunt-end cloned into the SmaI site of pGEM-4Z producing NS1/pGEM-4Z. The preceruloplasmin signal peptide encoding region was purified from the clone λhCP1 (Koschinsky et al. 1986) in the following manner. First, the 1.2 kb EcoRI cDNA was cut out of the clone and then re-ligated back in the opposite orientation allowing purification of a 150 bp HincII/SstI fragment. This DNA piece contains the upstream regulatory sequences as well as the sequence encoding the first 47 aa of preceruloplasmin. The 150 bp fragment was used to replace the 161 bp SstI/EcoRV fragment in NS1/pGEM-4Z. To move the new CPss/NS1 construct into the transfer vector, pAcYM1 (Matsuura et al. 1987), the 2.3 kb SstI/HindIII fragment was cut out and the ends filled-in with T4 DNA Polymerase allowing the fragment to be cloned into the SmaI site of pUC1813 (Kay and McPherson 1987). This step was the equivalent of placing BamHI linkers onto the ends, facilitating cloning of the BamHI CPss/NS1 fragment into the BamHI site of pAcYM1 (refer to Appendix Ib). This final vector was expected to encode a chimeric protein consisting of the preceruloplasmin signal sequence fused to NS-1 that is missing the first 41 aa of its amino-terminal. In vitro transcription and translation demonstrated synthesis of a protein slightly larger than full length MVM NS-1 (R. Russnak, pers. comm.).

The plasmid pAcYM1/NS-1 was constructed by Dr. Wei Chen of this laboratory. Briefly, the 2.3 kb Hgal fragment of MVM was isolated from a Hgal digest of pMM984. BamHI linkers were ligated to this fragment and the resulting fragment was then cloned into the BamHI site of pUC19. The NS-1 encoding fragment was then transferred to pAcYM1 by excision using BamHI and ligation at the unique BamHI site in the baculovirus transfer vector (refer to Appendix Ic).
2. Bacteria

A K-12 derivative, *E. coli* strain JM83 [ara, Δ(lac-proAB), rpsL(= strA), φ80, lacZΔM15] (Yannisch-Perron et al. 1985) was used to maximize expression of *lacZ* fusion proteins encoded by the pUC19 plasmid derivatives (Brown 1987). This strain was propagated in YT media (8 g/l bactotryptone, 5 g/l yeast extract, and 5 g/l NaCl) at 37 °C with vigorous shaking, or on YT plates (YT media plus 15 g/l bactoagar) incubated 16 hours at 37 °C. Bacteria containing pUC19 plasmids were selected using ampicillin (Amp) in the plates or media at a concentration of 50 µg/ml.

3. Hybridomas

Six Balb/c splenocyte:NS-l hybridoma cell lines secreting antibodies against the *lacZ/NS-l* fusion product encoded by the pUC19/D construct were isolated by G. Brown according to a modified method of Köhler and Milstein (Campbell 1984, Köhler and Milstein 1975). Two of the lines (CE10 and BE2) had been subcloned three times by limiting dilution while the other four (AC6, EA2, CH10, and BC4) had not been subcloned. All were supplied in 0.5 ml aliquots stored under liquid nitrogen (N2).

Cells were thawed in a 37 °C water bath and the contents transferred to a 15 ml conical tube containing 10 ml of (IMDM). Cells were spun down [300 x g, 5 min, room temperature (RT)], the supernatant removed, and the cell pellet resuspended in 5 ml of IMDM + 5% FCS with Balb/c splenocyte feeder cells (see following). Hybridomas were then grown in plates at 37 °C and 5% CO2 in a humidified incubator. Splitting of the cells was done by dilution using IMDM + 10% FCS without feeder cells.

Spleen feeder cells were prepared using the methods of Campbell (1984). Using sterilized instruments an 8 - 12 week old Balb/c mouse was killed by cervical dislocation, washed in 70% ethanol, and its spleen removed to a sterile 10 cm petri dish. Once in the sterile hood, the spleen was washed twice with IMDM. In 5 ml of fresh media, the spleen was teased apart with two 18-G needles. The cell suspension was drawn into a sterile 5 ml syringe, passed one-way through a 20¹/₂-G needle four times, and twice through a 22-G
needle with the cell suspension ending up in a sterile 15 ml conical tube. Feeder cells were spun for 5 min at 300 x g and then washed once with IMDM. Finally, the cell pellet was resuspended in 50 ml of IMDM + 10% FCS and incubated at 37 °C in a humidified CO2 incubator. The conditioned media and feeder cells were used either fresh or up to a maximum of seven days after preparation.

The four uncloned hybridoma lines were subcloned three times by limiting dilution to assure monoclonality. The day before the cloning, 100 μl of feeder cell suspension were laid down per well in 96-well plates. On the day of the cloning, the hybridomas were counted using a Nebauer haemocytometer and diluted to $10^4$ cells/ml in IMDM. This dilution was then serially diluted down to 10 cells/ml, 5.0 cells/ml, and 2.5 cells/ml using IMDM + 20% FCS. Each of these dilutions were plated out at 100 μl/well in the 96-well plates containing the feeder cells and then incubated at 37 °C under normal growing conditions for 7 - 12 days. Wells with a single visible colony per well were assayed by ELISA or Western blot against lacZ/NS-1 (see Materials and Methods sections E, F & G). Positive clones were gradually expanded up to >10⁷ cells and reduced down to a 10% FCS concentration requirement. At this point the cells were subcloned again and/or frozen away.

Hybridomas were frozen in IMDM + 20% FCS + 20% dimethyl sulfoxide in 0.5 ml aliquots at a concentration of 5 x 10⁶ to 5 x 10⁷ cells/ml. Aliquots were then placed at -20 °C for 1 hour, -70 °C overnight, and then transferred to liquid N₂ (-185 °C).

4. MVM and A9 cells

The prototype strain of MVM was grown in a continuous mouse L-cell derivative, both originally supplied by Dr. Peter Tattersall (Yale University). These cells are A9 ouabab11 cells, a ouabain resistant derivative of the HGPRT- mouse fibroblast cell line A9 (Tattersall and Bratton 1983). Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) + 5% FCS in a water jacketed incubator set to 37 °C and 5% CO2. Continuous cultures were split approximately once a week by trypsinization and dilution in DMEM + 5% FCS.

The A9 cells were grown asynchronously, or else synchronized by the double block
method of Cotmore and Tattersall (1987) as follows. The cells are grown in isoleucine
deficient (Ile-) DMEM + 5% dialyzed FCS for 48 hours causing the cells to accumulate in the
G₀ phase of the cell cycle. The cells are released from the isoleucine block while being
exposed simultaneously to aphidicolin at a concentration of 5 μg/ml in DMEM + 5% FCS. At
this point the cells can also be infected with virus at the appropriate multiplicity of infection
(MOI) for each experiment. The result of this second block is that the cells, still somewhat
asynchronous, leave G₀ and accumulate at the G₁-S boundary due to the inhibition of DNA
Polymerase alpha by aphidicolin. Virus is taken up by the cells but not replicated because of
the requirement for the cell to be in S phase. After 20 hours the cells are released from the
second block by three successive 5 minute washes with DMEM. The cells are then returned
to normal growing conditions as stated above. The start of the first wash is referred to as t =
0.

Asynchronous cells were infected by a different method. In this case, infection occurs by
overlaying the cells with virus at an appropriate MOI in the minimum volume necessary. The
virus is allowed to attach to the cells for 1 hour at 37 °C with occasional rocking. At the end
of this time, the overlay is aspirated off and the cells returned to normal growing conditions.
The time of infection here refers to the point when the virus was first added.

5. Baculovirus and Sf9 cells.
Sf9 cells (ATCC# CRL1711) are derived from a clonal isolate of Spodoptera frugiperda
IPLB Sf21 AE cells (Summers and Smith 1987). These cells were the generous gift of Dr. C.
Yong Kang (University of Ottawa) and of Dr. Frank Graham (McMaster University). The
media used was either TC-100, or TNM-FH. All were supplemented with 10% FCS. Cells
were propagated in closed tissue culture flasks or in plates placed inside a closed, humidified
container. Both types of closed vessels were kept inside a 27 °C incubator. Monolayer
cultures were split every two to three days as described by Summers and Smith (1987),
except that they were split 1:3 in media without FCS and were allowed to attach to the plate
or flask surface in the absence of FCS for 1 - 1½ hours. Alternatively, spinner cultures were
maintained for large scale infections as described by Summers and Smith (1987) with two exceptions. First, TC-100 media was used instead of TNM-FH and second, cells were split 1:3 or 4 instead of 1:5.

The wild type baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV) was the kind gift of Dr. C. Yong Kang (University of Ottawa). The recombinant baculoviruses AcSec and AcΔXho were constructed by Dr. Roland Russnak (this laboratory), and AcNS-1 by Gary Wilson (this laboratory). AcSec, AcΔXho, and AcNS-1 were produced by homologous recombination of the wild type virus DNA with the plasmids CPss/NS1/pAcYM1, pAcRP6/ΔX, and pAcYM1/NS-1 respectively.

Infection of Sf9 cells was similar to that described for MVM infection of A9 cells. Because there is no requirement of AcNPV for the cells to be in any specific phase of the cell cycle, the cells were always grown asynchronously. For infection of plates, the media was removed by aspiration and virus added for at least $1^{1/2}$ hours at 27 °C. For time course studies the virus overlay was then removed and the cells returned to normal media; otherwise, fresh media was added directly to the viral inoculum. Infection of spinner cultures was as described by Summers and Smith, 1987. The time of the addition of the virus is termed $t = 0$.

Viral stocks were made by infecting plates or spinner cultures of Sf9 cells with the appropriate virus at an MOI of 0.1 - 1.0. After 72 h.p.i. the extracellular virus was harvested by collecting the media in conical tubes. Cells and cellular debris were removed by centrifugation for 10 min at 500 x g. Techniques for plaque assaying the virus stocks are given elsewhere (Summers and Smith 1987).

For the labelling of Sf9 cells with $[^{35}S]$-methionine, approximately $1 \times 10^7$ cells in 60 mm tissue culture dishes were infected as described above. Prior to labelling, the infected cells were gently pelleted (300 x g, 5 min, RT), washed once with Met$^-$ TNM-FH media, pelleted again, and resuspended in 2.5 ml Met$^-$ TNM-FH + 10% dialyzed FCS. Cell-labelling grade $[^{35}S]$-methionine (>600 $\mu$Ci/ mmol) was added to a final concentration of 150 $\mu$Ci/ml. After a
3 hr labelling period at 27 °C, the cells were gently pelleted as before, resuspended in TC-100 +10% FCS, and incubated at 27 °C. The time of the labelling post-infection is given in the Figure legends.

C. Purification of lacZ/NS-1

1. Mini-lysates

The lacZ/NS-1 fusion proteins were expressed in E. coli JM83 as described (Brown 1987) except that only 1.0 ml of the 5.0 ml overnight culture was used to prepare the mini-lysates.

2. Large scale purification

Large scale purification of the fusion protein is also detailed by Brown, 1987 and was employed with the following modifications. First, the pooled cell pellet was washed once with STE buffer (10 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA) before being resuspended in lysis buffer. Second, after homogenization, the volume was increased to 10 ml with lysis buffer before collection by centrifugation. Third, after resuspension in sample buffer, the aliquots were incubated at 100 °C for 5 min and then stored at -20 °C. When needed, these aliquots were thawed and run on a 16 cm x 16 cm x 1.5 mm preparative, 4%/12% discontinuous SDS polyacrylamide gel at 100V for 18 - 20 hours at 4 °C. Fourth, after the lacZ/NS-1 band was excised, the gel piece was cut into four pieces, placed inside four 2" x 3/4" dialysis bags containing gel running buffer (123.8 mM Tris, 96 mM glycine), submerged in running buffer in a BRL Mini-Sub DNA Electrophoresis Cell, and electroeluted. The protein was subject to electroelution for 3 hours at 100V in a 4 °C cold room. The eluate was then concentrated in Centricon-10 microconcentrators at room temperature according to the manufacturer's instructions. Washes were performed with KBS buffer [3 mM KCl, 1.5 mM KH2PO4, 137 mM NaCl, 8.1 mM Na2HPO4, 0.05% (v/v) Tween 20, 0.02% (w/v) NaN3]. The yield of protein was quantified as described by Brown, 1987.

D. Production and Purification of Monoclonal Antibodies
1. Production of monoclonal antibodies

Monoclonal antibodies were collected either as tissue culture supernatants or as ascitic fluid by the methods of Harlow and Lane, 1988. Ascitic fluid was stored at -70 °C until ready for use.

2. Sub-typing of monoclonal antibodies

Monoclonal antibodies were sub-typed by ELISA using a mouse monoclonal sub-isotyping kit according to the supplier's instructions.

3. Purification of monoclonal antibodies

Purification of the monoclonal antibodies was performed in a two-step process involving ammonium sulfate [(NH₄)₂SO₄] precipitation and DEAE-Sephacel chromatography. All steps in the (NH₄)₂SO₄ precipitation were done at 4 °C as described by Harlow and Lane (1988) with the following alterations. First, ascitic fluid was thawed and diluted 1:3 with PBS buffer (KBS without Tween 20 and without NaN₃) to reduce the viscosity of the solution. Secondly, the final pellet was resuspended in PBS buffer, but dialyzed against three changes of 0.01M sodium phosphate buffer (pH 8.0) overnight instead of PBS buffer.

Ion exchange chromatography of the dialyzate was done according to the method of Zola (1987) using DEAE-Sephacel. On later purifications a 20 - 250 mM NaCl gradient was used rather than a 0 - 500 mM gradient. One tenth column volume fractions were collected and then analyzed by A₂₈₀, SDS polyacrylamide gel electrophoresis, and ELISA (see Materials and Methods sections E & F). Salt concentrations in the fractions were determined by conductivity measurements. Monoclonal antibody containing fractions were pooled and the protein concentration determined using a Bio-Rad microassay based the method of Bradford (1976) modified by Bio-Rad laboratories. The pooled fraction was then either coupled to CNBr-activated Sepharose 4B as described later (see Materials and Methods section K) or lyophilized after a small sample had been tested to show that the mAb was in fact stable to lyophilization. DEAE-Sephacel was regenerated through sequential 0.5 N HCl and 0.5 N NaOH washes and then stored at 4 °C under 20% ethanol.
E. ELISAs

For the ELISAs, 50 μl of *lacZ/NS-1* at 1 μg/ml in KBS buffer was added to the wells of a Falcon 96-well MicroTest III flexible plate and dried down for 2 hours at 65 °C. The plate was then washed once with distilled water. Wells were blocked with 200 μl of KBS + 5% FCS per well unless the antibody solution used was a tissue culture supernatant. If this was the case, blocking was not required due to the presence of FCS in the supernatant. Following one washing with KBS, 50 μl of antibody solution was added and incubated for 1 hour at room temperature. After washing the plate three times with KBS buffer, 50 μl of alkaline phosphatase-linked rabbit anti-mouse immunoglobulin (rmIg) was added, at the manufacturer's recommended dilution, for 1 hour. The plate was then washed as before and developed with 100 μl of p-nitrophenyl phosphate (p-NPP) solution [1 mg/ml in 10 mM diethanolamine, 0.5 mM MgCl₂, 0.02%(w/v) NaN₃]. Color development was stopped after 45 min by the addition of 50 μl of 2M NaOH. The A₄₀₅ was read in a Titertek microtitre plate reader using the p-NPP solution with 2M NaOH as the blank.

For titration ELISAs, the procedure was the same except that dilutions were done in the wells. To do this, 100 μl of antibody solution was added to the first well of a row and 50 μl of blocking solution in the rest of the wells of the row. Serial doubling dilutions were made by removing 50 μl of solution from the first well, mixing it with the contents of the next well, and repeating this process across the row, finally removing 50 μl from the last well of the row.

For competition ELISAs, the monoclonal antibody solutions were used at the dilution that gave 80% binding efficiency, as determined by the titration ELISA. One μl of the competitor at 50X final competitor concentration was added and mixed into the wells immediately after the monoclonal antibody solution. All steps following the addition of the primary antibody and the competitor were the same as for the regular ELISAs. Peptide 1 (H₃N-PLASDLEDLAPWSTPNTPVAGTA-COOH) and peptide 2 (H₃N-PNTKIDNWYFHYEQQHVRMLRLC-COOH) were the generous gift of Dr. Ian
Clark-Lewis (Biomedical Research Centre, University of British Columbia).

**F. SDS Polyacrylamide Gel Electrophoresis**

SDS-polyacrylamide gel electrophoresis was performed using a Bio-Rad Mini-Protean II gel apparatus according to the manufacturer's instructions based on the method of Laemmli (1970). Gels were 0.75 mm thick with a 12% separating gel and a 4% stacking gel, and run at 200V for 45 - 60 minutes at room temperature. Alternatively, 8 cm x 14 cm x 0.75 mm gels were run on a Hoeffer SE600 apparatus at 30 mA constant current for 4 hrs. Proteins gels were stained and destained using Coomassie brilliant blue, and then dried down at 80 °C for 1 - 3 hours.

**G. Immunoblots**

Samples were subjected to SDS-polyacrylamide gel electrophoresis as described previously and transferred onto nitrocellulose paper, or Optibind by electrophoretic elution (Towbin et al. 1979) using a Bio-Rad Mini-Trans Blot transfer cell or a Bio-Rad Trans Blot II transfer cell according to the supplied instructions. The transfer buffer used was 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Transfers were done at 150 mA constant current for 1 hour for mini-gels or 100 mA constant current for 21/2 hrs for the large gels.

Blots were immunostained as described by Harlow and Lane, 1988. Blocking solution was 5% FCS in KBS buffer. The primary antibodies used were unlabeled tissue culture supernatants. The secondary antibody was either alkaline phosphatase-conjugated rxmlg or biotinylated goat anti-mouse immunoglobulin, diluted as instructed by the manufacturer. If the biotinylated secondary antibody was employed, an extra 30 min incubation with streptavidin-alkaline phosphatase, according to the supplier's instructions, was included followed by washing with 5 changes of KBS. Development of the immunoblot was done using 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium.

**H. Immunofluorescence**

For immunofluorescence studies, cells had to be grown on or mounted on coverslips.
Acid-etched, 22 mm, circular coverslips were prepared by boiling in 1 M HCl for 10 minutes, washing thoroughly with 3 changes of distilled water, and finally storing under 95% ethanol until needed. When required, the coverslips were moved to the tissue culture hood, dried and sterilized by flaming, and placed inside sterile, 35 mm tissue culture dishes. For MVM and A9 cell studies, 1.0 x 10⁵ to 1.5 x 10⁵ cells were seeded per dish and incubated for 18 - 24 hours at 37 °C. Cells were then infected, or synchronized and infected, at an MOI of 0.1 to 1.0 pfu/cell. Parallel mock-infections were done using media without serum as the inoculum. For baculovirus and Sf9 cell studies, 1.0 x 10⁶ to 1.5 x 10⁶ cells were seeded and incubated for approximately one cell division. Again, cells were then infected at a MOI of 0.1 to 1.0 pfu/cell or mock-infected using media without FCS. Infection was allowed to proceed for the length of time stated in the Figure legends.

At the appropriate time point, cells were fixed, permeabilized, and immunostained. Dishes were rinsed once with PBS-C (PBS buffer with 0.7 mM CaCl₂, 0.5 mM MgCl₂) and then fixed with 3.68% (w/v) EM grade paraformaldehyde in PBS for 10 minutes. Cells were rinsed with three changes of PBS-C followed by the standard wash protocol consisting of three changes of wash buffer (PBS-C + 1% BSA) for 5 min each. Coverslips with the cells attached could be stored under wash buffer at 4 °C at this stage. The cells were permeabilized using 0.25% (w/v) Saponin in wash buffer. For permeabilization, the Saponin solution was left on top of the cells at room temperature for 45 minutes followed by the standard wash. Primary antibody in the form of a tissue culture supernatant was added for 1 hr at room temperature, again followed by the standard wash.

Immunofluorescent staining of the cells was performed in one of two ways. The first method used was a one-step process with a rhodamine-conjugated goat anti-mouse IgG, diluted 1:99 parts PBS-C after it had been centrifuged (5 min, 12,000 x g, RT) to remove unconjugated rhodamine particles. This secondary antibody was incubated for 1 hour at room temperature and unbound antibody was removed using the standard wash protocol. The second method was a two-step process involving the use of a biotinylated goat anti-mouse
IgG diluted according to the supplier's instructions. This antibody was incubated the same way as the rhodamine-conjugated antibody in the one-step method, washed, then incubated for 30 min with Texas Red-conjugated streptavidin, and washed again.

The coverslips were inverted and mounted on depression slides containing wash buffer. Coverslips were firmly seated onto the slide using a small amount of silicon grease, taking care not to trap air bubbles under the slide. Phase contrast light microscopy and fluorescence microscopy were done on a Zeiss Universal microscope equipped with an epi-fluorescence head.

I. Epitope Mapping

1. Basic Molecular Cloning Techniques

Many of the techniques used can be found in Maniatis et al. (1982) or Sambrook et al. (1989). These include methods used for: 1) isolation of single bacterial colonies, 2) growth, maintenance and preservation of bacterial strains, 3) small scale plasmid DNA isolation by alkaline lysis, 4) large scale plasmid DNA isolation by alkaline lysis, 5) purification of closed circular DNA by equilibrium centrifugation in cesium chloride-ethidium bromide gradients, 6) extraction of ethidium bromide with organic solvents, 7) preparation of frozen competent and calcium chloride competent E. coli, 8) electrophoresis of DNA in agarose and acrylamide gels, and 9) phenol:chloroform [50:50 (v/v)] extractions.

The plasmid DNA isolations had the following modifications added. For the small scale isolations, two phenol:chloroform extractions were included (Sambrook et al. 1989). In the large scale isolation (Maniatis et al. 1982), chloramphenicol and lysozyme were not used, the centrifugation after the potassium acetate addition was done in a Beckman Ti 45 rotor (30,000 rpm, 5 °C, 1 hr), and the final centrifugation was done in a Beckman Ti 70.1 rotor (60,000 rpm, 20 °C, 16 hrs).

DNA was precipitated using ethanol (Maniatis et al. 1982). A 1/10th vol of 3M sodium acetate was added, if necessary, to optimize the salt concentration of the DNA solution. This
was followed by 2 volumes of 95% ethanol. Precipitation was allowed to occur at 0 °C for 10 min after which the sample was spun down (12,000 x g, 7 min, 4 °C) and washed with 70% ethanol. The supernatant was discarded and the tube inverted on a paper towel to remove as much of the ethanol as possible. Finally, the DNA was dried in a vacuum desiccator for 5 minutes.

Restriction enzyme digests were performed according to the supplier's instructions. Reactions were stopped using an appropriate amount of Ficoll gel loading buffer (Sambrook et al. 1989). DNA bands were resolved on 1% agarose gels in TBE buffer (Sambrook et al. 1989).

### 2. Attachment of the Termination Oligomer

Two partially complementary synthetic oligomers were synthesized by Tom Atkinson (University of British Columbia) on an Applied Biosystems DNA Synthesizer. Oligomer #1 was a 16-mer with the sequence, 5'-p-GATCCTAAGTAATTAG-3'. Oligomer #2 was also a 16-mer, but with the sequence, 5'-AATTCTAATTACTTAG-3'. Crude oligomers were resuspended separately in 1 ml of TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA) + 500 mM NaCl. Two Sep-Pak C18 cartridges were equilibrated with 10 ml of acetonitrile followed by 10 ml of autoclaved, distilled water (dH2O). Each oligomer was applied to one of the two columns using a 5 ml syringe over a period of 3 - 5 min, washed with 5 ml of dH2O, and then eluted with 5 ml of 20% (v/v) acetonitrile. One ml fractions were collected.

Concentration of the oligomers was determined by A260. Cuvettes were cleaned beforehand using hydrogen peroxide for 15 min, followed by a thorough washing with dH2O. The molar extinction coefficients were calculated based on the sequence (Wallace and Miyata 1987) allowing spectrophotometric determination of oligomer concentrations. Fractions containing the oligomers were evaporated to dryness on a Savant Speed-Vac Concentrator for 2 hrs and stored at -20 °C.

Both oligomers were resuspended separately in dH2O at a concentration of 1 mM. Ten μl of purified oligomer #1 and 10 μl of purified oligomer #2 were added to 78.5 μl of dH2O, 1 μl of
1 M Tris-Cl (pH 8.0), and 0.5 µl of 200 mM EDTA, in a 500 µl Eppendorf tube. The solution was vortexed and placed in a 70 °C water bath for 5 min. The tube was left in the water bath as it was turned off. Both the tube and water bath were allowed to slowly cool down to room temperature after which 20 µl aliquots of the annealed 'termination linkers' were stored at -20 °C.

In order to replace the BamH1/EcoR1 fragment in pUC19/D with the termination linker, 3.75 µg of pUC19/D was subjected to a double digestion with 20 U of BamH1 and 20 U of EcoR1. After 2 hrs at 37 °C, the reaction mixture was heat-inactivated for 3 min at 68 °C and run on an 1% agarose TBE gel. The 3441 bp band was excised and the DNA was isolated using Geneclean following the supplier's instructions. The fragment was resuspended in 20 µl of dH2O. An aliquot of the annealed termination oligomers was thawed slowly at 4 °C to prevent strand separation, and ligated to the 3441 bp EcoR1/ BamH1 pUC19/D fragment using 2 µl of the fragment (~0.21 pmol), 2 µl of linkers (~20 pmol), 2 µl of 10 mM ATP, 2 µl of 150 mM DTT, 2 µl of 10X linker kinase buffer [660 mM Tris-Cl pH 7.6, 10 mM spermidine, 100 mM MgCl2, 2 mg/ml BSA], 9 µl of dH2O, and 1 Weiss Unit of T4 DNA ligase. The ligation was allowed to proceed for 4 hrs at room temperature. Frozen competent E. coli JM83 were transformed with 2 µl of the ligation mixture and plated on YT/Amp (50 µg/ml ampicillin). The plasmid DNA from the transformants was isolated and subject to restriction enzyme analysis to test for the loss of specific restriction enzyme sites. Positive confirmation of plasmids containing the termination linker was obtained by sequencing of the clones (see Materials and Methods section 1.4), now named pUC19/D.t

3. Nested Deletions

Unidirectional deletions were made in pUC19/D.t from the end encoding the carboxy-terminal of lacZ/NS-1. Because there were no convenient restriction sites that would leave a exonuclease III resistant 5' overhang, unidirectional deletions were made by protecting one end with an α-phosphorothioate deoxynucleotide analog (Guo and Wu 1982, Putney et al. 1981) as follows. Fifteen µg of pUC19/D.t was cut with 140 U of BamH1 for 2 hrs at 37 °C,
extracted once with phenol:chloroform, and precipitated with ethanol. The DNA was resuspended in 43.5 μl of dH2O and the BamH1 site was partially filled in using 5 μl of 10X NT buffer [500 mM Tris-Cl pH 7.2, 100 mM MgSO4, 1 mM DTT, 500 μg/ml BSA Pentax Fraction V], 1 μl of 8.46 mM α-phosphorothioate dATP, 1 μl of 10 mM dGTP, and 4.5 U of DNA Polymerase I Klenow fragment for 30 min at 37 °C. The reaction was terminated by the addition of 1 μl of 0.5 M EDTA. The volume was increased to 150 μl using TE buffer (100 mM Tris-Cl pH 8.0, 10 mM EDTA), extracted twice with phenol:chloroform, and precipitated with ethanol. For the second restriction enzyme digest, the DNA was resuspended in 100 μl of 1X React 3 and digested with 50 U of Sal I for 2 hrs at 37 °C. The enzyme was heat inactivated for 20 min at 68 °C, extracted twice with phenol:chloroform, and precipitated with ethanol.

Following incorporation of α-phosphorothioate dATP, deletions were made using exonuclease III. The DNA was resuspended in 54 μl of dH2O and 6 μl of 10X ExolIII buffer [660 mM Tris-Cl pH 8.0, 770 mM NaCl, 50 mM MgCl2, 100 mM DTT]. Eight 500 μl microfuge tubes were prepared containing 32 μl of ExolIII stop buffer [200 mM NaCl, 5 mM EDTA (pH 8.0)]. A total of 126 U of exonuclease III was then added to the DNA solution and 8 μl aliquots were removed at either 1 min or 30 sec intervals depending on the length of the deletion required. Aliquots were heat inactivated for 10 min at 68 °C, then precipitated with ethanol.

The DNA was then blunt-ended using S1 nuclease and DNA Polymerase I Klenow fragment. Aliquots were resuspended in 50 μl of S1 cocktail (250 mM NaCl, 30 mM KOAc pH 4.6, 1 mM ZnSO4, 5% glycerol, 67 U/ml S1 nuclease) and incubated for 30 min at room temperature. To each, 6 μl of S1 stop buffer (500 mM Tris-Cl pH 8.0, 125 mM EDTA) was added, followed by one extraction with phenol:chloroform, and precipitation with ethanol. Samples were resuspended in 20 μl of TE buffer, of which, 4 μl were subject to restriction digest to check the extent of the deletions, 6 μl was stored at -20 °C, and 10 μl was filled-in using DNA Polymerase I Klenow fragment as follows. A 10 μl volume of S1 nuclease-
treated DNA was added to 2.5 µl of 10X NT buffer, 1.0 µl of 2.5 mM dNTP mix, and 11.5 µl of dH₂O. This mixture was incubated with 4.5 U of DNA Polymerase I Klenow fragment for 30 min at 37 °C, and then heat inactivated for 15 min at 68 °C. Eleven µl of the blunt-ended DNA was added to 3.0 µl of 5X ligase buffer and 1 Weiss Unit of T4 DNA ligase, and ligation was allowed to proceed for 5 hrs at room temperature. A 1.4 µl aliquot of each of the time point's ligated DNA was used to transform frozen competent *E. coli* JM83 cells. All of the transformation mixture was plated on YT/Amp (see Materials and Methods section B2).

Colonies from each time point were subject to small scale plasmid isolation and restriction enzyme digest to roughly determine the deletion size. Accurate deletion sizing was obtained by sequencing of the individual plasmids as outlined below. *E. coli* mini-lysates were made, run on a SDS-polyacrylamide gel, and immunoblotted against the six mAbs.

4. Sequencing

The source of DNA for sequencing was either cesium chloride gradient purified DNA or DNA from small scale plasmid isolations. The latter had to be treated with 10 U of RNase T1 for 1/2 - 2 hrs at 37 °C followed by extraction with phenol:chloroform and ethanol precipitation before sequencing. Cesium chloride gradient-purified DNA was sequenced without pre-treatment.

Double-stranded plasmids were alkaline denatured and sequenced using Sequenase according to the protocol of Gatemann et al. (1988). The primer used was a 17-mer M13mp18 Universal Forward Primer. This method is basically the same as that recommended by the suppliers, except that the labelling/extension step was omitted allowing sequencing closer to the primer. The sequencing mixes were run on a 38 cm x 18 cm x 0.4 cm, 8% polyacrylamide gel containing 8M urea at 32W constant power for ~1 3/4 hrs until the bromophenol blue front was near the bottom of the gel. Autoradiography was done using Curix RP-1 film after the gel was dried down.
J. Immunoprecipitations

Most immunoprecipitations were done using Protein-A Sepharose CL-4B. Due to the poor affinity of Protein A for mouse IgG1 antibodies (Harlow and Lane, 1988), immunoprecipitations done using mouse monoclonal antibodies of this class were performed using řmIg coupled to Protein-A Sepharose CL-4B, and later using Protein G Sepharose 4 FF. Both Protein-A Sepharose CL-4B and Protein G Sepharose 4 FF were prepared according to the supplier's instructions, with the final resuspension being in KBS buffer (see Materials and Methods section C.2). The suspension was stored at 4 °C until needed. For coupling of the anti-mouse Ig antibody, 2.0 mg of řmIg was added to 1.0 ml of swollen Protein-A Sepharose CL-4B beads and rotated end-over-end overnight at 4 °C. The coupled beads were then washed with KBS buffer and stored in that buffer at 4 °C until required.

Cell lysates for immunoprecipitations were prepared as follows. Cells were washed three times with cold PBS-C buffer, transferred to a 1.5 ml Eppendorf tube (scraping if necessary), and then gently pelleted (300 x g, 5 min, 4 °C). The pellet was resuspended in 1 ml of cold RIPA buffer [10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40 (NP-40), 1% (w/v) sodium deoxycholate] followed by incubation for 30 min on ice. The lysate was cleared by centrifugation at 12,000 x g for 10 min at 4 °C, and then divided into 100 μl aliquots. The samples could be used immediately or quick-frozen using dry ice and stored at -20 °C.

For immunoprecipitations, lysate aliquots were pre-cleared with 100 μl of swollen beads. The pre-cleared supernatants and 100 μl of the mAb-containing tissue culture supernatants were then incubated for 4 - 12 hrs at 4 °C with end-over-end rotation. Following this, 50 μl of swollen beads were added and the mixture was incubated for a further 4 - 12 hrs. Immune complexes were pelleted by centrifugation (12,000 x g, 15 sec, RT), washed three times with 200 μl cold RIPA buffer, and then disrupted by boiling for 5 min in 10 - 25 μl of Laemmlli sample buffer. Samples were subject to SDS-polyacrylamide gel
electrophoresis as described before, dried down and autoradiographed on X-OMAT AR film.

For double immunoprecipitations, immune complexes were disrupted by boiling in 200 µl of RIPA buffer + 0.05% SDS + 5 mM β-Mercaptoethanol. Disrupted beads were removed by centrifuging for 12,000 x g at RT for 15 sec, with the supernatant being transferred to a fresh tube. This clearing step was repeated once more, after which the supernatant was subject to the second round of immunoprecipitation following the procedure given previously for the single immunoprecipitation.

K. Immunoaffinity column preparation

CNBr-activated Sepharose 4B was prepared according to the manufacturer's instructions. The pooled, purified antibody was dialyzed versus three changes of 0.5 M sodium phosphate buffer (pH 7.5) before coupling to the CNBr-activated beads according to the procedure of Harlow and Lane (1988).

L. Immunoaffinity Purification of CP/NS-1 expressed in Sf9 cells

The CE10 mAb column and the pre-column, containing glycine blocked CNBr-activated Sepharose CL 4B, were pre-equilibrated with Buffer I [50 mM Tris-Cl(pH 7.8), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, 50 µg/ml PMSF] without NaCl for 1 hr at a flow rate of 150 ml/hr.

2.2 x 10^8 Sf9 cells grown in 100 mm tissue culture dishes were infected with AcSec at an MOI of ~10. At 48 h.p.i. the cells were harvested as follows. The cells were resuspended in 10 ml of PBS-C by gentle pipetting and then pelleted (300 x g, 5 min, RT). The rest of the steps were performed at 4 °C. Cells were washed three times with cold PBS-C buffer and resuspended in 6 ml of hypotonic lysis buffer [10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 1.5 mM MgCl2] for 15 minutes on ice. Lysis was obtained by 30-60 passes with a tight-fitting Dounce homogenizer until 95% disruption occurred, as judged by phase microscopy. Nuclei and cellular debris were pelleted by centrifugation at 300 x g for 10 min at 4 °C and the supernatant transferred to a fresh tube. After the supernatant was adjusted to Buffer I
conditions, it was circulated through the pre-column four times.

The cleared lysate was applied to the CE10 mAb column and recirculated through the column for 1 hr at a flow rate of 100 ml/hr to allow complete binding. The first wash consisted of 150 ml of Buffer I at a flow rate of 100 ml/hr. The second wash consisted of 100 ml of Buffer II [50 mM Tris-Cl (pH 9.0), 1 mM DTT, 10% (v/v) glycerol, 50 μg/ml PMSF] at the same flow rate. Elution was achieved under strong alkaline conditions using one column volume of Buffer III [20 mM triethanolamine, 10% (v/v) glycerol, 50 μg/ml PMSF] followed by 100 ml of Buffer I. Fractions of 180 μl were collected in 20 μl of Buffer IV [500 mM Tris-Cl (pH 7.8), 10 mM DTT, 10% (v/v) glycerol] and analyzed by SDS polyacrylamide gel electrophoresis and immunoblot against the CE10 mAb. Fractions collected at various steps during the purification procedure were included in the analyses.

M. Immunoaffinity Purification of NS-1Ac expressed in Sf9 cells

1. Elution using peptide and alkali

The procedure used for the purification of NS-1 was similar to that of CP/NS-1 with the following exceptions. First, the Sf9 cells were grown and infected with AcNS-1 in a 100 ml spinner culture flask. Second, the volume of hypotonic lysis buffer the cell pellet was resuspended in was 8.5 ml instead of 6.0 ml and the final volume of the cell lysate after adjusting to Buffer I conditions was 10 ml. Third, application of the lysate to the pre-column matrix and to the CE10 mAb column matrix was done in batch suspension (end-over-end rotation at 4 °C for 1 hr) instead of in a column. After absorption to the pre-column matrix, the mixture was centrifuged at 900 x g for 3 min at RT and the supernatant added to the CE10 mAb matrix for binding in batch suspension as before. The beads were pelleted, washed once with Buffer I, poured into the column, and then washed with 150 ml of Buffer I as described before. Fourth, elution was first attempted with a two column volume 0 - 300 mg/ml peptide 1 gradient in Buffer II followed by 100 ml of Buffer II. Twenty-five 400 μl fractions were collected. After analysis of these fractions by SDS polyacrylamide gel electrophoresis, the
remaining bound material was eluted with alkali as described previously.

2. Elution using 6M guanidinium chloride

All steps were performed as for the peptide elution with the following alterations based on that of Tratner et al. (1990). First, PMSF was included in the hypotonic lysis buffer at a concentration of 50 μg/ml. Second, Buffer Ia (Buffer I with 500 mM LiCl instead of 100 mM NaCl) was substituted for Buffer I, and Buffer IIa [10 mM piperazine-N-N'-bis(2-ethanesulfonic acid) (pH 7.4), 5 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 50 μg/ml PMSF] for Buffer II. Third, elution was performed using 1 column volume of 6 M guanidinium chloride (~pH 6.2) followed by buffer Ia. Fractions (500 μl each) were dialyzed versus six changes of TM buffer [50 mM Tris-Cl (pH 7.9), 12.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol] before analysis.

3. Elution using acid

All steps were performed as for the peptide elution with the following alterations suggested by Harlow and Lane (1988). First, PMSF was included in the hypotonic lysis buffer at a concentration of 50 μg/ml. Second, Buffer Ib [10 mM phosphate (pH 8.7), 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, 50 μg/ml PMSF] was used in place of Buffer I, and Buffer IIb [10 mM phosphate (pH 6.8), 10% (v/v) glycerol, 50 μg/ml PMSF] in place of Buffer II. Third, elution was achieved using one column volume of Buffer IIIb [100 mM glycine (pH 2.5), 10% (v/v) glycerol, 50 μg/ml PMSF] followed by 100 ml of Buffer Ib. Fractions of 450 μl were collected in 50 μl of Buffer IVb [1 M phosphate (pH 8.0), 10% (v/v) glycerol].
IX. Results

A. Characterization of the mAbs

Attempting to generate monoclonal antibodies against the lacZ/NS-1 fusion protein, G. Brown identified six positive hybridomas secreting antibodies against the antigen after the first fusion. These were named CE10, AC6, BE2, EA2, CH10, and BC4 based on the plate letter and well number. The CE10 and BE2 mAb hybridomas were subcloned three times by C. Astell and the remaining four hybridoma cell lines were subcloned three times by the method of limiting dilution (see Materials and Methods section B.3) by P. Tam and myself. At this point, the mAb secreting cell lines were assumed to be monoclonal. Before these antibodies could be used in the detection of MVM NS-1, they had to be characterized with respect to iso-subtype in order to determine the route of production and purification.

Testing of the iso-subtype of the six monoclonal antibodies was done using a commercially available ELISA kit. Mouse monoclonal antibodies were subtyped based on the reaction of specific anti-heavy chain antibodies against each mAb. Of the six antibodies, CE10, AC6, BE2, EA2, and CH10 mAbs were found to be of the IgG\(_1\) class. The sixth mAb, BC4, was found to be of the IgG\(_{2a}\) class. Since each mAb reacted with only one anti-iso-subtype antibody each hybridoma cell line was assumed to be monoclonal, although the possibility that two or more antibody cell lines of the same subtype being present cannot be ruled out.

Next, the reactivity of the mAbs was determined. *E. coli* JM83 were transformed with pUC19 or pUC19/D. Total cell lysates of the transformed bacteria were prepared from liquid cultures and run on a discontinuous SDS-polyacrylamide gel. Figure 3 shows the Coomassie blue-stained gel and immunoblots of the lysates with the six mAbs. The lacZ/NS-1 band is visible in Figure 3A as a 30 kDa band in the pUC19/D lane only. On the immunoblots, all six mAbs react specifically against a 30 kDa band in the pUC19/D lanes (Figure 3B). Also visible in the same lane is an unidentified cross-reacting band at approximately 40 kDa.
Figure 3: Coomassie blue-stained gel of lacZ/NS-1 and immunoblots using the six monoclonal antibodies.

One ml aliquots of pUC19- and pUC19/D-transformed E. coli JM83 were lysed by boiling in Laemmli sample buffer. Five µl samples were run through a 4%/12% discontinuous SDS polyacrylamide gel and (A) stained with Coomassie-blue or (B) electrophoretically transferred to nitrocellulose. The blots were reacted with one of the six monoclonal antibodies (indicated below each blot) and developed. For further details refer to the Materials and Methods section.
Smearing of the bands is due to the presence of DNA in the total cellular lysates with the non-uniform concentration of the DNA in the sample producing the smearing in some of the blots while not in others.

The mAbs were tested for their ability to detect MVM NS-1. Asynchronous mouse fibroblast cells were infected with MVM or mock-infected. Cells were harvested at 24 h.p.i., total cellular lysates prepared, and aliquots run on a discontinuous SDS-polyacrylamide gel. Figure 4 shows a Coomassie blue-stained gel and immunoblots using all of the mAbs. Although overloaded, the stained gel shows two distinct bands in the MVM-infected lane not present in the mock-infected lysate at 83 kDa and 66 kDa. These two bands correspond to the expected sizes and proportions of the VP-1 and VP-2 proteins, respectively. On the immunoblots all mAbs reacted against an 83 kDa band present in the MVM-infected cells only. The CE10, AC6, and BE2 immunoblots show a cross-reacting 'smear' centered at 40 kDa in the MVM-infected cells only, likely due to degradation products of the 83 kDa band. As the antibodies were raised against an lacZ/NS-1 fusion protein and they recognize an 83 kDa band found in MVM-infected cells only, it was assumed that the antibodies recognized NS-1.

In order to test whether these six antibodies react against a more native form of the MVM NS-1 protein, the mAbs were used in indirect immunofluorescence studies of MVM-infected LA9 cells. A time-course immunofluorescence test was first done using only the CE10 antibody, to test the specificity of the CE10 mAb and determine the optimum time post-aphidicolin release for use in the immunofluorescence studies using the other mAbs. Synchronous LA9 cells were grown on glass coverslips, MVM-infected or mock-infected, and then fixed with para-formaldehyde at t = 2, 4, 6, 8, 10, and 12 hours post-aphidicolin release (h.p.r.). Cells were permeabilized with Saponin, incubated with the CE10 mAb, and detected with a Texas-Red fluorescence system. In this test the mock-infected cells showed no fluorescence (data not shown). In MVM-infected cells, fluorescence was found to be restricted to the nuclear region of infected cells. fluorescence was first visible at 2 h.p.r. and
Figure 4: Coomassie blue-stained gel of mock- and MVM-infected LA9 cell lysates, and immunoblots using the six monoclonal antibodies.
Asynchronous LA9 cells were mock- or MVM-infected at an M.O.I. of 10 and harvested at 24 h.p.i. The cell pellet was washed with PBS-C and then lysed in 100 μl of Laemmli sample buffer by incubation at 100°C for 5 minutes. Ten μl aliquots were run on a discontinuous SDS polyacrylamide gel and (A) stained with Coomassie-blue or (B) electrophoretically transferred to nitrocellulose. The blots were reacted with one of the six monoclonal antibodies (indicated below each blot) and developed. For further details refer to the Materials and Methods section.
increased in intensity in discrete foci in the nuclei through 4, 6, and 8 h.p.r. (see Figure 5). At 10 and 12 h.p.r., the fluorescence collected at the edge of the nuclear region while still increasing in intensity. From this experiment, the t = 8 h.p.r. time was chosen for the future study because of the ease of detection of the discrete foci.

The same procedure as before was used to test whether the other five mAbs could detect fluorescent nuclear foci in the MVM-infected cells fixed at t = 8 h.p.r. For the CE10, AC6, BE2, and EA2 mAbs, the results were as expected in that there was no fluorescence in the mock-infected cells (data not shown), and the nuclear foci were visible in the MVM-infected cells, albeit at differing intensities (Figure 6). With the other two mAbs, CH10 and BC4, a faint background fluorescence was detected in the mock-infected cells (data not shown). The fluorescent foci were still detectable against the background in the MVM-infected cells using the CH10 antibody, but not with the BC4 antibody.

B. Mapping of the epitopes

During the course of the characterization studies, R. Russnak isolated the recombinant baculovirus AcΔXho using the pAcRP6/ΔX construct. This recombinant virus was expected to produce a fusion protein containing 604 aa of NS-1 (Met1 - Leu604) fused to 71 aa of NS-2 caused by the reading frame shift at the filled-in XhoI site. On an immunoblot, he found that the CE10 mAb did not react against this protein, but that a polyclonal anti-NS-2 serum kindly provided by S.F. Cotmore and P. Tattersall (Yale University) did. Russnak interpreted this result to indicate that the CE10 mAb epitope resides in the 68 carboxy-terminal amino acids of NS-1 not present in the NS-1/NS-2 fusion protein. Of these 68 residues, only 18 were present in the lacZ/NS-1 fusion protein against which the CE10 mAb had been raised. It was concluded that the epitope of the CE10 mAb lay in these 18 residues (Russnak and Astell, unpublished results).

In order to test this hypothesis more fully, a set of carboxy-terminal deleted lacZ/NS-1 proteins were made by creating a set of nested deletions in pUC19/D and transforming E. coli
Figure 5: Immunofluorescence time-course study of MVM-infected, synchronous LA9 cells using the CE10 monoclonal antibody.

Synchronous LA9 cells grown on acid-etched, glass coverslips were mock- or MVM-infected at an M.O.I. of 10 and fixed with paraformaldehyde at 2, 4, 6, 8, & 10 h.p.r. Cells were permeabilized with Saponin, and then reacted with CE10. Detection was obtained with a biotinylated secondary antibody and a Texas Red-streptavidin conjugate. The top-most picture shows a bright field microscopy picture of LA9 cells at 10 h.p.r. under a 63X objective magnification. The lower pictures show the immunofluorescence observed in the MVM-infected cells at the various times post-release under the same magnification. For further details refer to the Materials and Methods section.
Figure 6: Immunofluorescence in MVM-infected LA9 cells using the CE10, AC6, BE2, EA2, and CH10 monoclonal antibodies. Refer to the Figure 5 legend. Cells were fixed with paraformaldehyde at 8 h.p.r. Detection was performed with the CE10, AC6, BE2, EA2, or CH10 mAbs as indicated below each picture. Development was obtained as described previously.
JM83 with these deleted plasmids. First, a termination linker containing stop codons in all three reading frames was placed at the carboxy-terminal encoding end of pUC19/D so deletions that changed the reading frame of lacZ/NS-1 would still have a termination codon at the deletion junction. Unidirectional deletions were made with exonuclease III utilizing an α-phosphorothioate dATP-protected BamHI site and an unprotected Sal I site (refer to Figure 7). The resulting DNA was religated and used to transform E. coli producing lacZ/NS-1 fusion proteins that had truncated carboxy-terminal ends.

Recombinant clones were first screened by restriction enzyme digest to identify appropriate sized deletions. Forty-eight suitable clones were identified and sequenced. From the DNA sequence at the deletion junction, the amino acid sequence of the fusion proteins was predicted, allowing calculation of the size of the carboxy-terminal deletion (see Appendix II). Of the forty-eight clones, twenty-two were used to make bacterial protein lysates for immunoblot analyses with each mAb. An example of these results is shown in Figure 8. Clones were scored on whether or not each mAb reacted against the β-galactosidase fusion protein of the predicted size (30 - 23 kDa). Data from all the analyses are compiled in Table 2.

The results indicated the presence of four antibody specificity groups. Table 2 shows that the CE10 and AC6 mAbs have the same recognition specificity, as do BE2 and EA2 mAbs within the limits of the deletions. CH10 and BC4 have the two other specificities. In all cases, once a particular region was deleted, antibody binding never occurred in the further deletions. Because the mAbs were raised against a heat-inactivated lacZ/NS-1 protein eluted from a SDS-polyacrylamide gel, it was assumed that the epitopes of the mAbs would be linear determinants rather than conformation-dependent determinants. Therefore, the deletion analysis would allow mapping of the carboxy-terminal end of the epitope. Using this reasoning, the locations of the epitopes relative to lacZ/NS-1 and MVM NS-1 were predicted (see Figure 9). The A, B, and C epitopes are clustered in a region at the carboxy-terminal
Figure 7: The construction of pUC19/D.t and its unidirectional deleted clones.

For the construction of pUC19/D.t, the EcoRI - BamHI fragment of pUC19/D was replaced with the termination oligomer

\[
\text{GATCCTAAGTAATTAG} \\
\text{GATTCATTAATCTTAA}
\]

To create unidirectional deletions in pUC19/D.t, the plasmid was cut with BamHI, protected by filling-in with the \(\alpha\)-phosphorothioate dATP analog, and then cut with Sal I. Deletions were then made using Exonuclease III, blunt-ended using S1 nuclease, filled-in with DNA polymerase I (Klenow fragment), and finally religated using T4 DNA ligase. (see Materials and Methods)
Figure 8: Immunoblots of the six monoclonal antibodies against thirteen deletion clones of pUC19/D.t.
Cellular lysates were made from overnight cultures of E. coli JM83 transformed with one of thirteen pUC19/D.t deletion clones. Aliquots of the lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose, and probed with one of the six monoclonal antibodies as indicated beside each blot (refer to Materials and Methods). Deletion clones used were 1) Δ40, 2) Δ52, 3) Δ81, 4) Δ90, 5) Δ109, 6) Δ121, 7) Δ157, 8) Δ170, 9) Δ184, 10) Δ191, 11) Δ219, 12) Δ230, 13) Δ250, and 14) undeleted pUC19/D.t (refer to Appendix 2 and the Materials and Methods for further details)
Table 2: Immunoblot reaction of carboxy-terminal deleted lacZ/NS-1 proteins versus the monoclonal antibodies.\textsuperscript{a}

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<th>AC6</th>
<th>BE2</th>
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\textsuperscript{a} see legends for Figure 5 and Appendix 1.

This table is a compilation of the sequence analysis presented in Appendix II and the immunoblot analyses of 22 of the deletion clones using the six mAbs, including the data presented in Figure 8.
Figure 9: Predicted positions of the epitopes of the six monoclonal antibodies on \textit{lacZ/NS-1} and on MVM NS-1

The top line diagram shows the MVM NS-1 protein. Aligned below that is a representation of the \textit{lacZ/NS-1} fusion protein with each expressed part of the molecule shade-coded. The positions of the four epitopes are shown based on the data compiled in Table 2. The A epitope corresponds to that for the CE-10 and AC-6 mAbs, B for the BE-2 and EA-2 mAbs, C for the CH-10 mAb, and D for the BC-4 mAb. An expanded view of the A, B, and C epitopes is shown on the lowest line using the one letter code for the amino acids of the carboxy-terminal of \textit{lacZ/NS-1}. Amino acids encoded by the multiple cloning site are denoted by italics and the underlined sequence indicates the region encompassed by peptide 1.
end in lacZ/NS-1 and the D epitope is slightly more toward the amino-terminus of the protein.

When the mAbs were tested on immunoblots versus lysates of AcΔXho-infected Sf9 cells, the results were consistent with those obtained in the epitope mapping study. AcNPV- or AcΔXho-infected Sf9 cells were harvested 48 h.p.i. Cell lysates were prepared, run on a discontinuous polyacrylamide gel, and subject to immunoblot analyses using the six mAbs. A Coomassie-blue stained gel and the immunoblots using the CH10 and BC4 mAbs are shown in Figure 10. The predicted product of the recombinant AcΔXho baculovirus is a NS-1/NS-2 fusion protein containing Met1 - Leu604 of NS-1 fused to the NS-2 carboxy-terminal starting at NS-2 Arg114 with two unrelated amino acids in the joining region. From the epitope map, the fusion protein is expected to contain the D epitope and possibly the C epitope, but not the A or B epitopes. The immunoblots show that the CH10 and BC4 mAbs did react to a 77 kDa protein, the predicted size of NS-1/NS-2, confirming the presence of the C and D epitopes (see Figure 10). The CE10, AC6, BE2, and EA2 mAbs did not react (data not shown), indicating that the A and B epitopes were not present as predicted. These results also allowed a finer mapping of the C epitope by showing that the MVM NS-1 Glu605 and Asp606 were not required for binding.

Before the epitope mapping work was completed, a 25 aa peptide was synthesized by Dr. I. Clark-Lewis to include the 18 amino acids R. Russnak predicted to contain the CE10 mAb epitope. This peptide corresponds to Pro599 - Ala623 of NS-1 (see Figure 9). Once the epitope mapping had been completed, it became evident that this peptide could be used to confirm the epitopes of four of the six monoclonal antibodies by testing whether the antibodies would bind to the peptide. Fifty millilitres of tissue culture supernatant were collected from each mAb cell line and a titration ELISA was performed on each. The purpose of these experiments was to determine the dilution of the supernatant required so that each would be relatively equal in terms of antibody concentration. Serial doubling dilutions were made of the supernatants and run in an ELISA (see Figure 11). From these results, the
Figure 10: Immunoblots of the CH10 and BC4 monoclonal antibodies using a NS1/NS-2 fusion protein.
Total cellular lysates were made of AcNPV- and AcΔXho-infected Sf9 cells (M.O.I. = 5) harvested at 48 h.p.i. as detailed in the Materials and Methods. Aliquots were run on a discontinuous SDS-polyacrylamide gel and then (A) stained with Coomassie blue, or electrophoretically transferred to Optibind. Blots were reacted against (B) the CH10 mAb, or (C) the BC4 mAb and developed as previously described. Two marker lanes are present in the A; the first being Sigma SDS6H unstained molecular weight markers and the second being the Bio-Rad pre-stained high molecular weight markers used on the immunoblots.
Figure 11: Titration curves of the six monoclonal antibody solutions used in the competition ELISAs. Tissue culture supernatants of each mAb secreting hybridoma were titrated using doubling dilutions. For complete details refer to the Materials and Methods.
dilution at which 80% of maximal binding occurred was determined. At the 80% binding
efficiency point, the amount of antigen coating the ELISA plate wells is theoretically slightly
in excess of the antibody. Once this dilution had been determined for each supernatant,
competition ELISAs were performed.

In the competition ELISAs, a soluble competitor molecule was added to the antibody
solution at differing concentrations. The added molecule competes with the surface-bound
antigen for binding by the antibody forming soluble antigen-antibody complexes instead of
surface-bound complexes. Three competitors were used: 1) lacZ/NS-1, as a positive control
competitor, 2) the 25 aa NS-1 peptide (peptide 1), and 3) a 28 aa peptide with no homology
to NS-1 (peptide 2), as a negative control competitor. Soluble antibody-competitor
complexes are washed from the plate and the ELISA then developed as normal. These
results are shown in Figure 12. For all antibodies the lacZ/NS-1 competitor demonstrates
positive competition, indicated by the decrease in \( A_{405} \) with increasing competitor
concentration, and the negative control no competition, indicated by the lack of change in \( A_{405} \)
with increasing competitor. The NS-1 peptide competition is expected to show one or the
other. Assuming epitopes consist of seven amino acids (Berzofsky and Berkower 1984, I.
Clark-Lewis, pers. comm.), then based on Table 2 the NS-1 peptide would be expected to
contain the complete A and B epitopes. The C epitope would be missing one amino acid in
the peptide and the D epitope would lie completely outside the peptide (refer to Figure 9).
Indeed, Figure 12 shows that the NS-1 peptide competes for the CE10 and AC6 mAbs
binding, indicating the presence of the A epitope. Also as expected, the peptide does not
compete for the CH10 and BC4 mAbs binding, indicating the absence of the C and D epitopes.
Unexpectedly, there was no competition shown for the BE2 and EA2 mAbs representing the
B epitope.

A possible explanation for the observed non-competition for the B epitope mAbs might
be that the epitope was too near the terminus of the peptide and required more amino acids to
the amino-terminal side of the epitope to induce the correct local structure for antibody
Figure 12: Graphs of the peptide 1 competition ELISAs showing absorbance at 405 nm versus the competitor concentration in each well.

Competitors were added at the same time as the antibody. The solid line indicates the competition against the NS-1 peptide 1. The positive competition control using the antigen used to coat the wells (●) and the negative competition control using a similar length peptide with no homology to NS-1 (○) are shown for each antibody on the respective graph (refer to Materials and Methods).
recognition. This could be proven or disproven by synthesizing a peptide with the epitope in the middle of the peptide and testing for competition again. The negative control also shows abnormally high readings for the two highest competitor concentrations due to a chromogenic compound in the peptide preparation.

C. Recombinant Baculoviruses Expressing NS-1

1. AcSec

In addition to AcΔXho, two other recombinant baculoviruses, AcSec and AcNS-1, were made in order to overexpress the NS-1 protein. The AcSec virus was isolated before the AcNS-1 recombinant and characterization of the protein produced by the AcSec virus was done until the AcNS-1 virus became available. The CPss/NS1/pAcYM1 plasmid was used by R. Russnak to generate the recombinant baculovirus named AcSec. After isolation of the virus, a high-titre viral stock was produced and used for protein expression studies. Infection of Sf9 cells by AcSec was expected to produce a 77 kDa CP/NS-1 fusion protein which has 47 aa of the amino-terminal of preceruloplasmin replacing 41 aa at the amino-terminus of the NS-1 protein. The inserted 47 aa include the preceruloplasmin signal sequence, the signal cleavage site, and 28 aa from the ceruloplasmin amino-terminus. The construct was generated in the hope that NS-1 might be secreted from the infected Sf9 cells, minimizing cytotoxic effects due to localization and simplifying purification.

Figure 13 shows the Coomassie-blue stained gel of total cellular lysates of AcNPV- and AcSec-infected Sf9 cells harvested 48 h.p.i. The 29 kDa polyhedrin protein is visible in the AcNPV-infected cells even though the polyhedra were not lysed under alkaline conditions. In AcSec-infected cells, an 77 kDa band corresponding to CP/NS-1 is visible. The CP/NS-1 protein appears to make up 30% of the total protein based on this gel. The immunoblot of these lysates using the CE10 mAb revealed a strong-reacting band at 77 kDa in the AcSec-infected cells only. This protein was later shown to migrate slightly faster than MVM NS-1 (data not shown). As well, several minor, smaller cross-reactive bands were
Figure 13: Coomassie blue-stained gel of mock-, AcNPV- and AcSec-infected total Sf9 cell lysates. Refer to the Materials and Methods section. Sf9 cells were mock-, AcNPV- or AcSec-infected at an M.O.I. of 10, harvested at 48 h.p.i., and lysed by boiling for 5 minutes in Laemmli sample buffer. Aliquots were run through a discontinuous SDS-polyacrylamide gel and then stained using Coomassie blue.
observed most likely representing CP/NS-1 degradation products. A strong cross-reacting band was also noted in both AcNPV- and AcSec-infected cell lysates, running at an apparent molecular weight of 75 kDa; the identity unknown.

Having shown that the CE10 mAb binds to a denatured CP/NS-1, indirect immunofluorescence was used to determine the cellular localization and show binding to native CP/NS-1. Previous studies had indicated that CP/NS-1 was not secreted into the extracellular media (R. Russnak, pers. comm.) and therefore was not looked for in that location. Sf9 cells were infected with AcNPV or AcSec, permeabilized, and reacted against the CE10 mAb. Immunofluorescence was examined using a rhodamine conjugated secondary antibody. The results of this study are shown in Figure 14.

The AcNPV-infected cells showed no fluorescence whatsoever (data not shown). In the AcSec-infected cells, fluorescence was not visible until 24 h.p.i. The fluorescence was restricted to the cytoplasm of the cells and the intensity of the fluorescence increased as the infection proceeded, reaching a maximum at 48 h.p.i. Fluorescence was clearly visible right through until the last time point at 72 h.p.i., at which time the bright field phase contrast microscopy revealed that the cells were lysed (data not shown). Bright field microscopy also revealed the infected cells to look unhealthy at 48 h.p.i. and have lost structural integrity of the plasma membrane at 60 h.p.i., following the normal course of cytopathy seen in baculovirus infection (G. Wilson, pers. comm.). From this immunofluorescence study, the localization of CP/NS-1 appeared to be cytoplasmic or membrane-associated although in a non-uniform distribution. Exact determination of the localization of CP/NS-1 was complicated by the fact that cells of this lineage have a small amount of cytoplasm relative to the size of the nucleus. That said, CP/NS-1 could be on the plasma membrane, in the cytoplasm, or attached to the outer nuclear membrane, but does not seem to be within the nucleus.

Under the assumption that CP/NS-1 was cytoplasmic, a trial run for the CE10 mAb immunoaffinity column was performed (see Figure 15 and later discussion). Cells were
Figure 14: Immunofluorescence time course study on AcSec-infected Sf9 cells using the CE-10 monoclonal antibody.

Sf9 cells were grown on acid-etched glass coverslips, AcNPV- or AcSec-infected at an M.O.I. of 0.1, and paraformaldehyde fixed at 12, 24, 36, 48, 60, and 72 h.p.i. Cells were permeabilized with Saponin, reacted with CE-10, and fluorescently tagged using a rhodamine conjugated secondary antibody as described in the Materials and Methods. Coverslips were mounted and viewed under a 63X objective magnification. The AcSec-infected cells immunofluorescence are shown above at various times post-infection indicated below each. The bright field microscopy photography of AcSec-infected Sf9 cells was taken at 24.
Figure 15: Coomassie blue-stained gel and immunoblot using the CE10 monoclonal antibody, of fractions taken during the immunoaffinity purification of CP/NS-1 expressed in insect cells. The method has been given in the Materials and Methods section. The fractions presented are as follows: B) cytoplasmic supernatant, BP) nuclei and cellular debris pellet, D) unbound flow-through from after application to CE-10 column, E) Buffer I wash flow-through, F) Buffer II wash flow-through, f1...f8) fractions collected after alkaline (pH 11.4) elution, and M) Bio-Rad pre-stained molecular weight markers. The Coomassie blue-stained gel is shown above, and the corresponding immunoblot against the CE10 mAb below.
infected with AcSec, harvested 48 h.p.i., pelleted, and then resuspended in a detergent lysis buffer containing 0.5% NP-40. Nuclei and cellular debris were pelleted and the supernatant applied to the immunoaffinity column. The Coomassie blue-stained gel and the corresponding immunoblot analysis against the CE10 mAb revealed that >90% of the CP/NS-1 was found in the nuclear pellet and cellular debris rather than the cytoplasmic fraction (see Figure 15). In order to confirm this result, Sf9 cells were mock-infected, or infected with AcNPV or AcSec, harvested at 48 h.p.i., pelleted, and resuspended in a hypotonic detergent lysis buffer containing 10 mM Tris-Cl (pH 7.6), 1% NP-40, and 1% NaDOC. The cells were allowed to lyse on ice for 30 minutes and then spun down in a microcentrifuge. Equal aliquots of the pellets and supernatants were run on a polyacrylamide gel, transferred to Optibind, and reacted with the CE10 mAb (Figure 16). Again, >90% of CP/NS-1 was found in the pellet fraction. The addition of NaCl to a final 500 mM concentration had little effect on the amount of CP/NS-1 in the pellet. The same was true for the addition of EDTA to a final 5 mM concentration (data not shown).

Lysis of the cells in the absence of detergents produced the same results as well. Infected cells were lysed by a method used for the preparation of nuclear extracts (Lee et al. 1988) in which the cells are placed in a hypotonic buffer for fifteen minutes and then forced through a narrow gauge hypodermic needle. The cell suspension was centrifuged yielding a cytoplasmic fraction and a nuclear pellet. A high salt (420 mM NaCl) extract was made of the pellet fraction which was then cleared of debris by centrifugation. Aliquots of the cytoplasmic extract, the nuclear extract, and the resuspended debris pellet were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot using the CE10 mAb (data not shown). CP/NS-1 was present in all three fractions, but >75% was still found in the pellet fraction.

2. AcNS-1

Studies on the purification of CP/NS-1 polypeptide were set aside when the AcNS-1 baculovirus was isolated by G. Wilson. Construction of the plasmid from which this virus
Figure 16: Immunoblot of mock-, AcNPV-, and AcSec-infected Sf9 cell fractionation study.

Sf9 cells were mock-, AcNPV-, or AcSec-infected at an M.O.I. of 10 and harvested at 48 h.p.i. Cells were washed twice with PBS-C buffer (see Materials and Methods), and then pelleted at 300 x g for 5 minutes. The cell pellets were resuspended in 1 ml of 10 mM Tris-Cl (pH 7.6), 1% (v/v) NP-40, and 1% (w/v) NaDOC. Lysis was allowed to occur on ice for 30 minutes. The cell suspension was spun for 2 min, 12,000 x g, at 4°C. The supernatant was carefully removed and saved. The pellet was resuspended in 1 ml Laemmli buffer. A 80 μl aliquot of the supernatant fraction was added to 20 μl 5X Laemmli buffer. Both the pellet fraction (P) and supernatant fraction (S) were denatured by boiling for 5 minutes. Aliquots of the mock-, AcNPV-, and AcSec-infected cell fractions were electrophoresed through a discontinuous SDS-polyacrylamide gel, transferred to nitrocellulose, and reacted with CE-10. The blot was developed as described in the Materials and Methods.
was made is described in the Materials and Methods section B1. The recombinant virus was expected to produce a full length NS-1 protein (designated NS-1\textsubscript{Ac} to distinguish it from the MVM NS-1 protein) as opposed to a fusion protein like CP/NS-1. For this reason, studies were switched to the NS-1\textsubscript{Ac} protein expression.

Sf9 cells infected with AcNPV, AcNS-1, or mock-infected were harvested at 48 h.p.i., and total cell lysates were prepared. Figure 17A shows the Coomassie-blue stained gel of these lysates. Of interest are the 29 kDa polyhedrin band in the AcNPV-infected cells, and the 84 kDa band in the AcNS-1-infected cells corresponding to the expected mobility of NS-1\textsubscript{Ac}. Comparison against MVM NS-1 from MVM-infected mouse cells showed the baculovirus expressed NS-1\textsubscript{Ac} running at a slightly higher mobility (data not shown).

Transfer of the lysates to Optibind and detection using the CE10 mAb identifies a major band at ~84 kDa and several smaller minor bands in the AcNS-1-infected Sf9 cells only (Figure 17B). These smaller bands likely represent degradation products of the NS-1\textsubscript{Ac} protein.

In order to see if this protein was more easily solubilized than CP/NS-1, crude localization studies were done. AcNS-1-infected and AcNPV-infected Sf9 cells were harvested at 48 h.p.i. and subject to lysis in 1% NP-40 at two salt concentrations (N.B. both conditions gave the same results and only the lower salt condition is presented in Figure 18). The cell suspensions were then centrifuged to produce a supernatant containing the solubilized proteins, and a pellet containing the cellular and nuclear debris. Aliquots of each fraction were subject to discontinuous polyacrylamide gel electrophoresis and stained with Coomassie blue (Figure 18A). Identification and quantification of NS-1\textsubscript{Ac} in the respective fractions was done by immunoblot analysis using the CE10 mAb. The immunoblot (Figure 18B) shows that >75% of NS-1\textsubscript{Ac} is present in the supernatant fraction, although a small amount is found in the pellet fraction. The NS-1\textsubscript{Ac} in the pellet may be due to the fact that the pellet fraction was not washed. Confirmation of the cellular location was obtained using indirect immunofluorescence.
Figure 17: Coomassie blue-stained gel of mock-, AcNPV-, and AcNS-1-infected Sf9 cell lysates, and immunoblots using the CE10 monoclonal antibody.

Mock-, AcNPV-, or AcNS-1-infected Sf9 cells (M.O.I. = 2) were lysed at 48 h.p.i. by boiling for 5 minutes in Laemmli buffer. Aliquots were electrophoresed through a 4%/12% SDS-polyacrylamide gel, and (A) stained with Coomassie blue or transferred to Optibind and reacted against the CE10 mAb. The blot was developed as described in the Materials and Methods. The arrow indicates the position of the NS-1Ac band.
Figure 18: AcNPV- and AcNS-1 infected Sf9 cell fractionation study

Refer to the legend for Figure 16. The cell resuspension buffer contained 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% (v/v) NP-40, and 0.2% (w/v) NaN₃. The Coomassie blue-stained gel (A) and the immunoblot against CE10 (B) are shown. As before, centrifugation yielded a supernatant fraction (S) containing the NP-40 solubilized proteins and a pellet fraction (P). The arrow indicates the position of NS-1ₐₙₖₐₜ.
A time course study using indirect immunofluorescence was done following the same procedure used for the AcSec study (Figure 14) but with the AcNS-1 virus. Cells were fixed at \( t = 12, 24, 36, 48, 56, 60, \) and 72 h.p.i. As before, AcNPV-infected cells showed no fluorescence at any of the time points. In the AcNS-1 infection, fluorescence was not visible until 36 h.p.i. (see Figure 19). At this time point, fluorescence was very intense and showed cytoplasmic localization. After this time point, the intensity decreased and by 60 h.p.i fluorescence was no longer visible. Like the AcSec infection, the Sf9 cells showed the cytopathic effects of baculovirus infection, but the process appears to occur sooner in the AcNS-1 infection. The cells swell to a maximum size by 48 h.p.i. and have completely lysed by 60 h.p.i. The decreasing and eventual loss of fluorescence by 60 h.p.i. in the AcNS-1-infected cells is most likely due to the loss of integrity of the plasma membrane allowing cytoplasmic leakage.

D. Immunoprecipitation of \([^{35}S]\)-methionine labelled NS-1\(_{Ac}\)

In order to test if the mAbs could be used to purify NS-1\(_{Ac}\) from insect cells, an immunoprecipitation experiment was performed. Sf9 cells were infected with AcNPV or AcNS-1, labelled with \([^{35}S]\)-methionine for 3 hours at 31 h.p.i., and harvested at 48 h.p.i. Cells were lysed in RIPA buffer, centrifuged and the supernatant aliquoted. Tissue culture supernatants containing each mAb were added to individual aliquots and incubated together overnight. Antigen-antibody complexes were isolated using Protein A-Sepharose CL-4B coupled to a rIgM antibody, washed, and then incubated at 100 °C for 5 minutes to release the precipitated material. Aliquots were subject to SDS-polyacrylamide gel electrophoresis. The autoradiograph of the gel is shown in Figure 20. As a control, an anti-rhodopsin monoclonal antibody 2B2 (MacKenzie et al. 1984) was used to show the non-specific bands immunoprecipitated (lanes 1 & 2). A 40 kDa band is visible in these lanes. Immunoprecipitations using the six mAbs all precipitated two major bands in AcNS-1-infected cellular lysates. The band at 84 kDa corresponds to the expected size of NS-1\(_{Ac}\)
Figure 19: Immunofluorescence time course study of AcNS-1-infected Sf9 cells using the CE10 monoclonal antibody.

AcNPV- or AcNS-1-infected Sf9 cells (M.O.I. = 0.1) grown on acid-etched glass coverslips were fixed in 3.68% paraformaldehyde at 12, 24, 36, 48, 54, 60, and 72 h.p.i. Cells were permeabilized with Saponin, reacted with the CE10 mAb, and detected using a biotinylated secondary antibody and a Texas Red-streptavidin conjugate. Cells were viewed under a 63X objective magnification. Bright field phase microscopy pictures from 24, 48, and 60 h.p.i are shown on top. Immunofluorescence microscopy pictures from 36, 48, and 56, h.p.i. are shown below. For further details refer to the Materials and Methods.
Figure 20: Immunoprecipitation of AcNS-1-infected cell lysates using the six monoclonal antibodies.

1 x 10^7 Sf9 cells were AcNPV- or AcNS-1 infected. At 30 h.p.i., the cells were washed with Met'-TNM-FH media and resuspended in the same media with 100 μCi/ml 35S-Met and 10% dialyzed FCS. After a 4 hour labelling period, the cells were returned to TC-100/10% FCS. At 48 h.p.i. the cells were harvested, washed 2X with PBS-C buffer, and the cell pellet was resuspended in 1 ml RIPA buffer. After 30 minutes on ice, the cell lysate was cleared by centrifugation (12,000 x g, 10 min, 4°C), and the supernatant divided into 100μl aliquots. AcNPV- and AcNS-1-infected cell lysates were incubated with 50 μl of the various mouse monoclonal antibodies as indicated above each pair, rotating end-over-end at 4°C for 10 hrs. Immune complexes were precipitated by the addition of 25μl 20% Ig-ProA-Sepharose (2mg 20% Ig/ ml swollen ProtA-Sepharose) rotating end-over-end at 4°C for 10 hrs. Complexes were washed 3X with RIPA buffer, resuspended in 20μl Laemmli sample buffer, and incubated at 100°C for 5 minutes. Samples were electrophoresed through a discontinuous SDS-polyacrylamide gel. The gel was dried down and subject to autoradiography.
and the second band, at 60 kDa, was of unknown origin. As well, two other very faint bands are seen in the immunoprecipitations of NS-1\textsubscript{Ac} at 43 kDa and 40 kDa. Bands of similar mobility, together with a 60 kDa band, were later shown to be immunoprecipitated from the wild-type infected cell lysates.

In an attempt to identify the immunoprecipitated bands, the lysates were subject to a double immunoprecipitation. Lysates were first immunoprecipitated as just described above, using the CE10 mAb. To disrupt any immunoprecipitated protein complexes, the beads were boiled in RIPA buffer + 0.5% SDS + 5 mM β-Mercaptoethanol. Sepharose beads were removed by centrifugation, and the supernatant was subject to a second immunoprecipitation with the CE10 mAb or a guinea pig anti-TP polyclonal sera (Chow et al. 1986). Single immunoprecipitations using the anti-TP sera or the CE10 mAb were also performed. All are shown in Figure 21. None of the original CE10 mAb immunoprecipitated bands were detected in the second round of immunoprecipitation using the anti-rhodopsin mAb or the anti-TP sera (Figure 21A). Using the CE10 mAb, only the 84 kDa band was detected. The unidentified lower bands in the previous immunoprecipitation were all found in the first round immunoprecipitations of the AcNPV-infected cells (see Figure 21B). These bands were not detected in the original autoradiogram (Figure 20) because the amounts of AcNPV-infected cell lysates loaded were lower than those of the AcNS-1-infected cell lysates. This does not explain why the anti-rhodopsin antibody did not detect the 60 kDa band in Figure 20 and may indicate that NS-1\textsubscript{Ac} is complexed to a 60 kDa protein distinct from the one faintly seen in the Figure 21B immunoprecipitations. The intensity of the 60 kDa band in the CE10 mAb/AcNS-1 lane was stronger than that in the CE10 mAb/AcNPV or anti-rhodopsin mAb/AcNS-1 lanes.

The anti-TP sera did not immunoprecipitate any labelled proteins under the conditions used (Figure 21B). The TP protein, a nucleolar antigen found in mouse cells and HeLa cells, would likely require more extensive extraction conditions to dissociate it from the nuclear
Figure 21: Immunoprecipitation of AcNS-1- and AcNPV-infected cell lysates using the CE10 mAb and an anti-TP sera, and double immunoprecipitation studies on CE10 mAb immunoprecipitated AcNS-1 infected Sf9 cell lysates using the CE10 mAb and an anti-TP sera.

Cells were labelled and harvested as described in the Figure 20 legend. First-round immunoprecipitation was performed using the CE10 mAb. Immune complexes were disrupted as described in the Materials and Methods section J and subject to second-round immunoprecipitation with anti-rhodopsin antibodies, the CE10 mAb, or the anti-TP sera. These complexes were washed, resuspended, denatured, and analyzed as in Figure 20. The first round immunoprecipitations with the CE10 mAb, anti-rhodopsin sera, and the anti-TP sera are shown in B.
lamina if it is actually present in the insect cells as well (Walton et al. 1989, Bodnar et al. 1989).

E. Purification of NS-1 using a CE10 immunoaffinity column

From the studies described above, the CE10 mAb was shown to recognize all forms of NS-1 and NS-1 fusion proteins, and have the highest A<sub>405</sub> in the ELISA. Therefore, this antibody was chosen initially to be used in the construction of a NS-1 specific immunoaffinity column. In order to construct the column, large quantities of the mAb had to be produced, purified, and then coupled to a support matrix.

A total of twelve spf Balb/C mice were inoculated with the CE10 hybridoma. The first three inoculated mice produced low quantities of ascitic fluid and developed solid tumors at the point of injection. Hybridomas from one of these mice's ascitic fluid was grown in cell culture, and these cells were then used to inoculate the latter mice. The average titre of ascitic fluid obtained from these latter mice was 8.1 mls and solid tumors were no longer detected. The monoclonal antibody was purified from the ascitic fluid by ammonium sulfate precipitation and DEAE-Sephacel chromatography, and coupled to CNBr-activated Sepharose CL-4B at 7 mg/ml swollen beads. A pre-column matrix was prepared by the same method used for coupling the mAb, but without adding the mAb.

As mentioned previously, the first test of the immunoaffinity column was performed using the CP/NS-1 protein based on a method used for the immunoaffinity purification of SV40 large T antigen expressed in insect cells (Lanford 1988, Murphy et al. 1988, Simanis and Lane 1985). Aliquots were taken at various points during the purification procedure as outlined in the Figure 15 legend, and separated on a SDS-polyacrylamide gel. CP/NS-1 was detected immunologically by immunoblot analysis using the CE10 mAb. Comparison of lanes B and BP, which were adjusted to be equal with respect to cells per ml, indicates that >90% of CP/NS-1 remained in the first centrifugation pellet and was never applied to the column. Although not easily seen in the Coomassie blue-stained gel, a small amount of CP/NS-1 was
solubilized and applied to the column (see immunoblot, lane B). Lane D shows the proteins not bound to the CE10 mAb column and the immunoblot of this fraction showed that not all of the applied CP/NS-1 was bound. The elution of the bound proteins by strong alkaline (pH 11.3) conditions showed that the column material was functional (see lanes f5 - f8) and the immunoblot of the eluted proteins indicates the presence of CP/NS-1. Unfortunately, a large number of proteins appear in the stained gel of the eluted fractions that did not react with the CE10 mAb on the immunoblot. Also, the CP/NS-1 band in the eluted fractions was not clearly visible on the stained gel and recovery of CP/NS-1 from the Sf9 cells was estimated at only 10% at best. At this point, efforts were directed to increasing the proportion of CP/NS-1 solubilized, but because a more efficient method was never found, refinement of the column conditions and immunoaffinity purification of CP/NS-1 was not pursued further.

With the isolation of a NS-1Ac expressing recombinant baculovirus, the column was put to use in attempts at purification of the unfused NS-1Ac protein. Having proved that peptide 1 was bound by the CE10 mAb, it was thought that this peptide could be used to elute bound NS-1Ac from the CE10 mAb column and provide a very specific elution method. The results of this purification attempt are shown in Figure 22. The immunoblot of the total cellular extract (lane A) shows a large band running at 84 kDa corresponding to NS-1Ac. There are also several lower molecular weight bands appearing on the immunoblot, including ones that had not been seen previously (cf: Figure 17). As there were no protease inhibitors added during the hypotonic lysis, these bands were assumed to be degradation products. Comparison of lane B to lane BP shows that, unlike the CP/NS-1 purification, ~90% of the NS-1Ac protein was solubilized. Lane C shows that pre-column clearing of the sample did not affect any of the immunoreactive bands; the change in intensity on the immunoblot being accounted for by a dilution not adjusted for when the gel samples were made. Not all of the NS-1Ac applied to the CE10 mAb matrix was bound to the column (see lane D). Addition of peptide 1 up to a concentration of 300 mg/ml had no effect on the bound NS-1Ac, as only an unidentified ~62 kDa protein appeared on the gel and not on the immunoblot. If this protein
Figure 22: Coomassie blue-stained gel and immunoblot using the CE10 monoclonal antibody of fractions taken during the immunoaffinity purification of NS-1Ac expressed in insect cells and eluted by peptide and alkali. The method has been given in the Materials and Methods section M.1. The fractions presented are as follows: A) total cellular lysate, B) cytoplasmic supernatant after hypotonic lysis, BP) nuclei and cellular debris pellet after hypotonic lysis, C) cytoplasmic lysate after pre-column clearing, D) unbound flow-through after cytoplasmic lysate application to CE-10 column, F1..F9) fractions collected after elution by a 0 - 300 mg/ml peptide 1 gradient, f1...f14) fractions collected after elution by alkali (pH 11.4), and M) Sigma SDS-6H molecular weight markers (for gel) or BRL pre-stained high molecular weight markers (for immunoblot). The Coomassie blue-stained gel is shown above, and the corresponding immunoblot against the CE10 mAb below.
were actually competed off the matrix-attached CE10 mAb by the peptide, it would be expected to react on the Western blot. As it did not, this protein was assumed to be non-specifically eluted.

After realizing from the SDS-polyacrylamide gel that NS-1Ac had not been released from the column, the remaining proteins were eluted by alkaline conditions (lane f1...f14) and collected in 400 μl fractions one column volume after the application of the elution buffer. The immunoblot of these fractions indicated that NS-1Ac had been eluted, but as a broad peak centered at fraction 6. Once again, the Coomassie blue-stained gel showed a large number of eluted proteins, only a small number of which react against the CE10 mAb on the Western blot. The eluted proteins (see lane f6) showed a new immunoreactive species migrating at an apparent molecular weight of 68 kDa (cf: lane C) and the five original immunoreactive bands each appeared with a second slightly lower molecular weight band following alkaline elution. This could indicate dephosphorylation of NS-1Ac on serine or threonine residues; phosphoserine and phosphothreonine residues being alkaline labile under the pH conditions employed in the elution method.

With the failure of the peptide elution method and the probable dephosphorylation caused by the alkaline elution, alternative methods for the release of NS-1Ac were sought. Elution with 6M guanidinium chloride has been used to immunoaffinity purify a functional c-fos protein expressed in insect cells (Tratner et al. 1990), hence this method was attempted for NS-1Ac (see Figure 23). Lane A showed the addition of the serine protease inhibitor, PMSF (50 μg/ml), to the hypotonic lysis buffer resulted in the loss of the 80 kDa immunoreactive band seen in the previous purifications (cf: Figure 22, lane A), but had no effect on the four other lower molecular weight bands. The Tratner protocol included 500 mM LiCl in the first wash buffer which results in a large number of proteins being washed off the column including a small proportion of NS-1Ac (lane E). The guanidinium elution itself caused several undesirable problems. First, the column was seriously denatured, indicated by the two very distinct bands at 50 kDa and 27 kDa in lanes F3...F18. These bands correspond to the heavy
Figure 23: Coomassie blue-stained gel and immunoblot using the CE10 monoclonal antibody, of fractions taken during the immunoaffinity purification of NS-1Ac expressed in insect cells and eluted by 6M Guanidinium chloride. The method has been given in the Materials and Methods section M.2. The fractions presented are as follows: A) total cellular lysate, BP) nuclei and cellular debris pellet after hypotonic lysis, C) cytoplasmic lysate after pre-column clearing, D) unbound flow-through after cytoplasmic lysate application to CE-10 column, E) Buffer Ia wash flow-through, F) Buffer IIa wash flow-through, F1..F25) fractions collected after elution by 6M guanidinium chloride, M1) BRL pre-stained high molecular weight markers, and M2) BRL high molecular weight markers. The Coomassie blue-stained gel is shown above, and the corresponding immunoblot against the CE10 mAb below.
and light chains of the CE10 mAb and appear on the immunoblot due to the cross-reaction with the anti-mouse Ig secondary antibody. Second, the guanidinium was difficult to dialyze away completely, adding an extra day to the purification protocol. The presence of the guanidinium affected the running of the polyacrylamide gel by causing smearing, streaking, and aberrant mobilities in some fractions.

A milder elution procedure using acidic conditions (pH 2.5) was attempted and shown to produce more encouraging results (Figure 24). In the hypotonic lysis prior to the column purification, the nuclear pellet was resuspended in RIPA buffer before gel loading (lane BP). This appears to release more NS-1Ac (cf: Figure 22, lane BP). Whether this represents a small nuclear population of NS-1Ac, or results from incomplete washing of the nuclei or incomplete lysis of the Sf9 cells is yet to be determined. As before, not all of the NS-1Ac protein was bound to the 3.0 ml CE10 mAb column (lane D), although the washes did not release any of the bound NS-1Ac (lane E & F). Elution at pH 2.5 resulted in NS-1Ac eluting from the column in a major peak centered at fraction 9 and a second minor peak at fraction 18 (lanes f1...f20). The second peak occurs after approximately 2½ column volumes have been eluted, long after all released proteins would have been expected to elute. This 'tailing effect' may be due to re-absorption and re-elution occurring as the elution buffer is chased through by Buffer Ib (Scopes 1987). Comparison of lane A and lane f9 in the immunoblot shows that no additional immunoreactive bands appear during the purification procedure. The gel of fraction 9 shows that several bands in addition to those identified on the blot are eluted; the major band being at 60 kDa that is not seen in any of the other elution methods. This band may be related to the one seen in the immunoprecipitations of NS-1Ac. The absence of this band in previous purifications is likely due to the different wash protocol used. The NS-1Ac band was the second major band and recovery of this protein, based on estimation of protein intensities from the Coomassie blue-stained gel, was approximately 18% at best.
Figure 24: Coomassie blue-stained gel and immunoblot using the CE10 monoclonal antibody, of fractions taken during the immunopurification of NS-1Ac expressed in insect cells and eluted by acid (pH 2.5). The method has been given in the Materials and Methods section M.3. The fractions presented are as follows: A) total cellular lysate, BP) nuclei and cellular debris pellet after hypotonic lysis, D) unbound flow-through after cytoplasmic lysate application to CE-10 column, E) Buffer Ib wash flow-through, F) Buffer IIb wash flow-through, f1...f20) fractions collected after elution by Buffer IIIb, M) BRL high molecular weight markers (for gel) or BRL pre-stained high molecular weight markers (for immunoblot). The Coomassie blue-stained gel is shown above, and the corresponding immunoblot against the CE10 mAb below.
X. Discussion

A. Monoclonal antibodies against MVM NS-1

Previous antibodies against MVM NS-1 have been obtained from polyclonal sera directed against NS-1 specific peptides, bacterial fusion proteins expressing portions of NS-1, or other members of the autonomous parvoviruses (Cotmore et al. 1983, Cotmore and Tattersall 1988). In this study, we describe the first characterization of monoclonal antibodies to NS-1. The six mAbs raised against a fusion protein containing Cys364 to Ala623 of MVM NS-1 were shown to be specific for the lacZ/NS-1 antigen by Western blot analyses and epitope mapping of the antibodies. Immunoblot analyses using the mAbs also showed their ability to detect an 83 kDa protein found only in MVM-infected mouse fibroblast cells. Uninfected cells did not express this protein. Therefore, the 83 kDa protein had to be a viral protein or a virus-induced host protein. Of the MVM proteins, only NS-1 and VP-1 migrate at an apparent mobility of 83 kDa. The predicted epitopes of the mAbs are found in NS-1 and not in VP-1, leading to the belief that the mAbs could be recognizing NS-1. Further evidence that the mAbs were, in fact, NS-1 specific was obtained from the indirect immunofluorescence studies of infected mouse cells.

The kinetics of expression of the protein detected by the CE10 mAb in the first indirect immunofluorescence study are consistent with that of NS-1. The immunoreactive protein was detectable at 2 h.p.r. in the nucleus of MVM-infected cells with the intensity of the fluorescence increasing through 4, 6, and 8 h.p.r. The kinetics of NS-1 expression have been investigated previously by Cotmore and Tattersall using Western blot analysis (Cotmore and Tattersall, 1987). They, too, first detected NS-1 at 2 h.p.r. and found that the levels of NS-1 expression increased through 4 and 6 h.p.r. In their study, NS-1 was said to reach maximal levels of expression by 6 h.p.r, but no time points were shown past 6 h.p.r. Secondly, Bodnar et al. (1989) have proposed that the interaction with the nuclear matrix proceeds until all nuclear matrix attachment sites have become saturated and involves the breakdown of the
nucleoli. In our study, the fluorescence is seen first as discrete foci, corresponding to the nucleoli, and then on the periphery of the nuclei, indicating nuclear matrix attachment. Unfortunately, this experiment was not extended past 12 h.p.r. as it would be of interest to see the pattern of fluorescence in the final stages of the infection. From the data presented in this study, it is reasonable to assume that the mAbs are NS-1 specific.

In the second immunofluorescence study, five of the six mAbs demonstrated nuclear staining in discrete foci. The MVM NS-1 protein is known to localize to the nucleus (Cotmore and Tattersall 1986a) and previous immunofluorescence studies by Walton et al. (1989) showed that MVM DNA and a 60 kDa host nucleolar antigen TP exhibit the same intranuclear organization as that of the protein recognized by the six mAbs. The Bodnar group have shown: 1) the foci to co-localize with nucleolar marker antigens using double-labelling experiments (Walton et al. 1989), and 2) the interaction of the MVM DNA with the nuclear matrix being mediated through the 5' end of the genome and the TP protein (Bodnar et al. 1989). Cotmore and Tattersall (1988) have shown 90-95% of MVM genomic DNA to be covalently bound through the 5' end to NS-1. Therefore, the association with the nuclear matrix is likely to involve a complex containing TP, NS-1, the 5' end of the MVM DNA, and possibly other proteins. The detection of fluorescence in the discrete foci within the nucleus using the mAbs further supports the assumption of NS-1 specificity and demonstrates the presence of NS-1 in the nuclear matrix attachment complex.

The epitope mapping studies showed four epitopes (A - D) for the six mAbs; the CE10 and AC6 mAbs recognize the same A epitope, the BE2 and EA2 mAbs recognize the B epitope, the CH10 mAb recognizes the C epitope, and the BC4 mAb recognizes the D epitope. Three of the epitopes (A, B, and C) were clustered in a 16 aa region near the carboxy-terminal of lacZ/NS-l. The reason for the high antigenicity of this region is not clear. Keeping in mind that the original antigen had been denatured by heat and SDS, Kyte-Doolittle hydropathy plots showed that this region was only slightly hydrophilic. There are two proline residues which may produce a protruding surface structure that would be
antigenic (Berzofsky et al. 1985). The D epitope, on the other hand, does not contain any distinctive structural features or hydropathic characteristics which would account for its antigenicity. The ability of the mAbs to detect MVM NS-1 was not surprising because the epitopes lay near the carboxy-terminal, and the hydropathy plot of MVM NS-1 predicts this end to be hydrophilic and, therefore, exposed to the aqueous environment. Of the two termini, the carboxy-terminal of MVM NS-1 is expected to be the more accessible, as the covalent linkage to the 5' end of the genome is thought to occur through an alkali-stable ester linkage near the amino-terminal rather than the carboxy-terminal (Cotmore and Tattersall 1988, Cotmore and Tattersall 1989). One disadvantage of these antibodies being specific for carboxy-terminal epitopes is that they would be unable to detect the 60 - 63 kDa, carboxy-terminal deleted forms of NS-1 which have been reported by Cotmore and Tattersall (1986b, 1987, 1988).

Out of the six mAbs, only five showed binding to the paraformaldehyde-fixed, MVM-infected cells. The inability of the sixth mAb, BC4, to bind may be attributed to two factors. First, the binding affinity of the BC4 mAb may be weaker than the other five IgG1 mAbs. The maximum A405 obtained in the titration ELISAs was 5- to 9-fold lower than that of the other mAbs. Unfortunately, the binding affinities of the mAbs could not be obtained from the competition ELISA data because the concentrations of the mAbs were not accurately known and not necessarily equivalent. Second, the D epitope may be inaccessible in the native protein. The antibodies which recognize epitopes closest to the carboxy-terminal of MVM NS-1 had the highest intensity of fluorescence, reaction on immunoblots, and absorbance readings in ELISAs; the antibodies with the epitopes furthest from the carboxy-terminal had the lowest. The termini of proteins usually have the greatest mobility and are often on the exposed surface of the protein (Lerner et al. 1984). It is possible that the D epitope is buried in the interior of the MVM NS-1 protein making detection of this epitope in the native protein impossible. This theory is not supported by hydropathy plots of the protein, which indicate the D epitope region to be hydrophilic.
B. Recombinant baculoviruses

In this thesis, three recombinant baculoviruses expressing MVM NS-1 or portions of NS-1 have been used. Initial attempts by R. Russnak to express a full length MVM NS-1 in insect cells using the pAcRP6 vector were unsuccessful, but the introduction of a frame-shift mutation in the NS-1 gene resulted in the isolation of the AcΔXho virus. The ease in which AcΔXho was isolated seemed to support the idea that NS-1 was cytotoxic to cells. Under this assumption, Russnak attempted to make the insect cells secrete NS-1, by fusing the preceruloplasmin signal sequence onto NS-1. The AcSec virus was thus isolated. The NS-1\textsubscript{Ac} expressing virus, AcNS-1 was the result of later work by W. Chen and G. Wilson.

Of the three recombinant viruses, the AcΔXho virus had the lowest level of expression of the inserted gene. The low level of NS-1/NS-2 seen in the Coomassie blue-stained gel may be due to the pAcRP6 vector not containing the -7 to -1 polyhedrin nucleotides. These nucleotides have already been implicated in the high level expression of foreign genes (Matsuura et al. 1987). As this protein was not overexpressed and was not a full length NS-1 protein, further characterization of this protein was not attempted. This protein may be more useful when more is known about the functions of NS-1 or NS-2.

The second recombinant virus, AcSec produced the CP/NS-1 protein, containing 47 amino-terminal amino acids from preceruloplasmin in place of the NS-1 amino-terminal. Human ceruloplasmin is a 132 kDa blood plasma protein synthesized in the liver and is the principal copper transport protein in plasma (Owen Jr. 1982). Preceruloplasmin contains a canonical signal sequence; the first 19 amino acids of the preceruloplasmin protein contain an 8 - 9 aa hydrophobic core, and alanine residues at the -1 and -3 positions (von Heijne 1981, von Heijne 1983, von Heijne and Abrahmsen 1989). Cleavage of the 19 amino acids produces ceruloplasmin. Unfortunately, the CP/NS-1 protein was not secreted into the extracellular media as expected. It was reasonable to believe that it would have been as there are several examples of human signal sequences being recognized in the Sf9 cells (Luckow and Summers
1988b) and of chimeric proteins containing non-homologous signal sequences being properly secreted (Zerial et al. 1987). The failure of this chimeric protein to be secreted by the insect cells may be due to the preceruloplasmin signal sequence not being recognized by the Lepidopteran protein processing mechanism. It would have been better to employ a signal sequence that was known to be cleaved by the signal recognition mechanism of these cells, although recognition of the signal sequence does not necessarily guarantee proper cleavage. Carbonell et al. (1988) reported the failure of a chimeric human β-interferon signal sequence/scorpion neurotoxin to be properly processed even though human β-interferon has been produced and secreted properly in insect cells (Smith et al. 1983). The problem was attributed to improper protein folding and post-translational modifications. Two other factors that may play a role in signal cleavage are the large size of the fusion protein, and the presence of a nuclear localization signal.

Regardless of the failure of the signal sequence to be cleaved, a 77 kDa CP/NS-1 protein was identified on polyacrylamide gels of AcSec-infected Sf9 cell lysates, and on immunoblots using the CE10 mAb. The pattern of immunofluorescence shown by the CP/NS-1 protein indicated that the polypeptide was membrane-bound. Fluorescence appeared in a distinct manner around the circumference of the cell and remained until late in infection, when the cells had presumably lysed and soluble cytoplasmic proteins had been lost. As would be expected of a membrane-bound protein, Dounce homogenization did not release the majority of CP/NS-1, but neither did lysis in the presence of 1% (v/v) NP-40. Franke et al. (1981) showed that 0.5% (v/v) NP-40 disrupted all cellular membranes leaving an intact nuclear matrix scaffold. If CP/NS-1 were membrane-bound why was it not solubilized? One simple explanation would be that the protein was released from the membrane, but aggregated upon solubilization and remained in the pelleted fraction. Subcellular fractionation will be necessary to confirm the localization of this polypeptide.

In searching for NS-1 function, the preceruloplasmin fusion protein had several drawbacks compared with MVM NS-1: 1) the CP/NS-1 protein had an altered cellular
location, 2) it contained 47 foreign amino acids, and 3) it did not contain a full length NS-1 protein; missing 41 aa from the amino terminal. Rather than simplifying the purification, the addition of the signal sequence actually complicated isolation of this protein. With all of these differences and purification problems, the use of this protein in determining the function of MVM NS-1 was not apparent. It did, however, serve as a useful test for the CE10 mAb column. With the isolation of the AcNS-1 virus, the further characterization of the CP/NS-1 protein became secondary to NS-1Ac studies.

The final recombinant virus presented in this thesis was the AcNS-1 virus which was expected to express a full-length NS-1 protein. Infection of the insect cells with AcNS-1 produces an 84 kDa protein not found in AcNPV-infected cells, and Western blots of the lysates using the CE10 mAbs confirmed identification of the protein as NS-1Ac. Expression levels for NS-1Ac were found to be intermediate between that seen for NS-1/NS-2 and CP/NS-1; the reasons for the differences in expression not being clear. The start codon sequence in this recombinant gene does not match the Miller consensus sequence for highly expressed baculovirus genes, being ACCATG as opposed to AANATG (Miller, 1987). Inexplicably, site-directed mutagenesis of this sequence to AACATG did not result in a noticeable increase in expression levels (W. Chen, pers. comm.). Both the AcSec and the AcNS-1 viruses were isolated using the pAcYM1 vector, which contains the -7 to +1 nucleotides of the polyhedrin gene. Removal of the upstream non-coding regions of the inserted gene has been shown to result in greatly increased levels of expression (C.Y. Kang, pers. comm.) and this is currently in progress for NS-1Ac to increase levels of expression to that of CP/NS-1 or higher.

The insect cell-expressed NS-1Ac is much more similar to native NS-1 than the preceruloplasmin fusion protein. It runs at an apparent molecular weight of 84 kDa and appears to be phosphorylated. MVM NS-1 runs at an apparent molecular weight of 83 kDa, but a highly phosphorylated form of MVM NS-1 has been detected running at 85 - 88 kDa (Cotmore and Tattersall 1986a). For the related autonomous parvoviruses, H-1 and PPV,
the predominant phosphorylated amino acid in the NS-1 protein was phosphoserine (Paradiso 1984, Molitor et al. 1985). Phosphoester bonds on serine and threonine residues are alkaline labile and alkaline elution of immunoaffinity purified NS-1Ac resulted in the appearance of lower molecular weight bands that may be the result of such dephosphorylation. An initial attempt at $[^{32}\text{P}]$-labelling of baculovirus-encoded NS-1Ac was unsuccessful although there were several problems with the protocol used that may have caused the failure of the NS-1 protein to be labelled (Yeung and Astell, unpubl. res.) The effect of the higher degree of phosphorylation on NS-1 is unknown, but levels of phosphorylation are known to affect activity and cellular location (Gerace and Blobel 1980, Sevaljevic et al. 1981, Roth et al. 1989). Caution must be taken when making speculations about NS-1Ac modifications with respect to MVM NS-1, in that it is not known if the post-translational modification mechanisms are similar in insect cells and mammalian cells.

An interesting observation about the insect cell-expressed NS-1Ac was its intracellular location. As mentioned previously, MVM NS-1 is a nuclear protein, but the immunofluorescence studies and the localization studies on the AcNS-1-encoded protein have shown NS-1Ac to be cytoplasmic. The altered locale may indicate mutations in the nuclear localization signal, improper folding causing masking of the signal, or perhaps, incorrect post-translational processing (Roberts et al. 1987, Hunt 1989). Another intriguing possibility is that NS-1 requires the presence of another viral product (e.g. MVM DNA or NS-2) for proper localization. Though the cellular localization makes purification of this protein easier, the effect on function is yet to be determined.

Indirect observations about the cytotoxicity of NS-1 can be inferred from the time-course study of the AcNS-1 infection. In this study, the bright field phase contrast microscopy pictures show that the cytopathic effects are seen earlier in AcNS-1 infection than in AcSec infection or wild-type AcNPV infection. The cytopathic effects (plasma membrane degradation and cytoplasmic release) are noticeable at 48 - 60 h.p.i in AcNS-1 infection but, at 60 - 72 h.p.i in AcNPV infection. As well, the number of recombinant baculoviruses
obtained from the pAcYM1/NS-1 co-transfection was lower than expected (G. Wilson, pers. comm.). In the co-transfection of Sf9 cells with wild-type AcNPV DNA and the transfer vector, expression of foreign genes can be detected by 24 hours post-transfection (Summers, pers. comm.). Therefore, a negative selection against recombinant baculoviruses expressing high levels of NS-1Ac would occur. Possibly, the isolation of a virus with an altered cellular location for NS-1Ac may be a result of this selection pressure. Both observations indirectly support, but in no way prove, the notion that NS-1 is cytotoxic to cells in high concentrations.

C. Immunoaffinity purifications

The main purpose of this project was the isolation and purification of the NS-1 protein. The monoclonal antibodies against NS-1 provided a specific probe and a means for its purification. Many references demonstrate the use of immunoaffinity chromatography as a single-step purification method yielding homogeneous and functional protein (Murphy et al. 1988, Simanis and Lane 1985, Tratner et al. 1990) and it was hoped that the CE10 mAb column would yield the same results. Several problems complicate the purification of NS-1 from MVM-infected LA9 cells; the main one being the very low level of NS-1 produced in infected cells. Secondary problems include the tight association of NS-1 with the nuclear lamina, the abundance of VP-1 which co-migrates with NS-1, and the probable cytotoxicity of NS-1. Over-production of NS-1Ac using the baculovirus expression system alleviated the problems of low amounts of starting materials, VP-1 contamination, and NS-1 cytotoxicity. Although partial purification of the polypeptide was achieved, the immunoaffinity purification of NS-1Ac did not yield a 100% pure preparation of the protein, and no biochemical activity of the polypeptide has yet been demonstrated.

The first problem with the purification procedure was the presence of other immunoreactive bands and non-specific bands that elute with the CE10 mAb-bound proteins. The cross-reactive bands detected by the immunoblot analysis appear to be degradation products of NS-1Ac as they can be minimized by the addition of PMSF. Other protease
inhibitors have not been tried, although it would be wise to include them to see if the number of lower molecular weight bands could be reduced further. Reduction of the number of proteins non-specifically eluted will require more work on optimizing the wash conditions. A 0.5M LiCl wash buffer removed several of these proteins but also affected the bound NS-1\textsubscript{Ac} (Figure 23). Ideally an elution method specific for NS-1\textsubscript{Ac} will have to be found.

Peptide elution was attempted in order to elute only molecules bound to the CE10 mAb at its antigen binding site. Even though the CE10 mAb had been shown to bind the peptide, this method did not result in the release of NS-1\textsubscript{Ac} from the column. The reason for the failure of the peptide elution is likely due to the respective binding affinities of the mAb for the NS-1\textsubscript{Ac} protein and the peptide; the affinity for the protein being higher than that for the peptide. Under the elution conditions used, the peptide was theoretically at a 5-fold molar excess over the antigen binding sites. A second explanation for the failure of the peptide to compete off the bound NS-1\textsubscript{Ac} molecules would be that there was not sufficient time for the replacement reaction to occur. Antibodies with strong binding affinities (K\textsubscript{a}) have a calculated t\textsubscript{1/2} on the order of 23 minutes for the dissociation reaction (Berzofsky and Berkower, 1984). A worthwhile option would be to re-test the peptide elution method using one of the other five mAbs as the immunoaffinity matrix and an appropriate peptide. Of the four elution methods attempted, the acid elution procedure appears to be best; not adversely affecting the post-translational modifications of the NS-1 protein or the CE10 mAb column stability. The effect of the acid elution on the activity of the NS-1 preparation is not known although it has been used in other immunoaffinity purifications yielding functional proteins.

A second problem was the recovery of protein. The column had the CE10 mAb bound at a concentration of 7 mg/ml swollen beads. Samples taken before and after binding of the mAb to the CNBr-activated Sepharose 4B indicated a 99.3% coupling efficiency of the mAb to the Sepharose beads, but when the AcNS-1 cellular lysate was added to this column material, not all of the NS-1\textsubscript{Ac} was bound even though it would theoretically amount to only 1/100th of the binding capacity of the column. The degradation products were not included in this
calculation, but would not be expected to account for all of the remaining binding sites. The CNBr method for coupling of antibodies to solid matrices can produce the wrong spatial orientation of the mAbs with the antigen binding site pointed in towards the matrix. This would lead to a reduced binding efficiency for the column (Schneider et al. 1982). Alternative methods for antibody coupling include the use of other chemical linkages, Protein A, or Protein G (Harlow and Lane 1988, Bio-Rad Laboratories 1988) and should be investigated.

The work presented in this thesis serves as a starting point for future studies. The mAbs characterized in this study will be of use in the detection of the MVM NS-1 expressed in infected cells or other systems. With the high degree of antigenic conservation of NS-1 polypeptides among the parvoviruses, it would be interesting to see if these mAbs could also detect NS-1 proteins from the other parvoviruses. Monoclonal antibodies have several advantages over the traditional polyclonal sera in terms of supply, homogeneity, and specificity. Already, we have demonstrated the usefulness of the antibodies in the specific purification of NS-1Ac, although the results presented here indicate that the purification protocol has not yet been fully optimized. Still, functional studies can be done on the partially purified NS-1Ac. The expression of NS-1 in the baculovirus system is the first documented case of the overexpression of this protein and will be of great value once protein activity can be demonstrated. Ultimately, the relevancy of the baculovirus expressed proteins will have to be proven by purifying MVM NS-1 and demonstrating activity similar to NS-1Ac. The mAbs and the NS-1Ac protein will hopefully lead to a more detailed understanding of the function of MVM NS-1 and its role in the MVM replication strategy.
XI. Literature Cited


termini regions of adenovirus and minute virus of mice DNAs are preferentially associated with the nuclear matrix in infected cells. *J. Virol.* **63**(10): 4344-4353.


Cotmore, S. F., and P. Tattersall. 1988. The NS-1 polypeptide of minute virus of mice is


Appendix la: Construction of pAcRP6/ΔX.

This clone, constructed by R. Russnak, encodes a NS-1/NS-2 fusion protein. The NS-1 gene contains a frameshift mutation as a result of the filling-in of the unique XhoI site within the NS-1 gene, shifting the reading frame to that for NS-2.
Cut with Hgal, isolate 2.3 kb Hgal fragment, & fill-in ends

Cut with Smal

Ligate together

Cut with HincII, HincII

Cut out insert with EcoRI, & religate in reverse orientation

Cut with HindIII

Cut with H-inc / SstI & isolate the 150 bp Sst / Hind fragment containing the hCP signal sequence

Cut with SstI / EcoRV & replace the fragment with the SstI / HincI fragment of hCP1.R

CPss/NS1/pGEM-4Z
Appendix Ib: Construction of CPss/NS-1/pAcYM1.

The strategy used to construct the human preceruloplasmin (hCP) signal sequence - MVM NS-1 fusion protein encoding baculovirus transfer vector is shown above. This was conceived and carried out by R. Russnak as described in the Materials and Methods section B2.
Appendix Ic: Construction of pAcYM1/NS-1.
W. Chen's construction of the baculovirus transfer vector for the expression of an unfused, un-mutated NS-1 is schematically shown above. For the method of construction, refer the Materials and Methods section B2.
Appendix II: Nucleotide sequences of the pUC19/D deletions at the junction, predicted carboxy-terminal protein sequence of the resulting lacZ/NS-1 fusion proteins, number of the last amino acid sharing homology with lacZ/NS-1, and the number of the corresponding amino acid in MVM NS-1.

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Deletion clones were made as described in the Materials and Methods section and named based on the time point they were taken from, followed by a distinct number for that time point. The 'a' designation indicates an earlier trial. Sequencing of the clones was performed as described in the Materials and Methods, allowing calculation of the size of the DNA deletion and prediction of the carboxy-terminal protein sequence. Amino acids are given by their single letter code with '*' designating a termination codon. This sequence was then compared against that of lacZ/NS-1 to determine amino acid sequences not present in MVM NS-1. Finally, the last amino acid of MVM NS-1 sharing homology with the carboxy-terminal-deleted lacZ/NS-1 proteins was determined from the primary protein sequence of MVM NS-1.

MVM sequences are in bold text, and termination linker sequences are given in plain text.